INFECTION AND IMMUNITY, May 1992, p. 1858–1863
0019-9567/92/051858-06$02.00/0
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Vol. 60, No. 5

Further Evidence of the Toxic Effect of Ammonia Produced by *Helicobacter pylori* Urease on Human Epithelial Cells

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Received 26 September 1991/Accepted 17 January 1992

Former studies have shown that *Helicobacter pylori* can induce vacuolation of epithelial cells in vitro and possibly in vivo, either by direct action of a cytotoxin or by the action of its strong urease, which breaks down the urea physiologically present in the stomach into cytotoxic ammonia. We have developed a test using HEp2 cells with adherent *H. pylori* bacteria in order to compare the effects of an *H. pylori* urease-negative variant with those of its urease-positive parent strain in the presence of 10 mM urea. The level of ammonia production as well as cell vacuolation and viability were monitored for 72 h. The ammonia produced (20 mM) was found to be the essential determinant of the degree of cell vacuolation and viability of HEp2 cells. However, the addition of acetohydroxamic acid (200 mg/liter), a potent urease inhibitor which inhibits ammonia production, did not completely restore cell growth, suggesting the difficulty of neutralizing the ammonia in the vicinity of the cells. Antibodies directed against *H. pylori* did not neutralize the urease activity. When *H. mustelae* was tested in the same manner, the detrimental effects were not observed because a lower quantity of ammonia (5 mM) was generated. This was due to a lower urease activity, although the adherence properties of *H. mustelae* were different from those of *H. pylori* both quantitatively (greater adherence) and qualitatively (localized instead of diffuse adherence). We conclude that *H. pylori*-induced ammonia is an essential determinant of its cell toxicity as well as its adherence properties, which allow a high concentration of ammonia at the cellular level.

Helicobacter pylori is the etiological agent of type B gastritis, a common gastritis, the prevalence of which increases with age in developed countries (27) and for which no special cause had been found until now (34). Type B gastritis and antral metaplasia in the duodenum are thought to be the two prerequisites for duodenitis and, subsequently, duodenal ulceration (42).

However, the pathogenetic pathways of *H. pylori* at the cellular level are not yet well established. A cytotoxin which causes vacuolation has been described and would be present in approximately 50% of the strains (6, 13, 21). Some enzymes produced by *H. pylori* have also been studied, such as mucinase (32) and phospholipase A (35), but the strong urease produced by *H. pylori* has been given most of the attention. It has been shown that the ammonia production facilitates the colonization of the gastric mucosa (11) by increasing the pH around the organisms (24). However, in addition, a toxic action of the ammonia produced on epithelial cells could also be involved.

In vivo, the adherence properties of *H. pylori* allow numerous organisms to remain in contact with the cells. We have recently presented an in vitro model using HEp2 cell monolayers which mimics the in vivo situation (30). Our goal was to test the action of the ammonia produced by *H. pylori* urease on the cells in this model.

MATERIALS AND METHODS

Strains used. The strain of *H. pylori* (Bx2) used was isolated in 1984 in Bordeaux, France, from a patient with antral gastritis. This strain has the classical characteristics of *H. pylori*. It is positive for urease, oxidase, catalase, alkaline phosphatase, and γ-glutamyl transferase and negative for nitrate reductase and hippuricase (26).

A urease-negative mutant of this strain, obtained after more than 50 subcultures of the parent strain and without specific treatment, was also used. Both strains were compared by restriction endonuclease DNA analysis with a combination of *Hin*dIII and *Bst*EII (20).

Whole-cell lysates of both strains were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% acrylamide separating gel and were stained with Coomassie blue (19).

The two strains were also compared for their cytotoxin production by the neutral red uptake technique of Cover et al. (7).

A strain of *Helicobacter mustelae* ($FCLO_3$), a urease-positive bacterium colonizing the stomach of ferrets, kindly provided by D. S. Tompkins, Bradford, United Kingdom, was also tested.

The organisms were kept frozen at −70°C before being tested.

Adherence test. In all experiments, the adherence test was performed as described previously (30). Briefly, a subconfluent monolayer of HEp2 cells was grown in 25-cm^2 flasks (Nunc, Roskilde, Denmark) in Eagle's minimal essential medium (Flow Laboratories, Puteaux, France) with 10% fetal calf serum (Seromed, Berlin, Federal Republic of Germany) and 1% nonessential amino acids (medium 1). A 5-ml suspension of urease-positive *H. pylori*, urease-negative *H. pylori*, or *H. mustelae* grown on agar for 48 h was added at a concentration of approximately 5×10^7 CFU of bacteria per ml. The culture medium used to grow the bacteria was Wilkins-Chalgren agar (Oxoid, Basingstoke, United Kingdom) supplemented with 10% human blood. CFU were quantitated by serial 10-fold dilutions, each dilution being plated on three plates. Plates with 20 to 100 colonies were used for counting. After 3 h, the time period allowing optimum attachment of *H. pylori*, the supernatant was discarded and the monolayer was washed three times with 5 ml of phosphate-buffered saline in order to eliminate nonadherent bacteria. The monolayer was then stained with

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bisbenzimide (0.05 μg/ml), and the number of cells with more than 20 bacteria adhering were counted. Percentages were determined by counting 200 cells.

Titration of ammonia produced by the strains. The amount of ammonia produced by the urease-positive *H. pylori* and its urease-negative derivative, as well as by *H. mustelae*, was determined by the method described by Ferrero et al. and is expressed in micrograms of ammonia produced per minute per milligram of protein (12).

Test of the effect of bacterially induced ammonia on the cells. The first part of the test was identical to that of the adherence assay, but after the 3-h incubation period, instead of staining the monolayer, a second medium (medium 2) composed of medium 1 plus 10 mM urea and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) was then added. Both media were at pH 7. Incubation was carried out at 37°C for a maximum of 3 days.

After 24, 48, and 72 h, the following tests were performed: (i) macroscopic observation of the monolayer (May-Grunwald-Giemsa stain); (ii) viable-cell count (trypan blue stain) in triplicate; and (iii) dosage of ammonia (in triplicate) with an ammonia enzymatic UV kit (bioMérieux, Marcy l'Étoile, France).

Experiments were also performed on the cell monolayer to determine the effects of the total number of *H. mustelae* added (adherent and nonadherent). In this case, medium 2 was used throughout the test.

Controls used in the assay included a noninfected HEp2 cell monolayer, a HEp2 cell monolayer infected with urease-positive *H. pylori* in medium 1, and a noninfected HEp2 cell monolayer with jack bean urease (0.01 mg of urease per ml, with an activity of 545 U/mg) in medium 2 (Sigma).

Inhibition tests. Inhibition tests were performed on urease-positive *H. pylori* in medium 2 with the following substances: acetohydroxamic acid, a urease inhibitor known to be noncytotoxic by itself (36), at a concentration of 200 mg/liter; and pooled hyperimmune human serum against *H. pylori* (dilution, 1:100).

The sera were detected by an immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA), with a sonicate as the antigen. Because high values were obtained, the sera were pooled and used in the inhibition test.

The *t* test was used for statistical comparison.

RESULTS

Characterization of *H. pylori* urease-negative mutant. We found no difference between the parent strain and the urease-negative mutant with regard to other phenotypic properties.

By restriction endonuclease DNA analysis, the two strains did not show any differences. However, by SDS-PAGE, a 66-kDa band which was present for the urease-positive strains was absent from the urease-negative mutant (Fig. 1).

A weak cytotoxic effect was found (maximum OD, 0.16) which was identical in both the parent strain and its urease-negative derivative.

Effect of bacterially induced ammonia on the cells. The evolution of ammonia production in relation to adherent urease-positive *H. pylori* is presented in Fig. 2. Strong production was obtained after 24 h and remained constant throughout the test. The same concentration of ammonia was obtained with jack bean urease but occurred in the first few hours (not shown). The pH was not significantly altered because of the strong buffer used (maximum pH observed, 7.7).

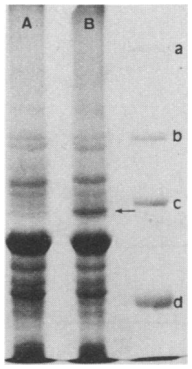

FIG. 1. SDS-PAGE of *H. pylori* Bx2. (A) Urease-negative derivative. (B) Urease-positive parent strain. Molecular mass markers (in kilodaltons): a, 200; b, 97; c, 68; and d, 43. A band of 66 kDa (arrow) is present for the urease-positive strain and absent for its urease-negative derivative.

In the controls with noninfected HEp2 cells, with urease-positive *H. pylori* but without urea in the medium (medium 1), as well as with the urease-negative *H. pylori* strain variant in medium 2, a low amount of ammonia was present.

After 1 day, the difference in HEp2 cell count between the test cultures and the controls was not significant (Fig. 2); however, vacuolation was detected in the test cultures, in which ammonia was determined to be present by microscopic observation of the stained monolayer. After 2 days, when a large quantity of ammonia was present, the cells did not grow and cell vacuolation increased (Fig. 3), while the cell count decreased (Fig. 2). No such effect was observed with the other controls ($P < 0.01$) except with jack bean urease.

The action of potential urease inhibitors is presented in Fig. 4. Acetohydroxamic acid was able to inhibit ammonia production, whereas serum from patients infected with *H. pylori* did not. The consequence of an elevated ammonia concentration on the aspect and viability of HEp2 cells was the same as in the previous experiment. Despite the absence of ammonia in the medium, cell growth was lower than in the control ($P < 0.05$), and some vacuoles were present, suggesting an incomplete neutralization by acetohydroxamic acid in the vicinity of the cells.

When *H. mustelae* was tested in the same model, differences were noted. The ammonia level increased but at a rate four times less than with urease-positive *H. pylori*, and furthermore, cell growth was not altered. In contrast, when nonadherent bacteria were also maintained in the medium in addition to adherent bacteria, the HEp2 cell number decreased ($P < 0.01$) (Fig. 5).

Adherence test. The percentage of cells with more than 20 bacteria per cell was similar for the urease-positive *H. pylori* strain and its urease-negative derivative, 57.2% ± 8.6% versus 55.2% ± 3.3% (± standard deviation), whereas it was higher for *H. mustelae* (72.5% ± 4.4%). However, the type of adherence was different; a diffuse adherence was present with both *H. pylori* strains, and a localized adherence was found with *H. mustelae*.

Ammonia titration. An important difference between the strains in the level of ammonia was observed. No ammonia was detected when the test was performed on the urease-negative *H. pylori*. In contrast, 350 μg of ammonia per min per mg of protein was produced by the urease-positive

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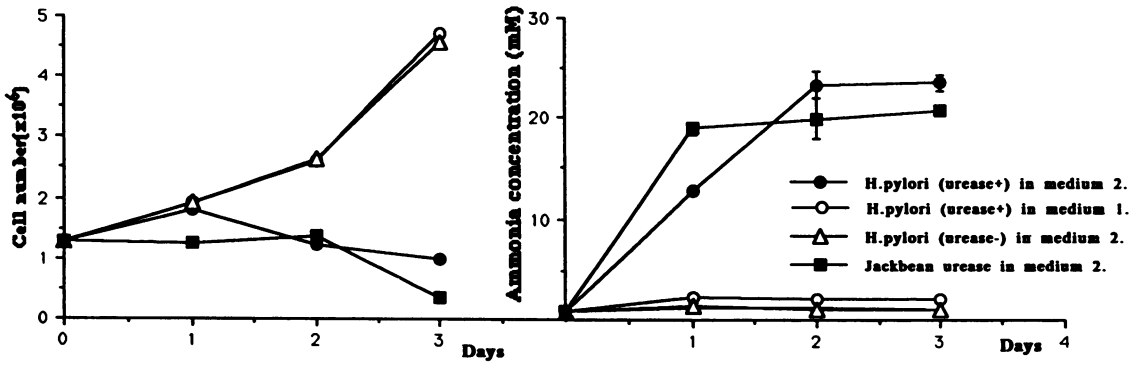

FIG. 2. Evolution of viable-cell count (left) and ammonia concentration (right) in HEp2 cells infected with *H. pylori* in a urea-containing medium. Medium 1 does not contain urea. Medium 2 is composed of medium 1 plus 10 mM urea and 20 mM HEPES. A control without *H. pylori* showed the same result as urease-negative *H. pylori* in medium 2. Cell number is expressed per 25-cm^2 flask.

strain, and three times less was produced by *H. mustelae* (118 μg/min/mg of protein).

DISCUSSION

There is now a consensus that *H. pylori* is the etiological agent of type B gastritis. Gastritis lesions consist of an infiltration of the mucosa by inflammatory cells, e.g., lymphocytes and polymorphs, which indicate the presence of gastritis. These infiltrates are observed after classical histological staining, such as with hematoxylin and eosin (8). However, when transmission electron microscopy is performed, alterations of the epithelial cells are also noted and consist of the destruction of villi at the site of adhesion, disruption of the intercellular junction, and the presence of vacuoles in the cells (3, 4, 39). By scanning electron microscopy, the cells look disordered and balloonlike (9). It is possible that these lesions are the consequence of the mediators of inflammation (23), but another alternative is the direct (e.g., cytotoxin) or indirect (e.g., products of metab-

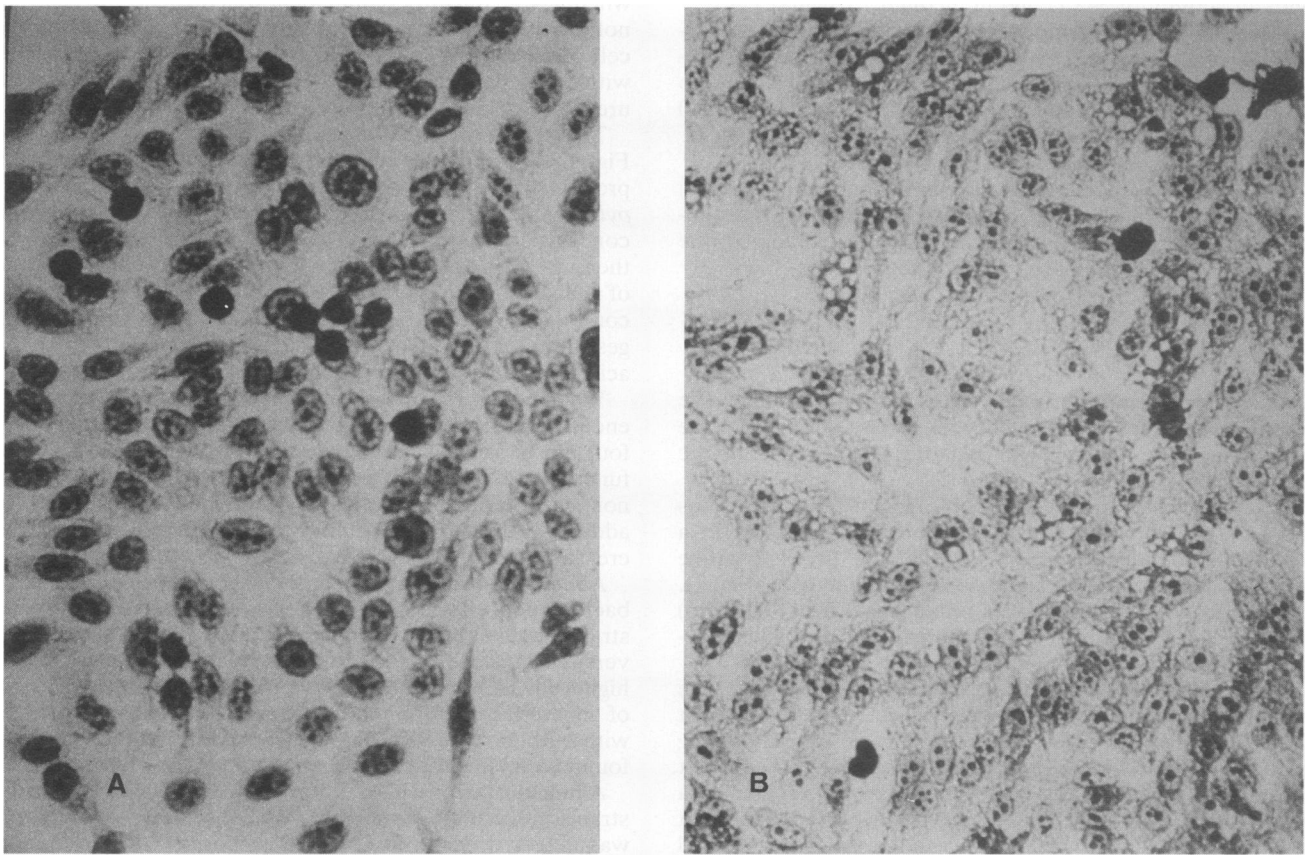

FIG. 3. Action of ammonia produced by adherent *H. pylori* on HEp2 cells in the presence of 10 mM urea after 48 h of incubation. (A) Control; (B) infected cells.

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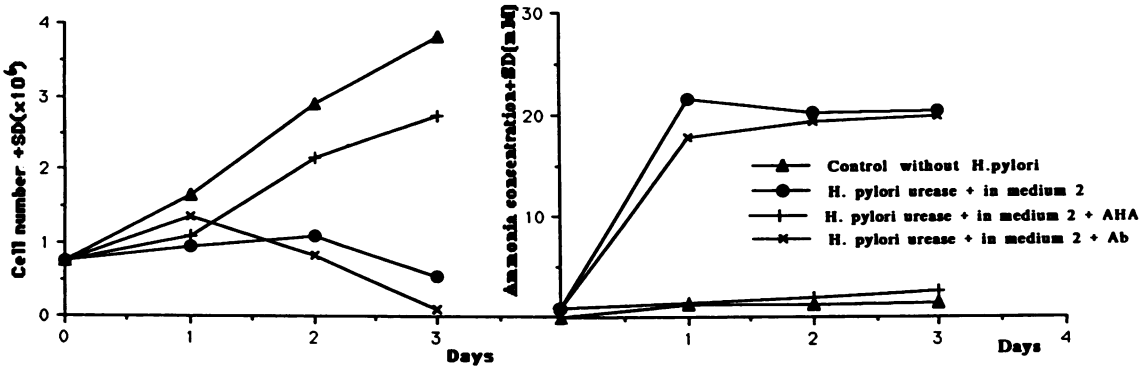

FIG. 4. Evolution of viable-cell count (left) and ammonia concentration (right) in HEp2 cells infected with *H. pylori* in a medium containing urea in the presence of different inhibitors. Medium 2 contains 10 mM urea and 20 mM HEPES. AHA, acetohydroxamic acid; Ab, *H. pylori* antibodies. Cell number is expressed per 25-cm^2 flask.

olism) action of *H. pylori* on the cells. *H. pylori* contains numerous enzymes (26), especially a strong urease, which has now been characterized (10, 12, 28). Urea is found in the gastric juice present in the stomach, probably by diffusion from the blood (16). Marshall and Langton found concentrations ranging from 1 to 6 mM in *H. pylori*-negative patients, whereas the concentration was always less than 1 mM in *H. pylori*-positive patients (25). The ammonia concentration in the stomach of infected patients is by contrast higher, 2.3 versus 0.4 mM, respectively, in the gastric juice of patients studied before and after *H. pylori* eradication (5), and 25 versus 5.8 mM in mucus samples from *H. pylori*-infected and noninfected patients, respectively (37).

In the intestine, ammonia is generated by proteolytic bacteria. Its toxic effect on the intestinal mucosa has been known for many years. The effects include alteration of nucleic acid synthesis, increase in the intestinal mucosal cell mass, and risk of viral infection. Ammonia has been implicated in carcinogenesis for populations that consume large amounts of protein (41). More recently, attention has been given to the question of ammonia toxicity on gastric mucosa by Japanese authors. Murakami et al., using rats, found a decrease in transmucosal electrical potential and a decrease in gastric mucosal blood flow after infusion of an ammonia solution into the stomach (29). The same group also found changes in gastric mucosal blood flow when experiments were performed in humans, while an increase in the degree of inflammatory cell infiltration was noted (18). Tsujii et al. noted a decrease in O_2 consumption and in the energy charge of the gastric cells in contact with ammonia (40). The impact of ammonia on epithelial cells was not considered, and therefore, we decided to investigate the action of urease in our model by using HEp2 cells and mimicking the in vivo situation. The adherence of *H. pylori* to HEp2 cells is morphologically similar to what is observed in vivo, and the glycolipid on antral cells that acts as a receptor has been found on HEp2 cells (22).

In the presence of a urea concentration in the range observed in vivo, the high level of ammonia generated by adherent *H. pylori* had a significant detrimental effect on the cell monolayer, i.e., vacuolation and decreased viability of the cells. These effects did not occur in the absence of urea or when the same strain did not produce urease. However, these effects occurred when purified urease was added to the medium. This effect was not linked to a change in pH because of the high buffering properties of the medium.

To clarify the role of ammonia in the observed findings, we also tested the effect of defined ammonium chloride concentrations by using the neutral red uptake technique of Cover et al. (6). A dose-dependent effect was obtained, and vacuoles occurred when a concentration of 8 mM was present (not shown).

Smoot et al. (36) also studied the action of ammonia produced by *H. pylori* urease on epithelial cells and obtained similar results. They used the CRL 1739 cell line, with a urea concentration twice that in this experiment and with total

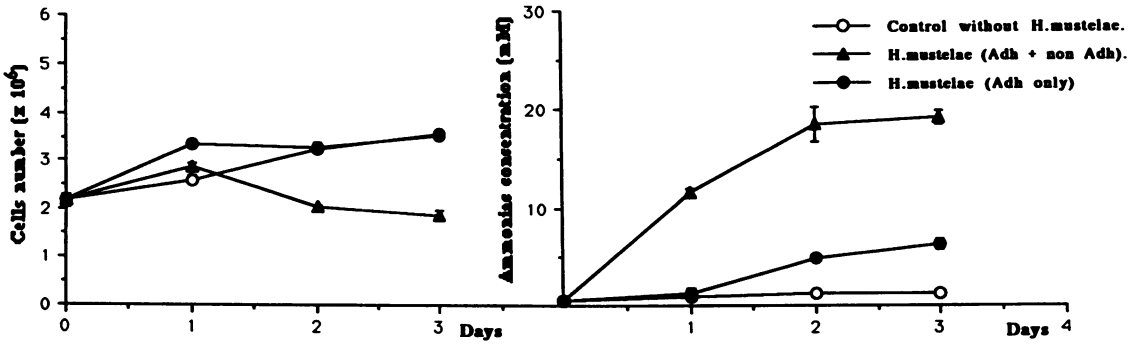

FIG. 5. Evolution of viable-cell count (left) and ammonia concentration (right) in HEp2 cells infected with *H. mustelae* in a medium containing urea. Adh, adherent (sessile); non Adh, nonadherent (planktonic). The same strain of *H. mustelae* was used, but in one case only the sessile bacteria were retained, whereas in the other case, both the sessile and the planktonic bacteria were left in the medium. Cell number is expressed per 25-cm^2 flask.

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bacteria (adherent and nonadherent) present (36). Another major difference was the use of a urease-negative mutant stemming from the urease-positive parent strain as the control in our experiment. Such a procedure is recognized as the best way to study the determinants of pathogenicity of a microorganism because otherwise, by comparing different organisms, one can never be sure that other determinants of pathogenicity are identical from one strain to another. However, compared with the use of genetically constructed isogenic mutants, this approach has the disadvantage that other possible mutations are not well controlled. These effects were not inhibited by high concentrations of *H. pylori* IgG antibodies, suggesting that, despite the antigenic properties of urease, antibodies do not bind to the enzymatic site. An inhibition was observed with acetohydroxamic acid, while cell growth was slightly decreased in the test flasks in comparison to the controls. Such a result can be explained by the fact that, as has been shown for toxins, ammonia delivered to the cell vicinity acts more efficiently on the target cell (44) and can escape neutralization (31).

Barer et al. (1) and Xu et al. (43) used another approach. They showed that bacteria-free supernatants from *H. pylori* grown in a urea-containing medium, tested on Vero and Int 407 cells, had either a vacuolating effect or a lytic effect, respectively, according to the ammonia concentration present.

The occurrence of such an effect with some strains of *H. pylori* has been reported by several authors under experimental conditions in which urea was not added and has been linked to the presence of a cytotoxin (6, 13, 21). However, the molecule has not been well characterized, and its potency is not important, since a concentrated broth culture supernatant must be used for its detection. Xu et al. proposed that this finding was due to the action of *H. pylori* urease on the low concentration of urea present in culture media because of the addition of fetal calf serum (5 mM urea in fetal calf serum used at 10% = 0.5 mM urea) (43). Ammonia is known to be a lysosomotropic agent which induces the inhibition of protein degradation (33). It induces vacuolation of lysosomes, which is observed microscopically. Recently, Cover et al., using the neutral red dye uptake test, presented data which confirmed the role of ammonia in vacuolation. There was a small but significant ($P < 0.05$) difference between a urease-positive parent strain and its urease-negative mutant. Nevertheless, the urease-negative mutant showed vacuolation, indicating the presence of another compound, which could be a cytotoxin (7). Smoot et al. also found that the viability of cells exposed to *H. pylori* was lower than that of cells exposed to jack bean urease for any given ammonia concentration in the medium, suggesting the production of a cytotoxin (36). We also found production of a cytotoxin in the urease-positive and urease-negative *H. pylori* strains, but this cytotoxin cannot explain our results because its level was low and was similar for the two strains.

H. mustelae, a bacterium colonizing the stomach of ferrets (14), has not been found in association with gastritis lesions in the strain of ferret bred in England (38), in contrast to what has been observed with the American strain (13). Since *H. mustelae* is also a urease producer, it was claimed that ammonia could not be the cause of the lesions observed with *H. pylori*. In fact, in the case of the ferret strain bred in England, it was mentioned that the bacteria were never as numerous as *H. pylori* found in human gastric mucosa (38).

In our experiments, we found that *H. mustelae* produces less ammonia than *H. pylori* and has a different pattern of adherence than *H. pylori*. Despite the fact that *H. mustelae* adheres more to HEp2 cells the localized adherence observed may not be significant in vivo. The arguments in favor of such a hypothesis are that we also found this type of cell association with *C. jejuni* subsp. *doylei* (29), which is not a significant pathogen in the stomach, and that *H. mustelae* is present in low numbers in the ferret stomach. We postulate that these two factors are complementary: adherence allows the bacteria to reach a high density in, and not be eliminated from, the mucus; and the urease, located at the periphery of the bacteria, generates ammonia close to the cells (3).

Lesions occur if the concentration of ammonia produced is sufficient. A threshold of 7 mM has been found for cell vacuolation, and a threshold of 20 mM has been found for cell lysis (43). This would explain why, in the antrum, *H. pylori* which adhere to mucus cells are usually abundant and induce lesions. In cases showing epithelial cell degeneration, Hessey et al. found a significant increase in the proportion of attached *H. pylori* (17); whereas in the body of the stomach—as for *H. mustelae* in the ferret stomach—*H. pylori* adheres poorly and is present in small numbers, and lesions are usually not observed.

Despite the fact that the ideal model, i.e., antral cells with a mucus layer, which is not yet available, was not used, we think that this model with HEp2 cells is useful for increasing knowledge of *H. pylori* pathogenesis.

ACKNOWLEDGMENTS

We thank P. Vincent (Laboratoire de Bactériologie, Faculté de Médecine de Lille, Lille, France) for comparing strains by restriction endonuclease DNA analysis and D. S. Tompkins (Department of Microbiology, Bradford Royal Infirmary, Bradford, United Kingdom) for providing us with an *H. mustelae* strain.

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