INFECTION AND IMMUNITY, Sept. 1994, p. 3604–3607
0019-9567/94/$04.00+0

Vol. 62, No. 9

Effect of Gastric pH on Urease-Dependent Colonization of Gnotobiotic Piglets by *Helicobacter pylori*

KATHRYN A. EATON* AND STEVEN KRAKOWKA

Department of Veterinary Pathobiology, The Ohio State University, Columbus, Ohio 43210

Received 2 May 1994/Returned for modification 19 May 1994/Accepted 10 June 1994

Thirty-seven gnotobiotic piglets from seven litters were infected with either *Helicobacter pylori* N6 or urease-negative *H. pylori* N6*ureG*::Km, which contains an insertion in the *ureG* gene and produces inactive urease. To produce achlorhydria, piglets were treated throughout the experiment with omeprazole (5 mg intravenously every 12 h) and ranitidine (75 mg orally every 6 h). Treatment resulted in elevation of gastric pH to 7.0 ± 1.1 throughout the experiment. Control piglets were not treated and remained normochlorhydric. Strain N6 colonized well in both normal and achlorhydric piglets. All 10 piglets were colonized, and colonization ranged from 4.4 ± 1.5 $\log_{10}$ CFU/g of gastric mucosa in normochlorhydric piglets sacrificed after 2 days to 6.9 ± 0.5 $\log_{10}$ CFU/g in normochlorhydric piglets sacrificed after 5 days. Strain N6*ureG*::Km did not colonize any of seven normochlorhydric piglets and was recovered only in low numbers (<100 CFU/g) from four of nine achlorhydric piglets. In the second experiment, piglets were coinoculated with both strains N6 and N6*ureG*::Km. Coinoculation did not affect colonization by urease-positive N6. Urease-deficient N6*ureG*::Km was unable to colonize even in the presence of urease-positive bacteria. These results confirm that urease enzymatic activity (and not urease protein) is essential for colonization, that this effect is independent of diffusible products of urea metabolism, and that gastric pH protection is not a major role of urease in promoting colonization by *H. pylori*.

Prominent bacterial urease activity is a characteristic feature of all gastric bacteria described to date, including *Helicobacter pylori* (12), *H. mustelae* (11), *H. felis* (20), *H. nemestrinae* (2), *H. acinonyx* (7), and "*H. heilmmanii*" (18, 23). *H. pylori* urease constitutes 5 to 10% of the bacterial protein (15). The universal presence of urease in gastric bacteria and the apparent importance of this enzyme to bacterial metabolism have led to the suggestion that urease promotes colonization of the gastric microenvironment (14). Our laboratory has shown that urease activity is essential for colonization of gnotobiotic piglets by *H. pylori* (6), and other studies have indicated that urease promotes colonization of *H. felis* in mice (17), but the mechanism whereby urease promotes such colonization is not known. It has been suggested that urease facilitates colonization either by producing a cloud of ammonia which protects the acid-sensitive bacteria in their transit across the gastric lumen to their final location below the gastric mucus (14) or by providing a nitrogen source to bacteria (14). Neither of these hypotheses has been tested in vivo. The goal of this study was to determine if urease-dependent colonization is due to either pH protection or to a diffusible product of urea metabolism.

MATERIALS AND METHODS

Bacterial strains. Two strains of *H. pylori* were used in this study. Strain N6 (wild type) is a urease-producing human isolate of *H. pylori* (8). Strain N6*ureG*::Km is a urease-negative strain derived from strain N6 by insertional mutagenesis (8). This strain contains an insertion in the *ureG* gene (one of several nonstructural genes required for expression of active urease) (9, 10) and fails to activate the urease apoenzyme. Both strains were kindly donated by Agnès Labigne.

* Corresponding author. Mailing address: Department of Veterinary Pathobiology, The Ohio State University, 1925 Coffey Rd., Columbus, OH 43210. Phone: (614) 292-9667. Fax: (614) 292-6473. Electronic mail address: keaton@magnus.acs.ohio-state.edu.

Bacteria were grown on blood agar plates or in *Brucella* broth with 10% fetal bovine serum at 37°C under microaerophilic conditions (19). Strain N6*ureG*::Km was grown on plates or in broth containing 25 μg of kanamycin per ml. Bacteria recovered from piglets were identified by urease activity and kanamycin resistance.

Animals. Thirty-seven outbred Yorkshire-cross piglets from seven litters were derived by cesarian section and maintained in sterile isolators (16). Piglets were inoculated with 10^9 CFU of bacteria at 1 week of age and sacrificed 2 or 5 days later. Bacterial inocula were grown in *Brucella* broth with 10% fetal calf serum (19). Strain N6*ureG*::Km was grown in broth containing 25 μg of kanamycin per ml.

Induction of achlorhydria. Previous experiments demonstrated that orally administered omeprazole alone (given once every 24 h) or omeprazole and cimetidine given three times daily did not result in consistently elevated gastric pH (not shown). Therefore, piglets were treated with omeprazole (5 mg given by intravenous injection) every 12 h and ranitidine (75 mg given orally) every 6 h. Treatment was started 12 h before bacterial inoculation and was continued until sacrifice. To determine the effectiveness of this procedure, gastric pH was determined every 6 h in one group of treated piglets with a pH probe (Synectics Medical, Irving, Tex.). Piglets were sedated, the probe was placed in the stomach and allowed to equilibrate, and the lowest pH reading was recorded. In all piglets, pH was determined at sacrifice both with a pH probe and with pH paper.

Experimental design. Piglets were randomly divided into nine groups (Table 1). Groups A (three piglets), B (three piglets), C (four piglets), D (three piglets), and E (seven piglets) were untreated. The other piglets were rendered achlorhydric as described above. Groups A, B, and F (four piglets) and group G (four piglets) were inoculated with *H. pylori* N6. Groups C, D, and H (five piglets) and group I (four piglets) were inoculated with N6*ureG*::Km. Group E was given 10^9 CFU of each bacterial strain. For this procedure, the two

TABLE 1. Colonization of normochlorhydric piglets by urease-positive and urease-negative *H. pylori*

Group	Strain	*n*	Sacrifice interval (days)	pH[a]	Log_{10} CFU/g	No. of pigs colonized/ total
A	N6	3	2	1.7 ± 0.6	4.4 ± 1.5	3/3
B	N6	3	5	ND[b]	6.9 ± 0.5	3/3
C	N6*ureG*::Km	4	2	1.5 ± 0.4	0	0/4
D	N6*ureG*::Km	3	5	2.3 ± 0.3	0	0/3
E	N6+N6*ureG*::Km	7	2	2.3 ± 1.0	3.9 ± 1.2	7/7

[a] pH at sacrifice as measured by pH probe.
[b] ND, not done.

TABLE 2. Colonization of achlorhydric piglets by urease-positive and urease-negative *H. pylori*

Group	Strain	*n*	Sacrifice interval (days)	pH[a]	Log_{10} CFU/g	No. of pigs colonized/ total
F	N6	4	2	7.1 ± 0.5	6.3 ± 0.3	4/4
G	N6	4	5	8.0 ± 0.4	6.5 ± 0.4	4/4
H	N6*ureG*::Km	5	2	7.1 ± 0.3	0.7 ± 1.0	2/5
I	N6*ureG*::Km	4	5	6.9 ± 0.8	0.8 ± 0.9	2/4

[a] pH at sacrifice as measured by pH probe.

bacterial strains were grown in separate flasks, enumerated by hemacytometer and plate count, and mixed immediately prior to administration. Groups A, C, E, F, and H were sacrificed 2 days after inoculation, and the other groups were sacrificed 5 days after inoculation.

At sacrifice, gastric pH was determined and one-half of the gastric mucosa was homogenized for quantitative culture (6). In piglets inoculated with both strains, colonization of the two strains was determined by making parallel dilutions of gastric homogenate on blood agar plates with 25 μg of kanamycin per ml (to enumerate colonization by N6*ureG*::Km) and without kanamycin (to enumerate colonization by both strains). The other half of the stomach was fixed by immersion in neutral buffered formalin for histologic examination.

Histologic examination. All tissues were fixed in neutral buffered formalin and embedded in paraffin. Six-micrometer sections were stained with hematoxylin and eosin to evaluate presence of gastritis. Gastritis was determined by grading of histologic sections. Lymphocytic and neutrophilic inflammation was graded as follows: 1 (focal infiltration), 2 (mild, diffuse infiltration), 3 (mild diffuse and moderate focal infiltration), 4 (moderate, diffuse infiltration), or 5 (moderate, diffuse and marked focal infiltration). Mucosal lymphoid follicles were enumerated. All sections were scored blind without knowledge of their source.

Statistics. Numerical data are expressed as means ± standard deviations. Groups were compared by the Mann-Whitney *U* test.

RESULTS

Gastric pH. Treatment with intravenous omeprazole and oral ranitidine produced consistent achlorhydria in neonatal piglets. In preliminary experiments, gastric pH in treated piglets was measured immediately prior to treatment every 6 h. Mean gastric pH in these piglets at all time points was 7.0 ± 1.1. Gastric pH in all piglets was measured at sacrifice. Mean gastric pH in all treated piglets at sacrifice was 7.3 ± 0.6, compared with 1.9 ± 0.5 in untreated piglets at sacrifice.

Colonization in normochlorhydric piglets. *H. pylori* N6 colonized all six untreated piglets, sacrificed either 2 or 5 days after inoculation. Mean colonization in piglets sacrificed 2 days after inoculation was slightly less than in piglets sacrificed 5 days after inoculation (Table 1), because in two piglets colonization was $<10^4$ CFU/g. This difference was not statistically significant, however ($P = 0.100$). Colonization in all other piglets was 10^6 CFU/g or higher. Strain N6*ureG*::Km did not colonize normochlorhydric piglets (Table 1).

Colonization of achlorhydric piglets. Strain N6 colonized all eight achlorhydric piglets sacrificed either 2 or 5 days after inoculation (Table 2). Colonization in all piglets was 10^6 CFU/g or higher, and mean colonization rates did not differ in piglets sacrificed 2 or 5 days after inoculation. In contrast, bacterial strain N6*ureG*::Km was recovered from only four of nine piglets and only at low levels. The number of bacteria in all four piglets was less than 100 CFU/g. There was no difference in the number of bacteria recovered from piglets sacrificed 2 and 5 days after inoculation.

Cocolonization by urease-positive and -negative *H. pylori*. Coinoculation with urease-positive *H. pylori* did not promote colonization by urease-negative *H. pylori*. In piglets inoculated with both strains, only strain N6 colonized. Absence of colonization by strain N6*ureG*::Km was indicated by failure of bacteria to grow on plates containing kanamycin and by positive urease reaction of the bacteria recovered on blood agar plates without kanamycin. The mean colonization rate in coinoculated piglets was 3.9 ± 1.2 CFU/g and ranged from 10^3 to 10^6 CFU/g. It did not differ statistically from the mean colonization rate in piglets inoculated with N6 alone and sacrificed 2 days after inoculation ($P = 0.833$).

Histologic lesions. Histologic gastritis was present in all four piglets colonized by strain N6 and sacrificed 5 days after inoculation. Gastritis was characterized by mild to moderate widespread lymphocytic inflammation (grades 2 to 5; four piglets), mild to moderate neutrophilic inflammation (grades 1 to 2; four piglets), hyperplasia of gastric mucosa-associated lymphoid tissue (three piglets), and globule leukocytes (two piglets). Mild lymphocytic infiltration (grade 2) was present in one piglet inoculated with strain N6*ureG*::Km and sacrificed 5 days later, but bacteria were not isolated from this piglet. Histologic lesions were not present in any other piglets.

DISCUSSION

The results of this study confirm previous findings that urease activity is essential for colonization of normochlorhydric piglets by *H. pylori* (5, 6). Colonization by strain N6 was comparable to colonization by other human strains of *H. pylori* (4, 5), although there was somewhat more variation in colonization rate by strain N6 (10^3 to 10^7 CFU/g of gastric mucosa) than by other strains (10^6 to 10^7 CFU/g of gastric mucosa) (4, 6). In contrast to strain N6, urease-negative N6*ureG*::Km did not colonize normochlorhydric piglets. No bacteria were present in the stomachs of inoculated pigs either 2 or 5 days after inoculation. This finding is compatible with previous studies (5) in which we showed that chemically derived urease-negative *H. pylori* persists in vivo only in low numbers and for less than 2 days (5). The data reported here demonstrate that inability to colonize is characteristic of urease-negative mutants derived by insertional mutagenesis as well as those derived by chemical mutagenesis. Furthermore, the inability of N6*ureG*::Km to colonize suggests that urease activity, and not simply the physical presence of the enzyme, is essential for

colonization in vivo. The urease-negative mutant used, N6*ureG*::Km, has an insertion mutation in the *ureG* gene and produces urease, but it fails to activate the enzyme and thus does not demonstrate urea-splitting activity.

The inability of urease-deficient *H. pylori* to colonize piglets confirms that urease has a major role in promoting colonization, but the mechanism of such promotion has not yet been determined. One suggestion has been that urease activity protects the acid-sensitive bacteria in transit across the gastric lumen to the alkaline pH of the gastric mucus where the bacteria colonize (14). This hypothesis predicts that (i) urease-deficient *H. pylori* should be able to colonize in the absence of gastric acid and (ii) once the urease-negative bacteria reach the alkaline mucus, they should proliferate and colonize at the same level as urease-positive bacteria. The results of this study demonstrate that neither of these predictions occurs. While urease-negative bacteria were isolated from four of nine achlorhydric piglets inoculated with this strain, bacteria were present only at very low levels, less than 100 CFU/g (approximately 100 CFU per stomach), which is just barely at the level of detection. It is likely that these trace numbers of bacteria reflect retention of *H. pylori* in the achlorhydric lumen rather than true colonization by *H. pylori* below the gastric mucus. Wild-type *H. pylori* colonizes the gastric mucus rapidly. Ultrastructural evidence of bacterial cell division in situ is present within 24 h of inoculation (22). While there are probably always some bacteria in the lumen, these are likely not independently detectable by quantitative culture because of the much larger number of bacteria below the gastric mucus. In the case of bacteria which do not colonize, however, a small number of luminal bacteria might be detected because they are the only bacteria present (there is no colonization in the normal location below the mucus). Thus, it is likely that the few urease-negative bacteria detected in achlorhydric piglets represent noncolonizing bacteria retained in the lumen. These bacteria are not present in normochlorhydric piglets given N6*ureG*::Km because the bacteria do not survive in the acid lumen. Because of the much greater total number of bacteria, they are not detected in piglets given N6. Thus, although urease-negative *H. pylori* may survive for several days in low numbers in the lumen of achlorhydric piglets, true colonization does not occur even in the absence of gastric acid.

This suggestion is supported by the fact that there was no difference in colonization 2 and 5 days after inoculation of strain N6*ureG*::Km. If the function of urease were only to protect the bacteria against luminal acid, the urease-negative bacteria would be expected to proliferate to normal levels once they achieved their normal location within the mucus. This did not occur, however, suggesting that pH protection is at best a minor function of *H. pylori* urease activity. Clearly, urease has some other function in promoting proliferation of *H. pylori* below the gastric mucus.

It has also been suggested that a role of urease may be to provide nutrition to *H. pylori*, thus promoting growth by promoting nitrogen metabolism (14). The products of urea metabolism, ammonia and carbonic acid, are highly diffusible. If urease promotes colonization by providing a nitrogen source from the products of urea metabolism, cocolonization with urease-producing bacteria would be expected to promote colonization by urease-deficient bacteria. In this study, promotion of growth of urease-negative bacteria by urease-positive bacteria did not occur, however, suggesting that urease-dependent colonization is not mediated by a diffusible factor and that physical association between urease activity and the bacterium is necessary to allow colonization.

These experiments demonstrate that (i) the primary function of urease is not either pH protection or provision of a soluble nutrient source and (ii) physical association between enzyme and bacterium is necessary to allow colonization. The mechanism by which urease promotes colonization remains unknown, but several potential roles for urease in promoting colonization remain to be investigated. *H. pylori* urease is associated with the bacterial outer membrane (1, 13). It is conceivable that this enzyme functions as an adhesin, promoting both colonization and persistence. This would be consistent with the minimal effect of gastric pH on colonization, the prominence of this enzyme in bacterial metabolism, and the necessity for physical association between urease and bacterium. It is less likely, however, because in this experiment the urease-negative mutant used did express urease enzyme but lacked only urease activity, suggesting that the enzymatic activity rather than the physical presence of urease is necessary for colonization. Another possible role for urease is to create an electrochemical gradient resulting in synthesis of ATP (3, 21). This mechanism is consistent with the requirement that urease be physically associated with the bacteria to promote colonization. Further study is necessary to determine if urease functions as an adhesin or contributes to energy metabolism by *H. pylori*.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants DK39570-01A3 and AI07938-02 from the NIH.

We thank Agnès Labigne for providing the bacterial strains N6 and N6*ureG*::Km. We also thank Judy Younger, Michelle Trumble, and Brian Kessler for excellent animal care.

REFERENCES

1. **Bode, G., P. Malfertheiner, M. Nilius, G. Lehnhardt, and H. Ditschuneit.** 1989. Ultrastructural localisation of urease in outer membrane and periplasm of *Campylobacter pylori*. J. Clin. Path. **42**:778–779. (Letter.)
2. **Bronsdon, M. A., C. S. Goodwin, L. I. Sly, T. Chilvers, and F. D. Schoenknecht.** 1991. *Helicobacter nemestrinae* sp. nov., a spiral bacterium found in the stomach of a pigtailed macaque (*Macaca nemestrina*). Int. J. Syst. Bacteriol. **41**:148–153.
3. **Dick, J. D.** 1990. *Helicobacter* (*Campylobacter*) *pylori*: a new twist to an old disease. Annu. Rev. Microbiol. **44**:249–269.
4. **Eaton, K. A., and S. Krakowka.** 1992. Chronic active gastritis due to *Helicobacter pylori* in immunized gnotobiotic piglets. Gastroenterology **103**:1580–1586.
5. **Eaton, K. A., and S. Krakowka.** 1992. The pathogenesis of urease-dependent colonization of gnotobiotic piglets by *Helicobacter pylori*. Ir. J. Med. Sci. **161**(Suppl. 10):8.
6. **Eaton, K. A., D. R. Morgan, C. L. Brooks, and S. Krakowka.** 1991. Essential role of urease in the pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect. Immun. **59**:2470–2475.
7. **Eaton, K. A., M. J. Radin, J. G. Fox, B. J. Paster, F. E. Dewhirst, S. Krakowka, and D. R. Morgan.** 1991. *Helicobacter acinonyx*, a new species of *Helicobacter* isolated from cheetahs with gastritis. Microbial Ecol. Health Dis. **4**(special issue):S104.
8. **Ferrero, R. L., V. Cussac, P. Courcoux, and A. Labigne.** 1992. Construction of isogenic urease-negative mutants of *Helicobacter pylori* by allelic exchange. J. Bacteriol. **174**:4212–4217.
9. **Ferrero, R. L., V. Cussac, P. Courcoux, and A. Labigne.** 1993. Construction of isogenic mutants of *Helicobacter pylori* deficient in urease activity, p. 179–182. *In* G. Gasbarrin and S. Pretolani (ed.), Basic and clinical aspects of *H. pylori* infection. Springer-Verlag, Berlin.
10. **Ferrero, R. L., and A. Labigne.** 1993. Organization and expression of the *Helicobacter pylori* urease gene cluster, p. 171–195. *In* C. S. Goodwin and B. W. Worley (ed.), *Helicobacter pylori* biology and clinical practice. CRC Press, Boca Raton, Fla.
11. **Fox, J. G., T. Chilvers, C. S. Goodwin, N. S. Taylor, P. Edmonds, L. I. Sly, and D. J. Brenner.** 1989. *Campylobacter mustelae*, a new

species resulting from the elevation of *Campylobacter pylori* subsp. *mustelae* to species status. Int. J. Syst. Bacteriol. **39**:301–303.
12. **Goodwin, C. S., J. A. Armstrong, T. Chilvers, M. Peters, M. D. Collins, L. I. Sly, W. McConnell, and W. E. S. Harper.** 1989. Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter pylori* and *Helicobacter mustelae* comb. nov., respectively. Int. J. Syst. Bacteriol. **39**:397–405.
13. **Hawtin, P. R., A. R. Stacey, and D. G. Newell.** 1990. Investigation of the structure and localization of the urease of *Helicobacter pylori* using monoclonal antibodies. J. Gen. Microbiol. **136**:1995–2000.
14. **Hazell, S. L.** 1990. Urease and catalase as virulence factors of *Helicobacter pylori*, p. 3–14. *In* H. Menge et al. (ed.), Helicobacter pylori. Springer-Verlag, Berlin.
15. **Hu, L.-T., and H. L. T. Mobley.** 1990. Purification and N-terminal analysis of urease from *Helicobacter pylori*. Infect. Immun. **58**:992–998.
16. **Krakowka, S., D. R. Morgan, W. G. Kraft, and R. D. Leunk.** 1987. Establishment of gastric *Campylobacter pylori* infection in the neonatal gnotobiotic piglet. Infect. Immun. **55**:2789–2796.
17. **Lee, A., E. Hegedus, J. O'Rourke, and H. Larsson.** 1992. Urease inhibitors not effective for treatment of helicobacter infection but do prevent colonisation. Ir. J. Med. Sci. **161**(Suppl. 10):4. (Abstr.)
18. **McNulty, C. A. M., J. C. Dent, A. Curry, J. S. Uff, G. A. Ford, M. W. Gear, and S. P. Wilkinson.** 1989. New spiral bacterium in gastric mucosa. J. Clin. Pathol. **42**:585–591.
19. **Morgan, D. R., R. Freedman, C. E. Depew, and W. G. Kraft.** 1987. Growth of *Campylobacter pylori* in liquid media. J. Clin. Microbiol. **25**:2123–2125.
20. **Paster, B. J., A. Lee, J. G. Fox, F. E. Dewhirst, L. A. Tordoff, G. J. Fraser, J. L. O'Rourke, N. S. Taylor, and R. Ferraro.** 1991. Phylogeny of *Helicobacter felis*, sp. nov., *Helicobacter mustelae*, and related bacteria. Int. J. Syst. Bacteriol. **41**:31–38.
21. **Romano, N., G. Tolone, F. Ajello, and R. LaLicata.** 1980. Adenosine 5′-triphosphate synthesis induced by urea hydrolysis in *Ureaplasma urealyticum*. J. Bacteriol. **144**:830–832.
22. **Rudmann, D. G., K. A. Eaton, and S. Krakowka.** 1992. Ultrastructural study of *Helicobacter pylori* in gnotobiotic piglets. Infect. Immun. **60**:2121–2124.
23. **Solnick, J. V., J. O'Rourke, A. Lee, B. J. Paster, F. E. Dewhirst, and L. S. Tompkins.** 1993. An uncultured gastric spiral organism is a newly identified *Helicobacter* in humans. J. Infect. Dis. **168**:379–385.