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The Urease Enzyme of *Helicobacter pylori* Does Not Function as an Adhesin

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Helicobacter pylori **urease is essential for colonization of the gastric mucosa irrespective of whether the stomach is acidic or hypochlorhydric. It has therefore been speculated that the enzyme functions as an adhesin. The aim of this study was to compare the adherence of *H. pylori* N6 with the adherence of an isogenic urease-negative mutant, strain N6(*ureB*::TnKm), to gastric cells. Strain N6 originated from a patient with gastritis. Strain N6(*ureB*::TnKm) is specifically modified in the gene which encodes the large subunit of urease, UreB, and hence does not form a UreA-UreB enzyme complex. We have used flow cytometry to assess the adherence of *H. pylori* to the cells. We have also used phase-contrast microscopy to assess the adherence of the organism to Kato III cells. In the absence of urea both strains bound to Kato III cells and to primary gastric cells. Binding of both strains to the cells occurred rapidly. The presence of urea in the incubation medium decreased the binding of strain N6 to the cells. This was due to a rise in the pH of the incubation medium, which caused loss of viability of the organism. Urea had no effect on the adherence of strain N6(*ureB*::TnKm). We conclude that the urease of *H. pylori* does not play a role in the adherence of the organism to gastric cells.**

Infection of the gastric mucosa of humans with *Helicobacter pylori* has been shown to cause type B chronic gastritis and to be strongly associated with duodenal ulceration (13, 16). Early acquisition of this infection is also associated with an increased risk of developing gastric cancer in later life (19). The organism is found within and beneath the gastric mucous layer as well as attached to gastric epithelial cells (15). Adherence of the organism to the gastric mucosal surface is thought to be an important step in colonization. Understanding the mechanism of adherence may be essential in determining how *H. pylori* causes disease.

The exact role that urease plays in the disease process is not yet known, although it is thought to play a role in protecting the organism from the effect of gastric acid (4, 17). *H. pylori* and *Helicobacter* species isolated from the gastric mucosa of animals possess a potent urease (23), whereas *Helicobacter* species that colonize the lower bowel often lack urease (21). The urease of *Helicobacter* species differs from other bacterial ureases in that it is composed of two rather than three subunits. UreA and UreB have deduced molecular weights of 26,000 and 61,000, respectively. A high level of homology exists among the N-terminal amino acid sequences of the enzyme subunits of four *Helicobacter* species studied by Turbett et al. (23). This suggests a common ancestral origin and an important role for urease.

Urease is the most important virulence factor of *H. pylori* identified to date. Urease activity has been shown to be essential for colonization by *H. pylori* of the nude mouse (22) and the gnotobiotic piglet (6, 7). Similarly, urease activity is essential for colonization of the ferret stomach by *Helicobacter mustelae* (1). Recent studies have shown that oral immunization of mice with the urease of *H. pylori* protects them from subsequent infection with *Helicobacter felis* (11, 18). The vital role of urease in promoting colonization does not appear to be simply due to its effect on the acid environment of the stomach. In achlorhydric piglets urease remains essential for colonization by *H. pylori* (7). Another unusual feature of the urease of *H. pylori* is that it has been located on the surface of the bacteria (2, 14). Because of its surface location, there has been speculation that the urease of *H. pylori* may function as an adhesin (7, 9). Fauchere and Blaser (9) reported that saline or water extracts of *H. pylori* inhibited the binding of the organism to HeLa cell membranes. Gel exclusion chromatography of the extracts showed that the fractions which contained the highest urease activity bound best to HeLa cell membranes. These fractions contained urease subunit antigens. They speculated that the adhering ligand may be urease. Gold et al. (12) found that a urease-deficient strain of *H. mustelae* showed a selective reduction in binding to the glycosphingolipid Gg4. When urease activity in this mutant strain spontaneously reverted during culture, wild-type binding was again demonstrated.

It has recently been shown that two isogenic urease-negative mutants of *H. pylori* were able to colonize gastric explants derived from neonatal germ-free piglets (8). Using flow cytometry we have shown that Kato III cells (cells from a gastric adenocarcinoma cell line) and cells isolated from gastric biopsy specimens are ideal for studying the adherence of *H. pylori* in vitro (3). The aim of the present study was to compare the binding of an isogenic urease-negative strain of *H. pylori* to primary human gastric cells and to Kato III cells with the binding of the wild-type urease-positive parent strain in order to determine unequivocally if the urease of *H. pylori* plays a role in promoting adherence of the organism to gastric epithelial cells.

An isogenic mutant of *H. pylori* N6 negative for urease activity, strain N6(*ureB*::TnKm) (an isogenic *ureB* mutant), and the wild-type parent strain, N6, were used in this study. Strain N6 originated from a patient with gastritis (10). Strain N6(*ureB*::TnKm) was constructed by allelic replacement. A region of cloned DNA containing the structural genes *ureA* and *ureB* was disrupted by insertion of a mini-Tn*3* kanamycin transposon (10). Strain N6(*ureB*::TnKm) is specifically modified in the gene which encodes the large urease subunit, UreB, and does not synthesize this subunit. These strains were generously donated to us by Agnes Labigne of the Pasteur Insti-

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tute in Paris, France. Strains were grown and held in storage at −70°C as described previously (5). For each assay a vial of bacterial cells was thawed and cultured on Columbia blood agar (GIBCO) plates containing 7% (vol/vol) defibrinated horse blood for 3 days at 37°C in an atmosphere of 5% O_2 and 10% CO_2. Strain N6(*ureB*::TnKm) was grown on plates which also contained kanamycin (25 μg/ml). Bacteria were confirmed as *H. pylori* on the basis of colony morphology, Gram stain, and the production of oxidase and catalase. Urease activity was tested by transferring growth from the agar cultures with a sterile loop into 100 μl of urea solution containing 2% (wt/vol) urea and 0.001% (wt/vol) phenol red in 0.01 M phosphate buffer (pH 6.8). A positive reaction was indicated by a change in color from orange to pink within 5 min. Bacteria were harvested by rinsing the surface of each plate with 5 ml of phosphate-buffered saline (PBS; Dulbecco's formula A, pH 7.3) and removing growth by scraping with a sterile swab. Bacteria were washed once in PBS by centrifugation at 3,600 × *g* for 15 min. Quantitation of bacteria in suspension was determined by optical density measurement at 450 nm and by viable counts. Appropriate dilutions of the bacterial suspensions were spread on Columbia blood agar plates, and after incubation of plates at 37°C under microaerobic conditions (5% O_2, 10% CO_2) for 5 days, CFU per milliliter were enumerated.

Kato III cells are gastric adenocarcinoma cells obtained from the American Type Culture Collection. They were grown in RPMI 1640 medium (Imperial Laboratories) containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer and 12 mM sodium bicarbonate supplemented with 10% (vol/vol) fetal calf serum. For adherence assays, cells were scraped from the base of the flask and centrifuged at 200 × *g* for 5 min. Cells were resuspended in RPMI 1640 medium and counted by microscopy using a hemocytometer. Primary human gastric epithelial cells were isolated from biopsy tissue by digestion with 0.1 mM EDTA and 0.1 mM dithiothreitol followed by digestion with 0.05% (wt/vol) collagenase as previously described (3).

The adherence assay used in these experiments has been previously described (3). Briefly, gastric cells and different concentrations of bacteria were incubated together at 37°C for 30 min under microaerobic conditions. Cells were stained with a whole-cell *H. pylori* antibody, washed, and subsequently stained with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Sigma). Adherence was assessed by phase-contrast microscopy and by flow cytometry. By using a dot plot display of forward and right-angle scatter, the flow cytometer (Becton Dickinson) was gated to include single cells and to exclude most cell debris, clumps of cells and nonadherent bacteria. A total of 10,000 gated events was collected, and analysis of the data was performed by using the Lysis software program from Becton Dickinson. This program produces histograms of each cell sample and calculates the mean channel fluorescence (mean channel number) of the cell population, which directly relates to the surface density of fluorescently labelled *H. pylori* adhering to the cell, i.e., the higher the mean channel number the more bacteria there are adhering to the cell. Mean channel numbers of cells with adherent bacteria and cells without bacteria were compared. Results are also expressed as the percent fluorescent cells (i.e., the percent positive events or the percentage of cells with bacteria attached) calculated from fluorescence frequency distribution histograms (i.e., the relative number of cells versus the relative fluorescence intensity). The threshold for positivity was set for each experiment by flow cytometric analysis of cells without adherent bacteria which had been stained with anti-*H. pylori* whole-cell antibody and the fluorescein isothiocyanate-labelled second antibody. Unless otherwise stated, results are expressed as the mean results of at least three separate experiments ± the standard errors of the means. Results were analyzed by using the Student *t* test.

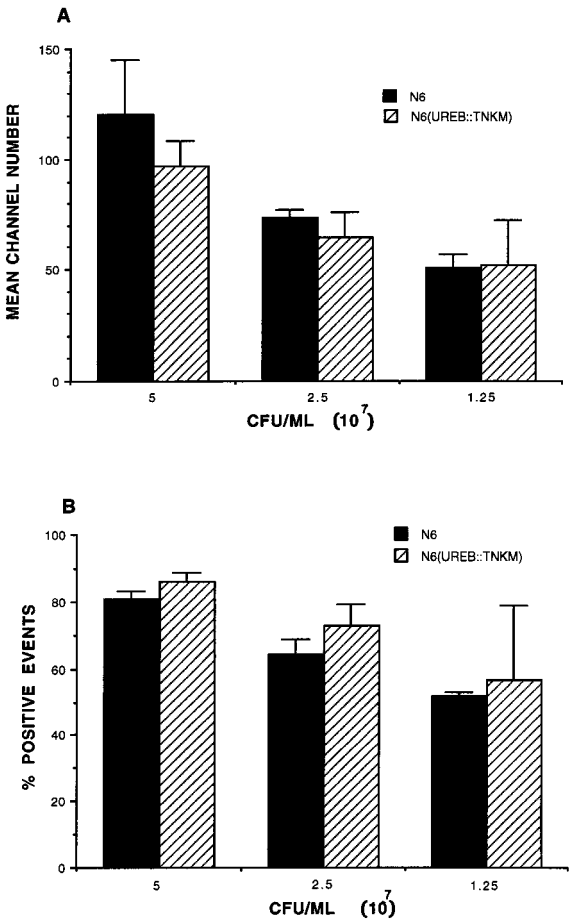

FIG. 1. Binding of *H. pylori* N6 and N6(*ureB*::TnKm) to Kato III cells. Different concentrations of bacteria were incubated with Kato III cells and adherence was assessed by flow cytometry as outlined in the text. The results are expressed as the mean channel number (A) and as the percent positive events (B) for each concentration. The results are means ± standard errors of the means for three experiments.

Strain N6 and the isogenic urease-negative mutant strain N6(*ureB*::TnKm) both bound to Kato III cells. The mean channel numbers and the percent positive events for the two strains were similar over the range of concentrations of bacteria tested (Fig. 1). There was no significant difference between the binding of strain N6 and that of strain N6(*ureB*::TnKm) to Kato III cells at any of the concentrations of bacteria tested ($P > 0.1$). Binding occurred rapidly and was complete within 30 min. There was no difference in the kinetics of binding within the first 30 min. There was no increase in fluorescence intensity when the bacteria were incubated with the cells for up to 2 h. The adherence of bacteria to the cells was confirmed by phase-contrast microscopy (Fig. 2). Both strains also bound to primary cells isolated from gastric biopsy specimens (Fig. 3). When urea (10 mM) was added to the incubation medium, there was a decrease in the adherence of strain N6 to primary cells but not in the adherence of strain N6(*ureB*::TnKm) (Fig. 3). A similar decrease in adherence to Kato III cells was noted

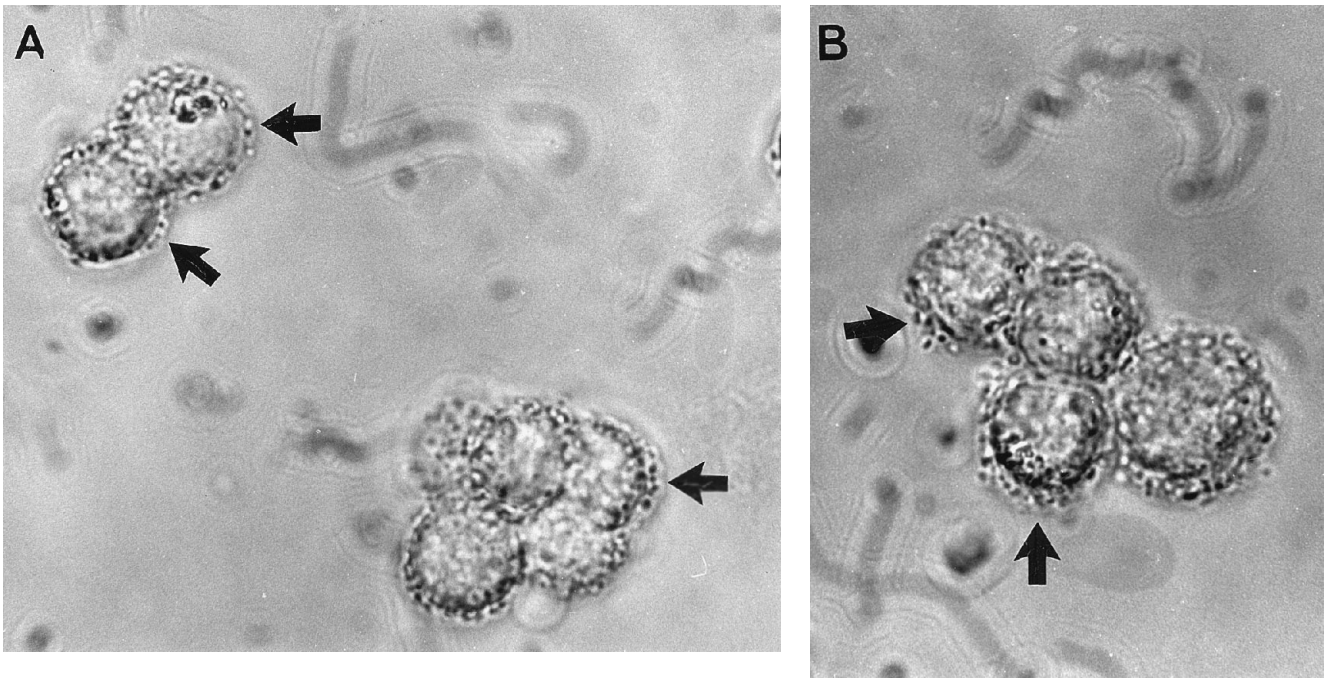

FIG. 2. Phase-contrast micrograph of *H. pylori* strain N6 (A) and strain N6(*ureB*::TnKm) (B) binding to Kato III cells. Arrows mark the sites of attachment of bacteria to the cells.

for strain N6 but not for strain N6(*ureB*::TnKm) when urea was added to the incubation medium (results not shown). Viability testing showed that strain N6 did not survive in solutions containing urea, whereas urea had no effect on the viability of strain N6(*ureB*::TnKm) (Fig. 4).

The mutant used in this study contains a disruption of the *ureB* gene and does not produce the UreB subunit on its surface or have any urease activity. The addition of urea to the incubation medium did not have any effect on the adherence of strain N6(*ureB*::TnKm) to the cells. We have recently shown that the addition of urea to a solution of neutral pH containing urease-positive *H. pylori* renders that solution alkaline, resulting in a loss of viability of the organism (4). In this study the pH of the solutions containing urease-positive *H. pylori* N6 and urea became alkaline (pH rose from 7.2 to 9.11) within 10 min of incubation. This explains the lack of viability of strain N6 when urea was added to the medium and the decrease in adherence of strain N6 to the cells.

This is the first study using an isogenic mutant deficient in urease production to show that the urease of *H. pylori* does not play a role in adherence of the organism to human gastric cells.

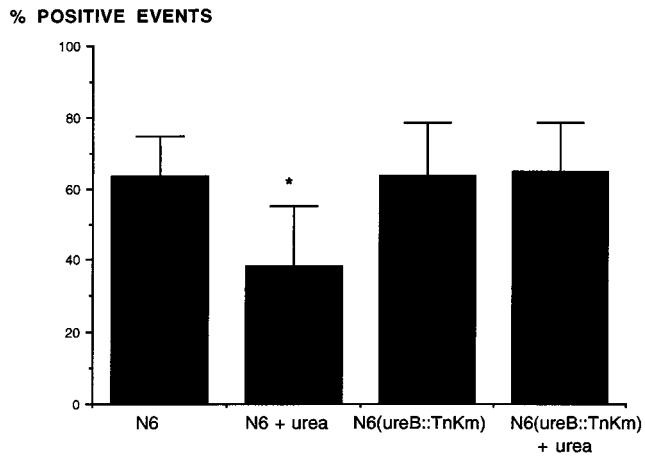

FIG. 3. Binding of *H. pylori* N6 and N6(*ureB*::TnKm) to primary human gastric epithelial cells. Adherence was assessed by flow cytometry as outlined in the text. The binding of strains N6 and N6(*ureB*::TnKm) was assessed both in the absence of urea and in the presence of 10 mM urea. The results are means ± standard errors of the means for four experiments. *, statistically significantly different from other results ($P \leq 0.05$).

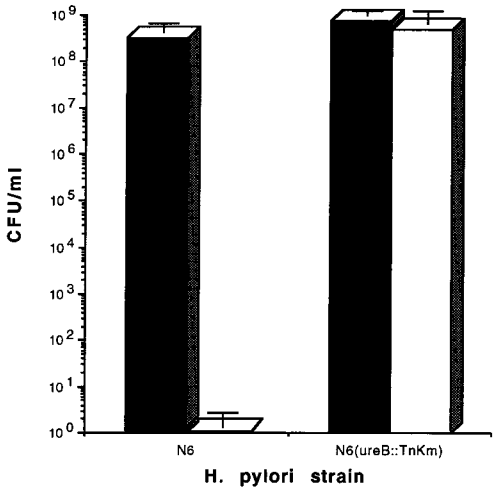

FIG. 4. Effect of urea on viability of *H. pylori* N6 and N6(*ureB*::TnKm). Bacteria were incubated in PBS, pH 7.2, containing either no urea (■) or 10 mM urea (□) for 30 min at 37°C under microaerobic conditions. Serial 10-fold dilutions of the bacterial suspensions were made, plated onto Columbia blood agar plates, and incubated for 5 days at 37°C under microaerobic conditions to determine the number of CFU per milliliter.

In the absence of urea, strain N6 and strain N6(*ureB*::TnKm) both bound to Kato III cells and to primary gastric cells, which shows that the physical presence of the UreA-UreB urease complex is not required for adherence of *H. pylori* to human gastric cells.

One of the reasons why the urease of *H. pylori* was speculated to be an adhesin was because it was found on the surface of the bacteria (2, 14). However, recently Phadnis et al. (20) have shown that, in common with other urease-producing bacteria, the urease of *H. pylori* is in fact a cytoplasmic enzyme. It may be found on the surface of *H. pylori* because some bacteria lyse and release the enzyme, which coats the surface of viable organisms. The fact that the enzyme is not exported to the cell surface suggests that it is unlikely to act as an adhesin and is consistent with the results of this study.

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