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Appearance of a Metronidazole-Resistant *Helicobacter pylori* Strain in an Infected-ICR-Mouse Model and Difference in Eradication of Metronidazole-Resistant and -Sensitive Strains

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We tested whether antibiotic-resistant strains appeared in vivo after the failure of treatment using the *Helicobacter pylori*-infected euthymic mouse model. The numbers of colonies isolated from 56 ICR mice 2 weeks after 4 days of treatment with metronidazole (3.2, 10, or 32 mg/kg of body weight) or amoxicillin (1, 3.2 or 10 mg/kg), with treatment started 4 days after *H. pylori* CPY2052 inoculation, were counted, and the isolated strains were tested for their sensitivities to two antibiotics to rule out the presence of antibiotic-resistant strains. One metronidazole-resistant strain was detected in a mouse treated with 10 mg of metronidazole per kg, and the MIC of metronidazole for this strain was 25 μg/ml, compared to a MIC of 1.56 μg/ml for the original strain. However, no resistant strain was detected in the amoxicillin treatment group. After the examination described above, mice challenged with a metronidazole-resistant or -sensitive strain isolated from the stomach of a mouse were treated with metronidazole or amoxicillin. The metronidazole-resistant strain was more difficult to eradicate in vivo than the sensitive strain after treatment with metronidazole but not after treatment with amoxicillin. Thus, a metronidazole-resistant *H. pylori* strain was selected by insufficient treatment, but no resistant strain was selected with amoxicillin. Eradication of a metronidazole-resistant *H. pylori* strain in vivo required a higher dosage than eradication of a metronidazole-sensitive *H. pylori* strain. These results may explain one of the reasons for *H. pylori* treatment failure.

Helicobacter pylori is considered to be associated with human gastritis, peptic ulcer, and gastric cancer (4, 12). Gastric and duodenal ulcers are chronic diseases in which frequent recurrences occur even after treatment with an acid-suppressing drug. However, antimicrobial treatment for the eradication of *H. pylori* has resulted in a low relapse rate (13, 31). Triple therapy with bismuth, amoxicillin (AMPC), and metronidazole (MNZ) has been a "gold standard" regimen, and omeprazole plus two antibiotics including AMPC, clarithromycin, or MNZ are standard regimens that also have high eradication rates (1, 3, 5, 27). However, it is reported that the presence of antibiotic-resistant *H. pylori* strains pre- and posttreatment resulted in treatment failure (9–11, 14, 22, 29).

Some microbes acquired resistance to MNZ with clinical use (17, 26). MNZ-resistant *H. pylori* exists at a high frequency worldwide (2, 8, 9). Therefore, the major problem of treatment failure and the difficulty of successful anti-*H. pylori* therapy seem to be strongly associated with MNZ-resistant strains. Despite its importance, studies of MNZ resistance with experimental animals have not been performed, and the mechanism of MNZ resistance is not yet known.

Several animal models of *H. pylori* infection are available; these include gnotobiotic piglets (21), athymic mice (18), germfree euthymic mice (19), euthymic mice (23), monkeys (30), and Mongolian gerbils (16, 24). We have also used a normal euthymic mouse model (20, 25) to study the pathogenesis of *H. pylori* or the actions of drugs in vivo. This mouse model has been shown to be a model of the continuous inflammation induced by *H. pylori* infection (18, 19, 25).

The purpose of this study was to investigate whether MNZ-resistant strains appeared in the ICR mouse model and whether a resistant strain is associated with the difficulty of anti-*H. pylori* therapy in vivo.

(Results of this study were presented in part at the 8th Workshop of the European *Helicobacter pylori* Study Group, Edinburgh, Scotland, 7 to 9 July 1995.)

MATERIALS AND METHODS

Animals, bacterial strain, growth conditions, and antibiotics. Four-week-old, specific-pathogen-free male ICR mice (Nippon SLC, Hamamatu, Japan) were used in this study. The mice were housed in animal facilities and were given food and water ad libitum. The study described below was approved by the Animal Experiment Committee of Fujisawa Pharmaceutical Co., Ltd. *H. pylori* CPY2052 was isolated from a patient with gastritis (18) and was identified by morphology; Gram staining; urease, oxidase, and catalase activities; and sensitivity to nalidixic acid and cephalothin. The strains isolated from the mice were stored at −70°C in brucella broth (Becton Dickinson and Company, Cockeysville, Md.) containing 15% glycerol and were cultured in brucella broth containing 10% fetal bovine serum for 24 to 30 h at 37°C under 10% CO_2 with shaking (120 rpm). MNZ (Aldrich Chemicals, Milwaukee, Wis.) and AMPC (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) were used in all studies.

Experimental design to test whether an MNZ-resistant strain appears. The experimental schedule used in this study was designed as described in Fig. 1. A total of 1.5 ml of 2×10^8 CFU of the *H. pylori* strain per ml was orally inoculated into euthymic ICR mice which had been fasted overnight. Four days after inoculation, 56 mice were orally treated with MNZ (at 3.2, 10, or 32 mg/kg of body weight) or AMPC (at 1, 3.2, or 10 mg/kg) (8 mice in each group) for 4 days twice a day. A control group of mice was not treated with any drug. All drugs were suspended in 0.5% (wt/vol) methylcellulose. The eight mice in each group were sacrificed 2 weeks after the final treatment (Fig. 1). The gastric mucosa was scraped and homogenized in 1 ml of 0.1 mol of phosphate-buffered saline per liter. Aliquots of 0.1 ml were inoculated onto a brucella agar plate containing 3% (vol/vol) horse serum, 2% (wt/vol) starch, Skirrow's antibiotics (Unipath Ltd., Basingstoke, United Kingdom), 10 μg of nalidixic acid (Nacalai Tesque Inc., Osaka, Japan) per ml, and 30 μg of bacitracin (Sigma Chemical Co., St. Louis,

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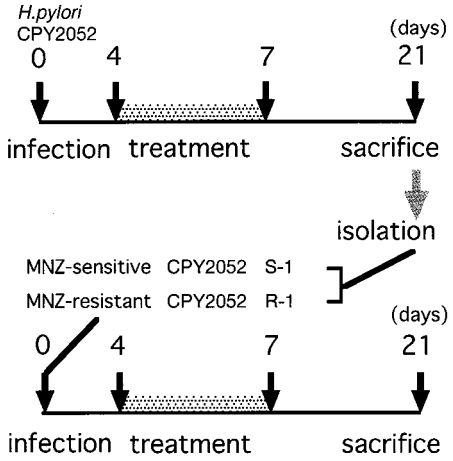

FIG. 1. Experimental design used in the study. Fifty-six mice were challenged with *H. pylori* CPY2052. In the first experiment, mice were treated with MNZ (3.2, 10, or 32 mg/kg), AMPC (1, 3.2, or 10 mg/kg), or no drug (control) for 4 days (group) (eight mice in each treatment group). Colonies were counted 2 weeks after the treatment. After determination of the MICs of AMPC and MNZ, strain S-1 was isolated from a control mouse as an MNZ-sensitive strain and strain R-1 was isolated from a mouse treated with 10 mg of MNZ per kg and was used as the MNZ-resistant strain. A parallel experiment was performed with both an MNZ-sensitive strain (strain S-1) and an MNZ-resistant strain (strain R-1). In the second experiment, MNZ (10, 32, or 100 mg/kg) or AMPC (0.32, 1, or 3.2 mg/kg) was used as the treatment.

Mo.) per ml. All plates were incubated at 37°C under 10% CO_2 for 5 days. Colonies were counted, and strains reisolated from each plate were grown on the brucella agar plates containing 3% (vol/vol) horse serum and 2% (wt/vol) starch and were stored at −70°C prior to testing the sensitivity of MNZ or AMPC. All colonies were identified as having urease activity by phenol red indicator paper, which was soaked in 0.01 mol of phosphate buffer per liter containing 10% (wt/vol) urea and 0.01% (wt/vol) phenol red.

Antibiotic susceptibility testing of *H. pylori*. MICs were determined by the agar dilution method. Brucella agar supplemented with 7% (vol/vol) horse blood was used. The original *H. pylori* CPY2052 strain and strains isolated from euthymic ICR mice were grown on the brucella agar plates containing 3% (vol/vol) horse serum and 2% (wt/vol) starch at 37°C under 10% CO_2 for 2 days. They were then suspended in brucella broth to a turbidity approximately matching that of a 1.0 McFarland standard, and 10-fold diluent of suspension in brucella broth was inoculated onto agar plates containing serial twofold dilutions of drugs prior to incubation at 37°C under 10% CO_2 for 3 days. The MICs were defined as the lowest drug concentration required to inhibit visible growth. A strain with acquired resistance was defined as having more than fourfold greater resistance to drugs compared to the level of resistance of isolates from control mice in which *H. pylori* CPY2052 was inoculated but that were not treated with MNZ or AMPC.

Challenge of both MNZ-resistant and -sensitive strains to the mice and evaluation of the efficacy of MNZ in challenged mice. One MNZ-resistant strain (strain R-1) was isolated from MNZ-treated mice by the method described above. One strain was selected from the control group not treated with of MNZ or AMPC and was used as an MNZ-sensitive strain (strain S-1). To test whether it was more difficult to eradicate in vivo the strain resistant to MNZ in vitro than the MNZ-sensitive strain, a parallel experiment was performed with both strains by the method described above (Fig. 1). MNZ (at 10, 32, or 100 mg/kg) or AMPC (at 0.32, 1, or 3.2 mg/kg) was administered to five mice challenged with the MNZ-resistant strain or five mice challenged with the MNZ-sensitive strain. Similarly, we counted the numbers of colonies from the mice challenged with either the MNZ-resistant or -sensitive strain mice after the described treatment (Fig. 1). The MICs of MNZ for the isolated strains were also determined as described above.

Statistical analysis. The eradication ratio was compared statistically by Fisher's exact test. All *P* values were calculated for two-tailed significance levels, and a *P* value of <0.05 was considered significant.

RESULTS

Evaluation of efficacy of MNZ or AMPC in vivo. *H. pylori* CPY2052 persistently infected all eight euthymic ICR mice not treated with any drugs (control group) during this experiment. The mean ± standard error number of colonies isolated from the control group was 4.42 ± 0.07 ($\log_{10}$ CFU/gastric mucosa specimen) and was similar to that reported for a previous experiment (25). The infiltration of inflammatory cells in the lamina propria and submucosa was observed in this study by histological examination (data not shown). For the groups to which MNZ was administered, organism eradication was achieved for none of eight (0%) mice in the 3.2-mg/kg group, three of eight (37.5%) mice in the 10-mg/kg group, and all eight (100%) mice in the 32-mg/kg group (Table 1). For the group to which AMPC was administered, organism eradication was achieved for three of eight (37.5%) mice in the 1-mg/kg group, seven of eight (87.5%) mice in 3.2-mg/kg group, and all of eight (100%) mice in the 10-mg/kg group.

TABLE 1. Eradication ratios for mice infected with *H. pylori* CPY2052 and the appearance of MNZ-resistant strain

Drug and dose (mg/kg)	Eradication ratio[a]
MNZ	
32	8/8 (100)
10	3/8[b] (37.5)
3.2	0/8 (0)
AMPC	
10	8/8 (100)
3.2	7/8 (87.5)
1	3/8 (37.5)
Control (0)	0/8 (0)

[a] The eradication ratio represents the number of mice from which *H. pylori* was eradicated/number of mice tested (percent).
[b] CPY2052 R-1 was isolated from one of the five infected mice.

MIC of MNZ or AMPC for strains isolated from MNZ- or AMPC-treated mouse mucosa and appearance of MNZ-resistant strains. We determined the MICs of both antibiotics for the original CPY2052 strain and strains isolated from CPY2052-challenged euthymic ICR mice by the agar dilution method and studied the frequency of resistance among strains isolated from the drug-treated mice. The MICs of MNZ and AMPC for the original CPY2052 strain were 1.56 and 0.05 μg/ml, respectively (Table 2). For strains isolated from MNZ-treated mice (3.2 or 10 mg/kg), AMPC-treated mice (1 or 3.2 mg/kg), and nontreated mice, the MICs of MNZ and AMPC were consistently similar to those for the original strain except for one strain isolated from a mouse treated with MNZ at 10 mg/kg. The MIC for the strain was 25 μg/ml, and we defined this strain as being an MNZ-resistant strain (strain R-1) (Table 2).

Evaluation of MNZ-resistant strains isolated in vivo. The colonization efficiencies of two strains (strains S-1 and R-1) isolated from ICR mice were similar to that for the original

TABLE 2. MICs of MNZ and AMPC for original CPY2052 strain and strains isolated from the gastric mucosa of mice after treatment

H. pylori strain[a]	MIC (μg/ml)	
	MNZ	AMPC
CPY2052	1.56	0.05
S-1	1.56	0.025
R-1	25	0.05

[a] Strain R-1 was isolated from mice treated with MNZ at 10 mg/kg (Table 1). Strain S-1 was isolated from a mouse not treated with drug.

TABLE 3. Eradication ratios for mice infected with *H. pylori* CPY2052 S-1 or CPY2052 R-1

Drug and dose (mg/kg)	Eradication ratio[a]	
	S-1	R-1
MNZ		
100	5/5 (100)	3/5 (60)
32	4/5[b] (80)	0/5[c] (0)
10	2/5 (40)	0/5 (0)
AMPC		
3.2	5/5 (100)	5/5 (100)
1	1/5 (20)	0/5 (0)
0.32	1/5 (20)	0/5 (0)
Control (0)	0/5 (0)	0/5 (0)

[a] The eradication ratio represents the number of mice from which *H. pylori* was eradicated/number of mice tested (percent).
[b] An MNZ-resistant strain was isolated from an infected mouse.
[c] $P < 0.05$.

CPY2052 strain, and the mean ± standard error numbers of colonies of *H. pylori* in the mice infected with strains S-1 and R-1 were 4.39 ± 0.07 and 4.29 ± 0.16 $\log_{10}$ CFU/gastric mucosa sample, respectively. For the group challenged with the MNZ-sensitive strain (strain S-1), organism eradication was achieved for two of five (40%) mice in the 10-mg/kg group, four of five (80%) mice in the 32-mg/kg group, and all five (100%) mice in the 100-mg/kg group. These results were consistent with the findings observed for the group challenged with the original CPY2052 strain. For the group challenged with the MNZ-resistant strain (strain R-1), organism eradication was achieved for none of five (0%) mice in the 10- and 32-mg/kg groups and three of five (60%) mice in the 100-mg/kg group (Table 3). The eradication ratio for mice challenged with the MNZ-resistant strain was significantly lower than that for mice challenged with the MNZ-sensitive strain 2 weeks after treatment with MNZ (32 mg/kg). The efficacy of AMPC in mice challenged with both the MNZ-sensitive strain (strain S-1) and the MNZ-resistant strain (strain R-1) was consistent with the findings observed in the group challenged with the original CPY2052 strain, and there was no significant difference ($P > 0.05$) between mice challenged with the MNZ-resistant and -sensitive strains 2 weeks after treatment with AMPC (0.32, 1, or 3.2 mg/kg). An MNZ-resistant strain was detected in one mouse in the group treated with 32 mg of MNZ per kg after challenge with the MNZ-sensitive strain through this experiment. The MIC of MNZ for this strain was 25 μg/ml.

DISCUSSION

MNZ use has generated resistance in *Trichmonas vaginalis* and *Bacteroides fragilis* during clinical use (17). In the case of *H. pylori*, it was reported that one of the main problems related to treatment failure was associated with MNZ-resistant strains (9–11, 14, 22, 29). To clarify this hypothesis in an animal model, we used the euthymic mouse model to test whether an MNZ-resistant strain appeared and whether the MNZ-resistant strain was more difficult to eradicate than the original strain. In this study, a drug-resistant *H. pylori* mutant was isolated from a mouse treated with 10 mg of MNZ per kg for 2 weeks, but not after AMPC treatment. This result indicated that *H. pylori* quickly develops resistance to MNZ in vivo as well as in vitro (15). In a parallel experiment, 32 mg of MNZ per kg completely eliminated colonization with *H. pylori* organisms, but no efficacy was observed with 3.2 mg of MNZ per kg. Both of these doses of MNZ did not select for an MNZ-resistant *H. pylori* strain. Therefore, insufficient treatment may explain the induction of MNZ-resistant strains in vivo. For organism eradication by AMPC, our inability to detect an AMPC-resistant strain in this study is consistent with information from another clinical report (10). AMPC at 10 mg/kg eradicated *H. pylori* from the mouse stomach. Then, the difference in the doses of MNZ and AMPC in vivo required to achieve 100% eradication was only threefold in comparison with the 30-fold required when assayed by MIC determinations (Table 1).

van Zwet et al. (32) reported that an MNZ-resistant *H. pylori* strain could lose its resistance characteristics by serial passage in vitro. However, our MNZ-resistant *H. pylori* strain continued to exhibit MNZ resistance after passage in vivo and in vitro (data not shown). Therefore, the MNZ-resistant strain may be stable in vivo and in vitro. The mechanisms of MNZ resistance in resistant strains from the mice in this study have not yet been identified (6, 7) and could be the subject of future studies.

The efficacy of MNZ in mice challenged with an MNZ-resistant strain was significantly decreased compared to that in mice challenged with an MNZ-sensitive strain. This result is consistent with the observation that a higher dose of MNZ is required to achieve higher eradication rates and to protect against the appearance of MNZ-resistant strains (28). The data imply that testing for susceptibility to MNZ is necessary before treatment for organism eradication is initiated.

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