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Eradication of *Helicobacter mustelae* from the Ferret Stomach: an Animal Model of *Helicobacter (Campylobacter) pylori* Chemotherapy

GLEN OTTO, JAMES G. FOX,* PAI-YEN WU, AND NANCY S. TAYLOR

Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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Colonization of the ferret stomach by *Helicobacter mustelae* has been suggested as a possible animal model for *Helicobacter pylori*-associated gastroduodenal disease of humans. Our study was designed to determine whether antimicrobial chemotherapy could eradicate *H. mustelae* from ferrets. Triple antimicrobial therapy combining amoxicillin, metronidazole, and bismuth subsalicylate was successful in eradicating the organism from 5 of 7 (71%) adult ferrets. Despite apparent in vitro susceptibility, neither chloramphenicol monotherapy nor a polytherapeutic regimen combining tetracycline, metronidazole, and bismuth subsalicylate proved effective in the eradication of *H. mustelae*. Several strains isolated after unsuccessful polytherapy showed markedly increased resistance to metronidazole. These preliminary findings are similar to results of *H. pylori* treatment trials with humans and suggest that the ferret may be a useful model for evaluating and comparing potential antimicrobial modalities for the eradication of *H. pylori*.

The organism previously known as *Campylobacter pylori* is a spiral bacterium which colonizes the human stomach and is associated with chronic active gastritis (16) and the relapse of duodenal ulcers (3, 24). The organism has recently been transferred to a new genus and is now named *Helicobacter pylori* (15). Numerous clinical trials have been undertaken in an attempt to identify a therapeutic regimen effective in eradicating the organism. Although results with any single drug have been poor, the use of multiple drug combinations has been more promising (4). Triple combinations with bismuth compounds in conjunction with two systemic antibiotics have been the most successful in eradication (3; G. Börsch, U. Mai, and W. Opferkuch, Gastroenterology **94**:A44, 1988). Attempts to compare the results of *H. pylori* treatment trials have proven difficult, especially since a variety of different physical or chemical forms of the active drugs have been used (29; C. A. M. McNulty, I. A. Eyrebrook, J. S. Uff, J. C. Dent, and S. P. Wilkinson, Program Abstr. 5th Int. Workshop Campylobacter Infect., abstr. no. 73, 1989).

An animal model of *H. pylori* chemotherapy would be of great benefit for evaluating therapeutic approaches and gaining further understanding of the factors involved in the successful eradication of gastric organisms (25). *Helicobacter (Campylobacter) mustelae* (9, 15) is a closely related organism which colonizes the ferret stomach in association with gastritis and ulcer disease, leading to the proposal of ferret stomach colonization by *H. mustelae* as an animal model of *H. pylori* infection (8, 11). The organism is an exceptionally efficient colonizer, with an approximate prevalence of 100% in many ferret populations (8, 35; L. J. Jeffries, D. E. Buckley, P. R. Blower, and J. N. Plumb, Letter, J. Clin. Pathol. **40**:1265–1267, 1987). The purpose of this study was to investigate the use of antimicrobial compounds in an effort to eradicate *H. mustelae* from the ferret stomach.

* Corresponding author.

MATERIALS AND METHODS

Animals. Fifteen domestic ferrets (*Mustela putorius furo*) were divided into three treatment groups prior to antimicrobial treatment. Animals in groups A and C were obtained from a commercial vendor (Marshall Farms; North Rose, N.Y.), while animals in group B were born in our facility. During and after therapy, animals were housed singly in stainless-steel cages, with the exception of pregnant jills, which were kept with their offspring after parturition. Animals were maintained in compliance with the standards of the American Association for Accreditation of Laboratory Animal Care, fed a commercial cat food diet once a day, and given water ad libitum.

Endoscopy. Gastric endoscopy and pinch biopsy were performed on anesthetized animals with a 5-mm-diameter flexible pediatric bronchoscope (Pentax Model FB-15H; Asahi Optical, Tokyo, Japan). Ferrets were premedicated with atropine at a dose rate of 0.04 mg/kg injected intramuscularly prior to being anesthetized with a ketamine-xylazine combination (40 and 3.0 mg/kg intramuscularly, respectively). Yohimbine (0.5 mg/kg intramuscularly; Antagonil; Wildlife Laboratories, Fort Collins, Colo.) was used to partially reverse anesthesia upon completion of the procedure (34a).

Urease assay. The presence of urease in mucosal samples was determined by a microwell urease test previously described for the rapid diagnosis of *H. pylori* (18). The use of this test to detect *H. mustelae* in the ferret has been validated (12). Biopsies were considered positive if test wells underwent a change to a dark-pink color within 24 h.

Microbiology. Colonization with *H. mustelae* was determined by gastric culture pretherapy or posttherapy or both. Gastric mucosal samples obtained at necropsy or by endoscopy were transported and homogenized in 0.5 ml of 0.01 M phosphate-buffered saline, plated on blood-based media, and incubated microaerophilically for 3 to 5 days as previously described (8). A minimum of one antral and one fundic biopsy was cultured from each ferret undergoing endoscopy or necropsy.

Antibiotic susceptibility assay. An agar dilution method

was used to determine the MICs of the antibiotics used in this study for selected *H. mustelae* strains. Bacterial cells were harvested from 72-h blood agar subcultures, suspended in 0.01 M phosphate-buffered saline, and adjusted to an optical density at 660 nm of 0.1. This suspension was inoculated onto agar plates by using a replicating system previously described (7). The media used was brucella agar (Difco Laboratories, Detroit, Mich.) containing 5% sheep blood and twofold dilutions of the antibiotics to be tested. A human isolate of *H. pylori* (BU23) and the type strain of *H. mustelae* (ATCC 43772) served as control strains. Growth was assessed after microaerophilic incubation at 37°C for 72 h.

Immunology. Serology was performed on sera from selected ferrets. An enzyme-linked immunosorbent assay (ELISA) was utilized to identify *H. mustelae*-specific immunoglobulin G in serum samples. Whole-cell sonic extracts from three *H. mustelae* strains were used as antigen in the assay, which follows a protocol previously described for *H. pylori* ELISA (10). Serum samples were tested at a dilution of 1:50 and were considered positive if their measured reactivity against *H. mustelae* antigen was ≥3 standard deviations above the mean of negative controls. The assay is specific for *H. mustelae* antibody in ferret serum (J. G. Fox, P. Correa, N. S. Taylor, A. Lee, G. Otto, J. C. Murphy, and R. Rose, Gastroenterology, in press).

Treatment groups. Group A (n = 3) consisted of 5-month-old ferrets which had been affected by a chronic enteritis diagnosed as proliferative colitis (13). They were given a course of chloramphenicol therapy appropriate for the treatment of this disease (21). An oral suspension of chloramphenicol palmitate was administered at a dose rate of 50 mg/kg twice daily for a period of 3 weeks. Therapy resulted in complete clinical recovery in all three ferrets. The animals were biopsied for bacterial culture 1-week posttherapy to determine the effect of chloramphenicol therapy on *H. mustelae* colonization.

Group B was a litter of 4-month-old ferrets from a colony known to have a high *H. mustelae* prevalence rate. Control animals (n = 2) were euthanized prior to any antimicrobial treatment, and their stomachs were cultured for *H. mustelae*. Experimental animals (n = 7) were given an oral polytherapeutic regimen which was formulated by using established small-animal drug dosages and which was based on a triple-therapy protocol which successfully eradicated *H. pylori* from humans (3). The combination consisted of tetracycline hydrochloride (25 mg/kg), metronidazole (20 mg/kg), and bismuth subsalicylate (2.1 mg/kg; Pepto-Bismol chewable tablets; Procter & Gamble, Cincinnati, Ohio). The metronidazole and bismuth subsalicylate tablets were crushed, mixed with the powdered tetracycline, and placed in a gelatin capsule (gelatin capsules no. 4; Eli Lilly & Co., Indianapolis, Ind.) for oral dosing. The drug combination was administered three times daily for 4 weeks (with the exception that metronidazole was discontinued after the first 10 days). One treated ferret was euthanized with carbon dioxide and was cultured for *H. mustelae* 2 weeks posttherapy. The remaining six animals were biopsied for bacterial culture at 2 weeks and 6 months posttherapy.

The triple antimicrobial combination was modified for use in group C, which consisted of pregnant jills in the last trimester (n = 5). Pregnant animals were used in an effort to obtain specific pathogen-free offspring (results to be discussed elsewhere). Since tetracycline is contraindicated during gestation, an oral amoxicillin suspension was substituted (10 mg/kg). The dose rate of metronidazole (20 mg/kg) was not changed, but the tablet was crushed, mixed with a palatable nutritional supplement (Nutri-cal; Evsco, Buena, N.J.), and given as an oral paste for the entire length of treatment. A liquid form of bismuth subsalicylate was used (Pepto-Bismol original formula; Procter & Gamble) and, after comparison to a published human pediatric dose (6), the ferret dose rate was increased to 17.5 mg/kg. All drugs were given three times daily for 3 (n = 2) or 4 (n = 3) weeks, beginning 2 weeks before the predicted parturition date. Animals in group C were biopsied for bacterial culture and bled from the jugular vein for serology at 72 h posttherapy. Those found to be culture negative for *H. mustelae* were subjected to follow-up endoscopy for bacterial culture and urease assay at 1, 2, 3, 4, and 6 months posttherapy.

Two of the previously treated ferrets from group A were subsequently placed on the amoxicillin-metronidazole-bismuth combination for 3 weeks. These animals were biopsied prior to therapy and again at 72 h posttherapy. Animals negative at this point were rebiopsied at 2 and 3 months posttherapy.

RESULTS

For the purposes of this study, the inability to isolate *H. mustelae* at a point 72 h to 2 weeks posttherapy was considered clearance. Eradication was defined as the elimination of the organism (as determined by negative mucosal urease assay and negative isolation attempts) for a period 8 weeks or more posttherapy, similar to the terminology used in human *H. pylori* eradication trials (T. Borody, P. Cole, A. Morgan, G. Ossip, J. Maysey, and S. Brandl, Gastroenterology **94**:A43, 1988).

Adverse effects from drug treatment were not observed in any of the treated animals or their offspring, nor were complications from endoscopic biopsy encountered. Administration of the oral compounds was successfully performed with a minimum of time and effort.

Chloramphenicol therapy had no detectable effect upon *H. mustelae* colonization. The organism was cultured from 3 of 3 ferrets in group A 1 week posttherapy. These results illustrate the high prevalence of *H. mustelae* in ferrets even at an early age.

Both control animals from group B cultured prior to the beginning of the experiment were positive for *H. mustelae*. At 2 weeks after tetracycline-metronidazole-bismuth treatment, the organism was not cultured from the gastric mucosa of the ferret which had been euthanized but was cultured from 5 of 6 animals biopsied. When biopsied 6 months posttherapy, all six remaining ferrets (including the previously culture-negative animal) were colonized with *H. mustelae*, indicating that eradication was not accomplished.

All animals given the amoxicillin-metronidazole-bismuth combination were colonized prior to therapy as determined by serology or biopsy. Serum samples from all six of the pregnant jills in group C were positive for anti-*H. mustelae* immunoglobulin G when tested by ELISA, indicative of prior colonization with *H. mustelae*. Four of the five animals were cleared of *H. mustelae* when cultured 72 h after cessation of the amoxicillin-metronidazole-bismuth combination. Periodic follow-up biopsies from these four jills remained negative by both bacterial culture and urease assay throughout the 6-month posttherapy evaluation period. The two additional ferrets (from group A) placed on the amoxicillin-metronidazole-bismuth combination were culture positive and urease assay positive for *H. mustelae* when biopsied immediately prior to therapy. One ferret remained

TABLE 1. MICs for control strains and for *C. mustelae* isolates obtained after or before antimicrobial therapy

Source of isolate (no. of isolates)	Antimicrobial agent	MIC for individual isolates[a] (μg/ml)
C. pylori BU23[b]	Amoxicillin	0.025
	Chloramphenicol	0.5
	Tetracycline	0.04
	Metronidazole	0.4
C. mustelae R85-13-6P[b]	Amoxicillin	3.2
	Chloramphenicol	2.0
	Tetracycline	4.0
	Metronidazole	3.2
Postchloramphenicol treatment (3)	Chloramphenicol	2.0
Post-TMB[c] treatment (4)	Tetracycline	2.0_2, 4.0_2
	Metronidazole	6.4, 51.2_3
Pre-AMB[d] treatment, ferret 3A	Amoxicillin	0.8
	Metronidazole	3.2
Post-AMB treatment, ferret 3A	Amoxicillin	1.6
	Metronidazole	51.2

[a] The inferior number is the number of isolates with the MIC indicated.
[b] Control strains.
[c] Tetracycline-metronidazole-bismuth subsalicylate.
[d] Amoxicillin-metronidazole-bismuth subsalicylate.

colonized (positive culture and urease assay) when biopsied 72 h posttherapy, but the other was cleared. The latter ferret remained negative as determined by urease assay and culture when rebiopsied at 2 and 3 months posttherapy. These results indicate that the oral amoxicillin-metronidazole-bismuth combination resulted in the eradication of *H. mustelae* from 5 of 7 (71%) of the ferrets treated.

H. mustelae strains subjected to MIC determination included posttherapy isolates from the three group A ferrets and four of the group B ferrets. Both a pretherapy and a posttherapy isolate were characterized from one animal (ferret 3A) which failed to clear after treatment with the amoxicillin-metronidazole-bismuth combination. The MICs for the strains evaluated are shown in Table 1. The control isolate of *H. mustelae* was susceptible to all of the antibiotics tested, although the MICs were higher than those for the control *H. pylori* strain. Posttherapy isolates of *H. mustelae* showed similar antibiotic susceptibilities with the exception that four of the strains tested were metronidazole resistant. The pretherapy and posttherapy isolates from ferret 3A showed a 4-dilution increase in the MIC of metronidazole (3.2 to 51 μg/ml), demonstrating that this resistance was acquired during the unsuccessful treatment period.

DISCUSSION

There are a number of similarities between our findings and the results of *H. pylori* treatment trials in humans. Chloramphenicol possesses extreme stability and excellent distribution into body tissues and secretions (34). Despite these characteristics and the apparent in vitro susceptibility of *H. mustelae* in our study, oral chloramphenicol monotherapy failed to clear the organism. This is consistent with human trials where monotherapy with a variety of agents did not successfully eradicate *H. pylori* (14, 23, 26–28, 30, 33). An additional similarity between *H. mustelae* and *H. pylori* chemotherapy is the development of marked resistance to metronidazole associated with treatment failure. Acquired resistance to metronidazole and the related drug tinidazole has been documented in *H. pylori* therapy trials (17, 19).

The 71% successful *H. mustelae* eradication rate of the amoxicillin-metronidazole-bismuth regimen falls within the range of long-term triple-therapy eradication success for *H. pylori* reported in humans, which varies from 65 to 94% (3, 32; Börsch et al., Gastroenterology **94**:A44, 1988; McNulty et al., Program Abstr. 5th Int. Workshop Campylobacter Infect., abstr. no. 73, 1989). Endoscopy for preculture was contraindicated in the five pregnant ferrets, but they were known to be *H. mustelae* positive on the basis of their ELISA results. Positive ELISA results indicate active *H. mustelae* colonization (Fox et al., in press); we have never encountered spontaneous clearance of *H. mustelae* from a ferret stomach with our current isolation protocols. Similarly, spontaneous clearance of naturally occurring *H. pylori* in the human rarely, if ever, occurs (22, 32).

No eradication was seen with the tetracycline-metronidazole-bismuth regimen. A number of differences exist between the successful and the unsuccessful polytherapy combinations used in our study. Liquid preparations were used in the successful combination, and it has been suggested that such formulations may show higher efficacy against gastric organisms than do capsules or tablets because of more uniform mucosal distribution locally (14, 29). In addition, the dose of bismuth subsalicylate was increased and the duration of metronidazole administration was prolonged. The final change which may have had an effect was the substitution of amoxicillin for tetracycline in the successful regimen. The relative importance of each of these differences can only be determined by further comparative trials in the ferret.

The results in our in vitro antibiotic susceptibility tests suggest that *H. mustelae* possesses less inherent susceptibility to certain antibiotics as compared with *H. pylori*, similar to the findings of an earlier report (Jeffries et al., Letter, J. Clin. Pathol. **40**:1265–1267, 1987). However, although the MICs for *H. mustelae* are relatively high when compared with those for *H. pylori*, the MICs for our control and pretreatment *H. mustelae* strains fell within the range considered to indicate susceptibility to the antibiotics commonly utilized in formulating *H. pylori* triple-therapy regimens. In addition, it has been shown that the MIC of bismuth is equivalent for *H. mustelae* and *H. pylori* (as the tripotassium dicitrato bismuthate form; Jeffries et al., Letter, J. Clin. Pathol. **40**:1265–1267, 1987). The successful eradication of *H. mustelae* in this study supports our rationale for the use of the ferret in future chemotherapy trials.

There are a number of other proposed animal models for *H. pylori*-associated human disease, each with its own advantages and disadvantages. Natural or experimental *H. pylori* colonization has been described in nonhuman primates (2, 5) and gnotobiotic piglets (20; J. Lambert, M. Borromeo, K. Pinkard, H. Turner, C. B. Chapman, and M. L. Smith, Letter, J. Infect. Dis. **155**:1344, 1987). A mouse model using a closely related non-*H. pylori* urease-positive microaerophilic gastric spiral has also recently been described (A. Lee, J. G. Fox, G. Otto, and J. Murphy, Klin. Wochenschr. **67**[Suppl. XVIII]:39–40, 1989). No single animal model is superior in all aspects, but we believe that the ferret possesses certain advantages for drug therapy experiments. The anatomic and physiologic similarities of the ferret stomach to that of the human have previously been established (1, 31). The gastric colonization and pathology associated with *H. mustelae* in the ferret are similar to that seen with humans (Fox et al., in press). Ferrets are readily available, easy to maintain, and relatively inexpensive (8). In our experience, oral dosing is straightforward and the animals tolerate anesthesia and endoscopy well. In addition, we

have demonstrated that *H. mustelae* can be eradicated from the ferret stomach by using antimicrobial regimens known to be effective in eradicating *H. pylori* from the human stomach. For these reasons, we believe that the *H. mustelae*-colonized ferret is a practical and relevant animal model of *H. pylori* chemotherapy and we propose the oral amoxicillin-metronidazole-bismuth combination used in this study as a standard regimen against which other potential anti-*Helicobacter* treatment protocols may be evaluated.

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