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Gastric Penetration of Amoxicillin in a Human *Helicobacter pylori*-Infected Xenograft Model

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The delivery of antibiotics into *Helicobacter pylori*-infected human stomachs is still poorly understood. Human embryonic gastric xenografts in nude mice have recently been proposed as a new model for the study of *H. pylori* infection. Using this model, we compared the penetration of amoxicillin, after intraperitoneal administration of a dose of 20 mg/kg of body weight, into the gastric mucosae of infected and uninfected xenografts. The concentrations of this drug in serum and superficial gastric mucosae were determined at 20 min and 1 and 3 h after injection. Ten mice with *H. pylori*-infected grafts ($n = 5$) or uninfected grafts ($n = 5$) were studied. Mucosal samples were obtained by cryomicrotomy. The concentrations in serum were similar to those obtained in the serum of humans after oral administration of 1 g of amoxicillin. The mean area under the tissue concentration-versus-time curve from 0 to 3 h obtained for mice with infected grafts was significantly higher than that obtained for the animals with uninfected grafts ($P = 0.01$). These results suggest that the penetration of amoxicillin into the superficial gastric mucosa may be substantially increased in the case of *H. pylori* infection. Thus, human xenografts in nude mice represent a new, well-standardized model for investigation of systemic delivery of drugs into *H. pylori*-infected gastric mucosa.

In vitro, *Helicobacter pylori* is naturally susceptible to most antibiotics. Unfortunately, when administered to a human, no single-antibiotic therapy is able to achieve a high eradication rate (1, 10, 20, 27, 32, 34). This lack of clinical efficacy may be explained by acquired resistance, poor compliance, insufficient antibiotic penetration into the site of infection, and/or a low level of drug stability at this location. The relative ineffectiveness of administration of single antibiotics has empirically led to the use of triple therapies that consist of combinations of two antibiotics (amoxicillin, clarithromycin, or imidazoles) with an antisecretory drug (proton pump inhibitor or H_2-receptor antagonist) and that have been shown to be the most effective (8). However, today, these recommended therapies do not result in eradication in all patients. The search for optimal *H. pylori* treatment was essentially based on the results of a great number of clinical trials. Until now, no pharmacological approach has been systematically used to improve existing therapeutic regimens or to search for new treatments. This may be explained by the absence of a convenient and suitable experimental model (18). The study of gastric penetration of antibiotics should ideally be performed with patients with *H. pylori* infection. However, gastric pharmacokinetic studies with humans have involved the use of gastric biopsy specimens. These biopsy specimens may include deep, vascularized layers, which increases the risk of contamination of the specimen with drugs from the systemic circulation, and do not target precisely the ecological niche of *H. pylori*. Moreover, these studies with humans may also be difficult for ethical reasons. On the other hand, extrapolation to humans of pharmacokinetic results obtained with uninfected guinea pigs, which is the only animal model that has been used to study the gastric penetration of antibiotics (35–37), remains difficult. An in vitro model consisting of gastric mucosae obtained from rats and mounted in Ussing chambers has recently been developed (12). This is a convenient model for investigation of the characteristics of local or systemic delivery of drugs to the stomach, although it may have a poor correlation to the situation in humans, particularly when systemic delivery is predominant. In such a model, it may be difficult to mimic the in vivo pharmacokinetics observed in humans. Moreover, the use of human gastric mucosa infected with *H. pylori* would be more appropriate for extrapolation to humans.

The severe combined immunodeficient mice, in which human fetal thymic and liver tissues have been implanted, was recently proposed as a model for determination of the in vivo effectiveness of different therapeutic agents on immunodeficiency virus infection (28). We have previously developed a new model of *H. pylori* infection in nude mice using human gastric xenografts which exhibit differentiated human gastric epithelium (19). The aim of this work was to investigate the usefulness of this model for the study of amoxicillin penetration into the infected human stomach after parenteral administration.

MATERIALS AND METHODS

***H. pylori* infection model.** Ten pangenic, 6- to 8 week-old, Swiss nude mice purchased from Iffa Credo (Lyon, France) were used. They were housed in individual cages, fed a commercial rodent diet, and given water ad libitum. All animal experimentation was performed in accordance with the institutional guidelines and approval of the Service Vétérinaire de la Santé et de la Protection Animale (Direction Générale de l'Alimentation du Ministère de l'Agriculture et de la Forêt).

This model has previously been described in detail (19). Briefly, human embryonic stomachs (gestational age, 6 to 8 weeks) were obtained after legal abortion. They were stored at 4°C in a sterile isotonic glucose solution and within 4 h were grafted into mice that were under general anesthesia induced with ketamine (Ketalar; Parkes-Davies, Courbevoie, France) administered intraperi-

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toneally (0.1 g/kg of body weight). Anesthesia could be prolonged if required by repeated administration of ketamine (one-fourth of the initial dose every 20 min). Mice were placed in a sterile environment and were subjected to surgery under aseptic and microsurgical conditions. The skin of the abdominal wall was opened at the midline by a xiphopubic incision and was then loosened from the underlying musculoaponeurotic layer. The anterior aponeurosis was opened, and the musculus rectus abdominis was detached from the epigastric vessels and the parietal peritoneum. A pouch was built between the epigastric vessels and the parietal peritoneum at the back and the abdominal muscle layer in front. The entire stomach, which measured about 3 by 2 by 1 mm, was introduced into this cavity in such a way that its back was in close contact with the epigastric vessels. The pouch and the abdominal wall were then closed with successive single-layer sutures. All 10 stomach implants were successful.

Eighty days after implantation, mice were anesthetized as described above. The abdominal skin was disinfected and then opened. The human stomach, which measured at this time about 2 by 2 by 3 cm, was punctured, and the gastric juice was aspirated. The gastric wall was opened, and a reference biopsy specimen was taken for histological examination (hematoxylin-eosin) to ensure that all grafts exhibited human gastric epithelium. A Silastic catheter with an outer diameter of 600 μm (Lambert Rivière, Fontenay-sous-Bois, France) was introduced into the stomach, which was then closed with interrupted sutures. The catheter was slid under the thoracic skin and came out at the nape of the neck, to which it was securely attached. The observance of rigorous standards of hygiene permitted maintenance of the catheter for 3 months. Thus, gastric juice aspiration could be performed through the catheter twice a day (1 to 1.5 ml/day) during the whole experimental time to avoid fistulization.

At 1 to 3 days after catheter implantation, bacterial challenge was performed. The catheterized graft of each animal was aspirated and gastric juice was sampled for pH determination (pHG-1 pHmeter; Physitemp Instruments Inc., Clifton, N.J.). This permitted us to ensure that the gastric juice was acid, since the pH ranged from 1.5 to 2 for all grafts studied. Five randomly assigned grafts were inoculated, through the gastric catheter, two times at 3-day intervals with 0.6 ml of a bacterial suspension (approximately 10^8 organisms/ml in tryptose soy broth [Oxoid, Basingstoke, United Kingdom]) of *H. pylori* LB1, which was originally isolated from a patient with duodenal ulcer and severe gastritis (19). Three months after inoculation, each animal was anesthetized as described above. After disinfection and incision of the abdominal wall, each graft was microsurgically opened and two biopsy specimens were taken from adjacent sites in the gastric antrum for culture and histology. Finally, the gastric and the abdominal walls were closed. One biopsy specimen was fixed in 10% (wt/vol) buffered formalin (16 to 24 h) for histological examination, and the second was immediately placed in a semisolid agar transport medium (Portagerm pylori; bioMérieux, Marcy l'Etoile, France) for culture. This sample was transferred to 0.5 ml of brucella broth (Difco, Detroit, Mich.) and was homogenized for 1 min with an Ultra Turrax grinder (Labo-Moderne, Paris, France) before inoculation onto selective and nonselective agars (19). Formalin-fixed specimens were processed by standard methods, embedded in paraffin, sectioned, stained with hematoxylin-eosin, and examined for histopathological changes. All inoculated xenografts were considered infected on the basis of positive culture results. In these grafts, widespread erythematous areas were visible at the surface of the antrum and were associated with minimal hemorrhagic points. No visible gastric erosions or ulcerations were seen. Histological examination of the gastric mucosa showed mild inflammation and polymorphonuclear leukocyte infiltration. Mucosal edema was always present and was associated with capillary dilatation and proliferation. In contrast, in the other five uninoculated and culture-negative xenografts, macroscopic and histological examination revealed no abnormalities.

Study design and sampling. The pharmacokinetic study was performed 3 to 5 days after the evaluation of the infection. At this time, mice with infected (mean ± standard deviation [SD] weight, 30.5 ± 3.76 g) and uninfected (mean ± SD weight, 29.8 ± 4.31 g) xenografts were anesthetized, and a catheter in Teflon was microsurgically placed in the femoral artery for blood collection. The grafts were then microsurgically opened as described above, and gastric juice was taken for pH determination. For the kinetic study, animals were maintained under anesthesia. Each mouse was given a single intraperitoneal dose of amoxicillin (20 mg/kg). This permitted attainment, as observed in a preliminary study (unpublished data), of a maximum measured concentration in serum (measured C_{max}) and an area under the serum concentration-versus-time curve from 0 to 3 h (AUC_{0-3}) close to those calculated from data obtained for humans after the administration of a 1-g single oral dose of amoxicillin (6). Blood samples (50 to 100 μl) were collected prior to dosing and at 20 min and 1 and 3 h after intraperitoneal administration. All blood samples were immediately centrifuged at 1,600 × *g* at 4°C. The serum was then removed and was stored at −80°C until analysis. Concomitantly, large gastric antral biopsy specimens (4 by 4 by 1 mm) were surgically obtained from areas devoid of any hemorrhagic lesions and were rinsed in 0.1 M phosphate buffer (pH 7.5). Then, the superficial gastric mucosa was immediately removed at a depth of 300 μm by standardized cryomicrotomy at −20°C (Cryomicrotome HM 500M; Microm, Francheville, France) as described previously (17), with sections of 3 μm put into preweighed Eppendorf caps. The caps containing the mucosal material was reweighed without delay and were stored at −80°C until drug assays. The weights varied between 10 and 20 mg.

TABLE 1. Concentrations of amoxicillin in serum of mice with uninfected or infected xenografts

Time post-injection[a]	Mean ± SD concn (μg/ml) in mice with:		*P* value[b]
	Uninfected xenografts (n = 5)	Infected xenografts (n = 5)	
20 min	18.76 ± 5.57	19.03 ± 2.16	>0.05
1 h	13.92 ± 4.03	12.12 ± 2.29	>0.05
3 h	5.14 ± 2.28	4.52 ± 2.05	>0.05

[a] After administration of 20 mg/kg intraperitoneally.
[b] Determined by using the paired Student *t* test.

Drug assay. (16). All chemicals and solvents used were of high-performance liquid chromatography (HPLC) grade. Titrated powder of sodium amoxicillin (SmithKline Beecham Laboratories, Nanterre, France) was dissolved in ultrapure water to give a stock solution of 100 mg/liter. The latter was further diluted in ultrapure water and human blank pooled serum (1:9 [vol/vol]) to obtain calibration standards with concentrations of 0.1, 1, and 10 μg/ml for the determination of concentrations in serum. For the determination of concentrations in the mucosa, standard solutions with concentrations of 0.1, 0.5, and 5 μg/ml were prepared in 0.1 M phosphate buffer (pH 7.5).

Two hundred microliters of either the standard amoxicillin solutions or diluted unknown samples was mixed with an equal volume of acetonitrile in 5-ml screw-cap glass tube. After vortex mixing for 15 s and shaking by rotation (20 rpm) for 10 min, the samples were centrifuged at 1,600 × *g* for 10 min at 4°C. The supernatant was transferred to another screw-cap glass tube, and 2 ml of methylene chloride was added. After shaking and centrifugation as described above, the upper aqueous layer was removed before injection into the HPLC system.

Gastric mucosal material was suspended in 200 μl of 0.1 M phosphate buffer (pH 7.5). After vortex mixing for 5 min, this solution was kept at 4°C for 2 h. Then, mucosal samples as well as standard solutions were processed in the same way described above for serum samples.

The isocratic HPLC system consisted of a 110 A solvent delivery module (Beckman, Fullerton, Calif.), a model 210 sample injection valve equipped with a 50-μl sample loop (Beckman), and a model 160 variable-wavelength detector (Beckman). Chromatograms were processed with a Beckman recording data processor with Gold, version 6.01, software. Separations were performed on a high-speed analytical column (inner diameter, 75 by 4.6 mm) packed with 3-μm-diameter particles (Ultrasphere XL-ODS; Beckman). The mobile phase consisted of 2 M ammonium acetate–0.1 M tetrabutylammonium–acetonitrile–water (0.75:5:13:81.25 [vol/vol/vol/vol]) adjusted to pH 7.5 with sodium hydroxide. The flow rate was set at 1 ml/min, and the eluent was monitored at 227 nm.

Serum and tissue samples obtained before dosing were used as blank samples. For serum and gastric mucosa, the standard curves displayed excellent linearity, and r^2 values generally exceeded 0.999. The interassay reproducibility was assessed by using the standard solutions. The between-group variances, as determined with concentrations of 0.1, 1, and 10 μg/ml for control serum samples and 0.1, 0.5, and 5 μg/ml for control mucosal samples, were 1.8, 2, 4.5, 2, 3.5, and 5%, respectively. The lower limit of quantitation was 0.01 μg/ml for serum.

Pharmacokinetic analysis. The measured C_{max} was obtained by direct observation of the individual kinetic profiles. The AUC_{0-3} was calculated by using the trapezoidal rule and included all datum points obtained for serum or mucosa. Results are given as mean ± SDs. Mean values were compared by the paired Student *t* test (Stat.ITCF 5 software; Institut Technique des Céréales et des Fourrages, Paris, France). A *P* value of less than 0.05 was considered significant.

RESULTS

As shown previously (19), the gastric juice pH was consistent with that found in *H. pylori* infection since it was increased in all infected xenografts (pH range, 5 to 7.5) and remained low in uninfected xenografts (pH range, 1 to 2). No macroscopic blood contamination was evidenced in mucosal or gastric juice samples.

In mice with uninfected xenografts, amoxicillin concentrations in serum decreased from 18.76 ± 5.57 μg/ml at 20 min to 5.14 ± 2.28 μg/ml at 3 h (Table 1). These concentrations were not statistically different from those observed at the same time in mice with infected xenografts. The mean measured C_{max} in the serum of mice with infected (19.03 ± 2.16 μg/ml) or uninfected (19.12 ± 5.10 μg/ml) xenografts (Table 2) were similar to those observed in humans after administration of a 1-g oral single dose of amoxicillin (19.7 ± 5.4 μg/ml) (6). C_{max} was

TABLE 2. Pharmacokinetic parameters for serum and human mucosa from mice with uninfected and infected xenografts[a]

Graft infection status	Serum		Gastric mucosa		AUC_{0-3} for mucosa/ AUC_{0-3} for serum
	AUC_{0-3} (μg · h/ml)	C_{max} (μg/ml)	AUC_{0-3} (μg · h/ml)	C_{max} (μg/ml)	
Uninfected ($n = 5$)	33.29 ± 8.85	19.12 ± 5.10	10.69 ± 3.70	5.14 ± 1.89	0.31 ± 0.09
Infected ($n = 5$)	30.04 ± 5.65	19.03 ± 2.16	22.58 ± 5.10	11.94 ± 2.75	0.75 ± 0.07
P value[b]	>0.05	>0.05	0.01	>0.05	0.01

[a] Data are means ± SDs.
[b] Determined by the paired Student *t* test.

reached in serum at 20 min in all mice except for the serum of one mouse with an uninfected xenograft, which exhibited a C_{max} at 1 h. Moreover, the mean AUC_{0-3}s observed for the serum of both groups of mice (infected mice, 30.04 ± 5.65 μg · h/ml; uninfected mice, 33.29 ± 8.85 μg · h/ml) were similar to the mean AUC_{0-3}s calculated from data obtained for humans after administration of the same oral dose mentioned above (AUC_{0-3}, 36.94 μg · h/ml) (6).

The highest concentrations of amoxicillin in superficial gastric mucosa were measured in the samples of either the uninfected or infected xenografts taken at 1 h except for one graft in each group (taken at 20 min) (Table 3). Amoxicillin concentrations in the uninfected antral mucosae were significantly lower than those observed in serum at 20 min ($P = 0.0003$) and at 1 h ($P = 0.004$), with ratios of the concentration in the mucosa to that in serum (mucosa/serum ratio) at the two times ranging from 0.07 to 0.29 and 0.15 to 0.48, respectively. In the infected grafts, the mean concentration in serum was significantly higher than the mean concentration in the mucosa at 20 min ($P = 0.005$). Mean amoxicillin concentrations in the mucosa were not statistically different ($P = 0.7$) from those observed in serum at 1 h in the infected group (range of mucosa/serum ratios, 0.60 to 1.16) and at 3 h in both groups (infected group, $P = 0.3$ [range of mucosa/serum ratios, 0.77 to 1.50]; uninfected group, $P = 0.1$ [range of mucosa/serum ratios, 0.41 to 1.20]). In infected xenografts the mean concentrations in the mucosa were at least twofold higher than those in uninfected xenografts at each time point. The mean of the AUC_{0-3} for mucosa to that for serum (mucosa/serum AUC_{0-3} ratio was also significantly higher for infected xenografts than for uninfected xenografts ($P = 0.01$) (Table 3).

DISCUSSION

Until now only a few studies of gastric penetration of antibiotics have been performed with *H. pylori*-infected patients (5, 11, 33). This may be partially explained by the fact that in these studies the number and the nature of regimens that can be ethically used are limited. Other in vivo studies have always been performed with uninfected animals (35–37). However, gastric *H. pylori* infection may modify the gastric penetration of drugs by altering the mucus, the mucosal circulation, and/or the pH gradients in the stomach (14, 29, 30). This renders necessary the use of *H. pylori*-infected models for the study of the gastric pharmacokinetics of antibiotics. Thus, the current model represents an interesting new alternative to pharmacokinetic studies with humans. However, in experimental infection in animals, particularly rodents, it is well known that the drug kinetics differ from those observed in humans (4). Thus, the half-lives of β-lactam antibiotics are significantly longer in humans than in animals (31). In our model, the use of the intraperitoneal route (20 mg/kg) allowed us to circumvent this difference in half-life and resulted in mean C_{max} and AUC_{0-3} values for serum similar to those observed for the serum of humans after oral intake of 1 g of amoxicillin. Nevertheless, because the sampling period lasted for only 3 h, it cannot be concluded that half-lives are actually identical in humans and the animal model. Only C_{max} and the AUC_{0-3} are similar. Additional sampling times would have resulted in more precise profiles of the pharmacokinetics of amoxicillin in mice. However, in our study, the number of blood samples was limited in order to avoid a substantial decrease in the total blood volume of the animals, which would have affected the pharmacokinetic behavior of amoxicillin.

In our study, the mean amoxicillin concentrations observed in the gastric superficial mucosa ranged from 2.05 to 5.06 and from 4.92 to 10.14 μg/g in uninfected or infected grafts, respectively. These values are lower than those reported in human gastric mucosa (entire mucosal biopsy specimens) obtained 47 min to 2 h (15 to >322 μg/g) after the administration of a single 500-mg oral dose of amoxicillin (21). This may be due to the fact that when amoxicillin is given orally, its concentrations in the gastric mucosa may reflect both local absorption and diffusion from the systemic circulation. Another explanation for this difference may be that the concentration measured by using biopsy specimens may correspond to the amoxicillin concentration present locally and that present in the systemic circulation since biopsy specimens may also include the deep, vascularized layers of the gastric wall. In the stomach, *H. pylori* lives in the mucus layer and also adheres to gastric epithelial cells, especially at the intercellular junctions. Moreover, it has been shown that *H. pylori* may invade epithelial cells in vivo (3, 19, 25). The superficial gastric mucosa may therefore more adequately represent this microniche. Westblom et al. (35) used scraping with a glass slide to remove this portion from the stomach of adult guinea pigs to study the intragastric penetration of clindamycin. However, the distance between the luminal surface of the gastric mucosa and the muscularis mucosa may vary in the same stomach because of gastroplication. Freezing allows better stretching of the gastric

TABLE 3. Concentrations of amoxicillin in superficial gastric mucosa of uninfected and infected xenografts and mucosa/serum ratios

Time post-injection[a]	Mean ± SD concn (μg/g) in mucosa in:		Mucosa/serum ratio (range) in:	
	Uninfected xenografts	Infected xenografts	Uninfected xenografts	Infected xenografts
20 min	2.05 ± 1.04	7.98 ± 5.35	0.07–0.29	0.11–0.76
1 h	5.06 ± 2.02	10.14 ± 2.90[b]	0.15–0.48	0.60–1.16
3 h	2.91 ± 1.01	4.92 ± 2.19	0.41–1.20	0.77–1.50

[a] After intraperitoneal administration of 20 mg/kg.
[b] Statistically significantly different from mean concentration in uninfected grafts (paired Student's *t* test).

mucosa, which minimizes gastroplication. This may explain why cryomicrotomy may represent a more reproducible way to obtain gastric superficial mucosa (17). However, in guinea pigs, pharmacokinetic studies were performed with the superficial mucosa of the whole stomach, which permitted retrieval of enough material to detect even low levels of antibiotics, but one animal was killed at each time point. In our study, the pharmacokinetics of amoxicillin in the gastric mucosa have been studied at all time points with the same animal. The use of large biopsy specimens was necessary to be able to detect concentrations above the detection threshold, and so specimens from only a limited number of time points could be studied.

Antibiotics are usually given orally to eradicate *H. pylori*, but they may act after local or subsequent systemic delivery. The latter would play a role in therapeutic efficacy (18). Our results suggest that this penetration may be enhanced by *H. pylori* infection, since the concentrations of amoxicillin in the mucosa were significantly higher in mice with infected xenografts than those with in uninfected xenografts. This could also be due to contamination of mucosal samples with blood, the risk of which would be increased by the important neoangiogenesis observed in all the infected xenografts. However, this seems unlikely or at least negligible since no macroscopic blood contamination was detected at the time of sampling and since cryomicrotomy prevents any significant contamination from interstitial tissue and plasma (17). Amoxicillin is unstable at normal gastric pH (pH 1 to 2) (9). Thus, the lower concentrations observed in the superficial mucosa of uninfected grafts, in which the gastric juice pH was low, may be due to the hydrolytic degradation of amoxicillin in vivo but also ex vivo for acid-containing samples. However, all biopsy specimens were immediately rinsed with phosphate buffer (pH 7.5) and frozen. This renders any ex vivo degradation unlikely. The increased amoxicillin concentrations in the infected mucosa may be due to enhanced diffusion because of local vessel proliferation, capillary dilatation, and/or a better stability of amoxicillin at higher intraluminal pH.

Irrespective of the infection status, the concentrations of amoxicillin in the mucosa were always above the reported MICs at which 90% of isolates are inhibited (0.06 to 0.25 μg/ml) for *H. pylori* at neutral pH (13, 15) and were also at least 10-fold higher than the minimal bactericidal concentration (0.1 μg/ml), as determined by Mégraud et al. (22). Some investigators (2, 22) have suggested that the bactericidal activity of amoxicillin against *H. pylori* may be concentration dependent. Thus, the high concentrations obtained at 1 h in the gastric mucosa may be sufficient to obtain a good bactericidal effect. If our results were extrapolated to humans, they would suggest that the lack of eradication observed after therapies with amoxicillin in combination with proton pump inhibitors (26) would not be explained by low levels of antibiotic penetration but would more likely be explained by amoxicillin resistance (7) or other not well known mechanisms, including the possible intracellular localization of *H. pylori* (3, 19, 25). However, as the nature of the bactericidal effect of amoxicillin against *H. pylori* is still controversial (23, 24), it remains hazardous to draw any conclusion about this point and the nature of the effect requires further studies.

Our model permits study of the gastric penetration of xenobiotic agents into infected human gastric mucosa. In this study, it has been applied to the study of the systemic delivery of amoxicillin. However, it could also be used to study the local delivery of drugs in the superficial gastric mucosa, the penetration of antibiotics into tissue, and the effects of antibiotics against *H. pylori* in vivo. Therefore, it represents a new well-standardized model for the investigation of new anti-*H. pylori* agents.

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