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Evaluation of Nitrofurantoin Combination Therapy of Metronidazole-Sensitive and -Resistant *Helicobacter pylori* Infections in Mice

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The main objectives of this study were to determine whether the nitroreductase enzyme encoded by the *rdxA* gene of *Helicobacter pylori* was responsible for reductive activation of nitrofurantoin and whether a triple-therapy regimen with nitrofurantoin was able to eradicate metronidazole-sensitive and -resistant *H. pylori* infections from mice. The susceptibilities to nitrofurantoin of parent and isogenic *rdxA* mutant strains (three pairs), as well as a series of matched metronidazole-sensitive and -resistant strains isolated from mice (30) and patients (20), were assessed by agar dilution determination of the MIC. Groups of mice colonized with the metronidazole-sensitive *H. pylori* SS1 strain or a metronidazole-resistant *rdxA* SS1 mutant were treated with either metronidazole or nitrofurantoin as part of a triple-therapy regimen. One month after the completion of treatment the mice were sacrificed and their stomachs were cultured for *H. pylori*. The nitrofurantoin MICs for all strains tested were between 0.5 and 4.0 μg/ml. There was no significant difference between the susceptibility to nitrofurantoin of the parental strains and those of respective *rdxA* mutants or between those of matched metronidazole-sensitive and -resistant *H. pylori* isolates. The regimen with metronidazole eradicated infection from all eight SS1-infected mice and from one of eight mice inoculated with the *rdxA* mutant ($P \leq 0.001$). The regimen with nitrofurantoin failed to eradicate infection from any of the six SS1-infected mice ($P \leq 0.001$) and cleared infection from one of seven mice inoculated with the *rdxA* mutant. These results demonstrate that, despite the good in vitro activity of nitrofurantoin against *H. pylori* and the lack of cross-resistance between metronidazole and nitrofurantoin, eradication regimens involving nitrofurantoin are unable to eradicate either metronidazole-sensitive or -resistant *H. pylori* infections from mice.

Helicobacter pylori is a gram-negative, microaerobic, spiral bacterium that colonizes the stomachs of approximately one-half the world's population (54). Infection with *H. pylori* is associated with chronic gastritis and peptic ulceration, and the bacterium is also considered a risk factor for the development of gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (3, 41, 42). Although the 5-nitroimidazole metronidazole is an important component of many currently used *H. pylori* eradication regimens, resistance to this class of antibiotics is relatively common. It has been estimated that 10 to 30% of clinical strains isolated in western Europe and the United States are metronidazole resistant, and this prevalence is far higher in developing countries and in certain immigrant populations (11, 13). Although there have been conflicting reports concerning the clinical impact of metronidazole resistance in *H. pylori*, many studies have now demonstrated that resistance to the 5-nitroimidazoles reduces the efficacy of eradication regimens involving metronidazole and is therefore an important predictor of treatment failure (5, 23, 27, 43, 51). Several reports also suggest that the prevalence of metronidazole resistance is rising and is likely to become an increasingly important problem in the clinical management of *H. pylori* infection (34, 52). This, combined with the expense of currently used antimicrobial regimens, means that there is a need to evaluate alternative antibiotics for combination therapy of *H. pylori* infections. We have previously used the *H. pylori* SS1 mouse model to characterize the evolution of metronidazole resistance by *H. pylori* in vivo and to examine the contribution of underlying resistance mechanisms (26, 27). This model system may also be used to assess the efficacy of novel anti-*H. pylori* agents in vivo and to determine optimal regimens for the eradication of resistant strains.

Recently it was demonstrated that loss of oxygen-insensitive NADPH nitroreductase activity resulted in the development of resistance to metronidazole in *H. pylori* (18). It was proposed that this enzyme reduces the nitro group of metronidazole to active metabolites that are toxic to the bacterium and that resistance arose from mutational inactivation of the underlying gene, *rdxA* (HP0954 in the *H. pylori* genome database [49]). Subsequent studies have suggested that, while the development of metronidazole resistance in *H. pylori* is highly associated with mutational inactivation of the *rdxA* gene, other mechanisms of resistance are likely to exist in this bacterium (26, 48; D. H. Kwon, D. Y. Graham, and F. A. K. El-Zaatari, Gut **43**(Suppl. 2)**:**A6).

The nitrofuran group of compounds, which includes furazolidone and nitrofurantoin, appears a particularly promising source of alternative agents for metronidazole in *H. pylori* eradication regimens (7, 46, 57). Like that of the 5-nitroimidazoles, the biological activity of these nitroaromatic compounds is largely derived from reductive metabolism of the parent compound's nitro moiety by oxygen-insensitive nitrore-

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TABLE 1. *H. pylori* strains used in this study

Strain(s)	Characteristics	Reference or source
SS1	*H. pylori* wild-type strain; metronidazole sensitive; colonizes mice	32
G27	*H. pylori* wild-type strain; metronidazole sensitive	8
HAS-141	*H. pylori* wild-type strain; metronidazole sensitive	25
SS1-*rdxA*	*H. pylori* SS1 isogenic *rdxA* deletion mutant	This work
G27-*rdxA*	*H. pylori* G27 isogenic *rdxA* deletion mutant	This work
HAS-141-*rdxA*	*H. pylori* HAS-141 isogenic *rdxA* deletion mutant	This work
SS1-1 to SS1-10	Mouse-derived metronidazole-sensitive *H. pylori* SS1	27
SS1-11 to SS1-19	Mouse-derived metronidazole-resistant *H. pylori* SS1 with defined mutations in *rdxA*	27
SS1-20	Mouse-derived metronidazole-resistant *H. pylori* SS1 with no defined mutation in *rdxA*	27
SS1-21 to SS1-30	Mouse-derived metronidazole-resistant *H. pylori* SS1 (*rdxA* gene not sequenced)	27
T1S/T1R to T10S/T10R	Paired metronidazole-sensitive and -resistant clinical *H. pylori* isolates	48

ductases (2, 36, 55). A number of recent clinical trials have demonstrated that short-term triple therapies with furazolidone are effective in the treatment of *H. pylori* infection (10, 33, 46, 57). Based on nitrofurantoin's in vitro activity, there is evidence that it might also be a suitable alternative agent in combination antimicrobial therapy, particularly against metronidazole-resistant *H. pylori* strains (7). Although nitrofurantoin has failed to eradicate *H. pylori* in a limited number of clinical trials, in all cases the agent was given either as a monotherapy or in combination with bismuth subsalicylate and not as part of a triple-agent regimen (4, 21, 24, 39, 44).

The aims of this study were to (i) determine whether the *rdxA* gene of *H. pylori* is responsible for reductive activation of nitrofurantoin, (ii) evaluate the in vitro activity of nitrofurantoin against a series of matched metronidazole-sensitive and -resistant *H. pylori* isolates, and (iii) determine the efficacy of a triple-therapy regimen with nitrofurantoin in eradicating established metronidazole-sensitive and -resistant *H. pylori* infections from mice.

MATERIALS AND METHODS

Bacteria and growth conditions. *Escherichia coli* strain MC1061 (6) was used as the host for plasmid cloning experiments and was grown at 37°C in L broth (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter, pH 7.0) or on L agar plates (1.5% agar) at 37°C. Antibiotics were used at the following final concentrations: 100 μg of spectinomycin (Upjohn Laboratories, Paris, France) and 25 μg of kanamycin (Serva, Frankfurt, Germany)/ml.

H. pylori strains (Table 1) were routinely cultured on a blood agar medium (blood agar base no. 2 [Oxoid, Lyon, France]) supplemented with 10% horse blood (bioMérieux, Marcy L'Etoile, France) and the following antibiotics: 10 μg of vancomycin (Dakota Pharmaceuticals, Creteil, France)/ml, 2.5 IU of polymyxin (Pfizer Laboratories, Orsay, France)/liter, 5 μg of trimethoprim (Sigma Chemicals, Saint-Quentin Fallavier, France)/ml, and 4 μg of amphotericin B (Bristol-Myers Squibb, Paris, France)/ml. The plates were incubated at 37°C under microaerobic conditions in an anaerobic jar (Oxoid) with a carbon dioxide generator (CampyGen; Oxoid) without a catalyst. For the selection of metronidazole- and nitrofurantoin-resistant colonies and their subsequent subculture, the medium was additionally supplemented with 8 μg of metronidazole (Sigma) or nitrofurantoin (Sigma)/ml, respectively. *H. pylori* cells that had undergone chromosomal allelic exchange were selected on medium supplemented with 25 μg of kanamycin.

To determine viable counts of *H. pylori*, samples to be tested were serially diluted in sterile saline and then plated in duplicate onto blood agar plates supplemented with either 10% horse blood or fetal calf serum (Gibco BRL, Cergy Pontoise, France) and 10 g of agar (bacteriological agar no. 1; Oxoid)/liter, 200 μg of bacitracin/ml, and 10 μg of nalidixic acid (Sigma)/ml. After 5 days of incubation, colonies with *H. pylori* morphology were identified using standard criteria (morphology on Gram staining and the presence of catalase, oxidase, and urease enzyme activities) and enumerated (14).

General molecular-biology techniques and construction of a defined mutation in the *H. pylori rdxA* gene. The alkaline lysis procedure was used for small-scale plasmid preparation (45). MIDI columns (Qiagen, Courtaboeuf, France) were used for large-scale plasmid preparation. Genomic DNA from individual *H. pylori* strains was extracted using the QIAamp tissue kit (Qiagen) according to the manufacturer's instructions. Standard procedures for cloning and DNA analysis were used (45).

H. pylori strains with a defined mutation in the *rdxA* gene were generated by allelic exchange. For this purpose, a recombinant plasmid was constructed in *E. coli* MC1061 as follows. Oligonucleotide primers were designed to amplify a fragment of 510 bp from the 5′ end (HP0954-1 and HP0954-2) and 490 bp from the 3′ end (HP0954-3 and HP094-4) of the *rdxA* gene (Table 2). The two generated fragments were restricted with *Eco*RI and *Bam*HI and with *Pst*I and *Bam*HI, respectively, and were cloned simultaneously into the plasmid vector pILL570-1 (30) linearized with *Eco*RI and *Pst*I. The resulting plasmid was restricted with *Bam*HI, and the *Bam*HI-digested kanamycin cassette from pILL600 (31) was introduced to generate the final construct.

Resultant recombinant plasmids were introduced into *H. pylori* for allelic exchange by natural transformation. *H. pylori* strains were naturally transformed with circular plasmid DNA (~2 μg per transformation) using a modification of the technique of Wang et al. (53). Briefly, bacteria were inoculated as 1-cm patches and grown for 5 h before the addition of 10 μl of supercoiled plasmid DNA. After further incubation for 18 h, the bacteria from each individual patch were harvested and plated directly onto a single plate of selective medium containing 25 μg of kanamycin/ml.

To determine whether natural transformation of *H. pylori* was followed by allelic replacement of the intact chromosomal gene by the mutated gene, chromosomal DNA was isolated from paired *H. pylori* parent and mutant strains. Genotypic analysis was performed by PCR using a single pair of oligonucleotide primers that flanked the point of insertion of the antibiotic resistance cassette (HP0954-1 and HP0954-4) (Table 2).

Susceptibility testing. Susceptibility to metronidazole and nitrofurantoin was assessed by agar dilution determination of the MIC. Inoculates yielding 10^4 CFU/spot were inoculated onto plates of IsoSensitest agar (Oxoid) enriched with 10% horse blood containing doubling dilutions of metronidazole or nitrofurantoin. The MIC was defined as the lowest concentration of antibiotic inhibiting growth when the plates were read after 72 h of incubation under microaerobic conditions (generated as described above) at 37°C. Isolates were considered resistant to nitrofurantoin or metronidazole if the MIC of either was ≥8 μg/ml (56).

Infection of mice with *H. pylori* SS1. Six-week-old specific-pathogen-free Swiss mice (Centre d'Elevage R. Janvier, Le-Genest-St-Isle, France) were housed in polycarbonate cages in isolators and fed a commercial pellet diet with water ad libitum. All animal experimentation was performed in accordance with institutional guidelines. Mice were inoculated intragastrically with a suspension of either *H. pylori* SS1 ($n = 24$; Table 4) or the *rdxA* mutant, SS1-11 ($n = 25$), which had been harvested directly from 48-h plate cultures into peptone-trypsin broth (Organotéchnique, La Courneuve, France). SS1-11 is a mouse-derived *H. pylori* SS1 isolate that is resistant to metronidazole (MIC of 32 μg/ml) and whose *rdxA* gene contains frameshift mutations at positions 90 and 159, resulting in the creation of two translational stop codons within the gene (26, 27). Each animal was administered a single 100-μl aliquot of an inoculating suspension of 10^5 CFU/ml (equivalent to 100 times the 100% infectious dose [14]) on two consecutive days. This was administered with polyethylene catheters (Biotrol, Paris,

TABLE 2. Oligonucleotide primers used for PCR and cloning

Primer	Oligodeoxynucleotide sequence (5′–3′)[a]
HP0954-1	ggaattcCTGATTGTGGTTTATGGTTTGGGG
HP0954-2	gcggatccATAGAGATTTTGCATGTAGTGGCCG
HP0954-3	gcggatccCTGTGGGGCAAATTTGCATGGGCG
HP0954-4	aaaactgcagAATCCCTAAATATTTATTATTAACAGGG

[a] Underlining, *Eco*RI (GAATTC), *Bam*HI (GGATCC), and *Pst*I (CTGCAG) sites; lowercase letters, nucleotides that were added to the 5′ end to create a restriction site.

France) attached to 1-ml disposable syringes. A control group of mice ($n = 10$) was given peptone-trypsin broth alone.

Antimicrobial chemotherapy. Mice were administered antimicrobial chemotherapy 7 weeks after infection (Table 4). All solutions were administered intragastrically in a final volume of 100 μl via polyethylene catheters as previously described. The *H. pylori* SS1-colonized mice in group 1 ($n = 10$) and the *H. pylori* SS1-11-colonized mice in group 2 ($n = 10$) were treated for 7 days with peptone-trypsin broth. The *H. pylori* SS1-colonized mice in group 3 ($n = 8$) and the *H. pylori* SS1-11-colonized mice in group 4 ($n = 8$) were treated for 7 days with the mouse body weight equivalent of a recommended *H. pylori* eradication regimen of 20 mg of omeprazole (0.0086 mg; Astra Hassle AB, Mölndal, Sweden), 250 mg of clarithromycin (0.107 mg; Abbott Laboratories, Saint-Rémy-sur-Avre, France), and 400 mg of metronidazole (0.171 mg; Rhône-Poulenc Rorer, Vitry sur Seine, France) twice daily for 1 week (12). The *H. pylori* SS1-colonized mice in group 5 ($n = 6$) and the *H. pylori* SS1-11-colonized mice in group 6 ($n = 7$) were treated for 7 days with the mouse body weight equivalent of 20 mg of omeprazole (0.0086 mg), 250 mg of clarithromycin (0.107 mg), and 200 mg of nitrofurantoin (0.086 mg; Proctor & Gamble Pharmaceuticals, Staines, United Kingdom) twice daily for 1 week.

Assessment of *H. pylori* infection in mice. Colonization with *H. pylori* was assessed 1 month after the completion of each treatment regimen as recommended by recent guidelines (56). The animals were sacrificed, the stomach of each mouse was removed, and serum was recovered in microtubes (Sarstedt France, Orsay, France). The presence of *H. pylori* infection was determined by biopsy urease, quantitative culture, and serology. Stomachs were washed in physiological buffered saline and divided longitudinally into tissue fragments so that each fragment contained the cardia, body, and antrum. For each stomach, one fragment was immediately placed in urea-indole medium and another was immediately placed in peptone-trypsin broth. The presence of urease activity in tissue fragments was detected in urea-indole medium incubated for 24 h at room temperature (14). For the performance of quantitative bacterial cultures on stomach samples, tissue fragments were homogenized in peptone-trypsin broth using disposable plastic grinders and tubes (PolyLabo, Strasbourg, France). The homogenates were serially diluted in sterile saline and plated directly onto blood and serum plates for enumeration and onto selective plates containing 8 μg of either metronidazole or nitrofurantoin/ml. To increase the sensitivity of detection of metronidazole- and nitrofurantoin-resistant strains, all colonies that grew on the two enumeration plates were pooled and subcultured onto plates containing 8 μg of either metronidazole or nitrofurantoin/ml, respectively. *H. pylori* colonies were identified using standard criteria and were enumerated as described above.

Serum samples were tested for the *H. pylori* antigen-specific immunoglobulin antibody by a previously described enzyme-linked immunosorbent assay technique (14). Briefly, 96-well Maxisorb plates (Nunc, Kamstrup, Denmark) were coated with 25 μg of a sonicated whole-cell extract of *H. pylori* SS1. Serum samples were diluted 1:100 and were added in 100-μl aliquots to coated microtiter wells. To allow for nonspecific antibody binding, samples were also added to uncoated wells. Bound *H. pylori*-specific antibodies were detected by using biotinylated goat anti-mouse immunoglobulin and streptavidin-peroxidase conjugate (Amersham, Les Ulis, France). The readings for uncoated wells were subtracted from those for the respective test samples. A cutoff value was determined from the mean optical density value ± 2 standard deviations for the corresponding samples from naive uninfected mice. Samples with optical density readings greater than this cutoff value were considered positive for *H. pylori*-specific antibodies.

Statistical analysis. Differences in the eradication rates between the groups of mice were determined by Fisher's exact probability test. Differences in bacterial loads were determined by the Mann-Whitney U test (two-sided). A P value of ≤0.05 was considered significant.

RESULTS

Construction of the *H. pylori rdxA* mutant. In order to determine whether the oxygen-insensitive NADPH nitroreductase of *H. pylori*, RdxA, was responsible for susceptibility to nitrofurantoin as well as metronidazole, an isogenic mutant in the *rdxA* gene was constructed in three strains. To do this, a plasmid with the *aphA3* kanamycin resistance gene (50) inserted in *rdxA* was constructed in *E. coli. H. pylori rdxA* mutants derived from strains SS1, G27, and HAS-141 were then produced by allelic exchange following natural transformation with a concentrated preparation of the recombinant plasmid. The genotypes of the constructed mutants were verified by performing PCR with primers that flanked the point of insertion of the antibiotic resistance cassette (HP0954-1 and HP0954-4). The PCR products obtained with genomic DNA of these strains were of the correct size and consistent with insertion of the kanamycin cassette (1,400 bp) and an engineered deletion of 114 bp within *rdxA*: for parental strains SS1, G27, and HAS-141, 1,114 bp; for mutant strains SS1-*rdxA*, G27-*rdxA*, and HAS-141-*rdxA*, 2,400 bp (Fig. 1).

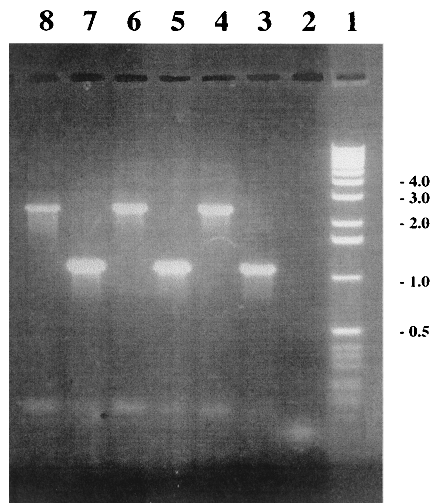

FIG. 1. Genotypic confirmation of the *H. pylori rdxA* mutant by PCR. Lane 1, 1-kb molecular weight marker (Gibco BRL); lane 2, negative control; lanes 3 to 8, SS1, SS1-*rdxA*, G27, G27-*rdxA*, HAS-141, and HAS-141-*rdxA* amplification products, respectively, with primers HP0954-1 and HP0954-4.

In vitro activity of nitrofurantoin against metronidazole-sensitive and -resistant *H. pylori*. To determine if there was cross-resistance between nitrofurantoin and metronidazole in *H. pylori*, the MICs of nitrofurantoin and metronidazole for the *rdxA* mutants and a series of matched metronidazole-sensitive and -resistant strains were determined using an agar dilution method (Table 3). The metronidazole MICs for *rdxA* mutant strains SS1-*rdxA*, G27-*rdxA*, and HAS-141-*rdxA* were significantly higher than those for the respective parental strains (Table 3). Although the MIC for SS1 rose from 0.0625 μg/ml for the parent strain to 2 μg/ml for the mutant, the MIC for the mutant was not sufficiently high for this strain to be considered resistant by standard criteria (56). The susceptibilities to nitrofurantoin of the *rdxA* mutants and respective parental strains were identical (Table 3).

The metronidazole and nitrofurantoin MICs for 10 mouse-derived metronidazole-sensitive SS1 isolates (SS1-1 to SS1-10) (27) were between 0.0625 and 0.125 μg/ml and between 0.5 and 1 μg/ml, respectively. The MICs of metronidazole and nitrofurantoin for 20 mouse-derived metronidazole-resistant SS1 isolates (SS1-11 to SS1-30) (27) were between 8 and 64 μg/ml and between 0.5 and 2 μg/ml, respectively. These strains included 10 in which the *rdxA* gene had previously been se-

TABLE 3. In vitro activity of metronidazole and nitrofurantoin against *H. pylori*

Strain	MIC[a] (μg/ml) of:	
	Metronidazole	Nitrofurantoin
SS1	0.0625	0.5
SS1-*rdxA*	2	0.5
G27	1	1
G27-*rdxA*	64	1
HAS-141	1	1
HAS-141-*rdxA*	64	1

[a] Mean obtained from three independent experiments.

TABLE 4. Comparison of regimens with metronidazole and nitrofurantoin for the eradication of metronidazole-sensitive and -resistant *H. pylori* from mice

Group	No. of mice	Inoculating suspension	Treatment[a]	No. of mice infected with:		
				H. pylori[b]	MTZ-resistant *H. pylori*	NF-resistant *H. pylori*[d]
Control	10	PTB	PTB	0	0	0
1	10	*H. pylori* SS1	PTB	10	0	0
2	10	*H. pylori* SS1-11	PTB	10	10	0
3	8	*H. pylori* SS1	OCM	0[e]	0	0
4	8	*H. pylori* SS1-11	OCM	7[e]	7	0
5	6	*H. pylori* SS1	OCNf	6[e]	0	0
6	7	*H. pylori* SS1-11	OCNf	6	6	0

[a] PTB, peptone-trypsin broth; OCM, omeprazole, clarithromycin, and metronidazole; OCNf, omeprazole, clarithromycin, and nitrofurantoin.
[b] Number of mice infected with *H. pylori* 1 month after the completion of treatment.
[c] Number of mice from which metronidazole (MTZ)-resistant strains were isolated.
[d] Number of mice from which nitrofurantoin (NF)-resistant strains were isolated.
[e] Statistically significant difference ($P < 0.001$).

quenced; in 9 the *rdxA* gene contained one or more mutations, and in 1 the gene sequence was identical to that of the parental SS1 strain (26). The susceptibilities to nitrofurantoin and metronidazole of 10 paired metronidazole-sensitive and -resistant clinical strains were also determined (48). The metronidazole and nitrofurantoin MICs for the 10 metronidazole-sensitive strains (T1S to T10S) were between 0.5 and 2 μg/ml and between 0.5 and 4 μg/ml, respectively. The MICs of metronidazole and nitrofurantoin for the 10 corresponding metronidazole-resistant strains (T1R to T10R) were between 8 and 64 μg/ml and between 0.5 and 4 μg/ml, respectively.

In vivo activity of nitrofurantoin against metronidazole-sensitive and -resistant *H. pylori*. In the control group, none of the 10 mice inoculated with peptone-trypsin broth were infected with *H. pylori* 1 month after the completion of treatment (Table 4). In contrast, all 10 SS1-inoculated mice in group 1 that were treated with peptone-trypsin broth (Table 4) were infected, with bacterial counts of between 2.2×10^4 and 7.0×10^6 CFU/g of tissue. In group 2, quantitative cultures of gastric tissue samples taken from all 10 *rdxA* mutant-inoculated mice 1 month after completion of treatment were positive for *H. pylori* (Table 4). The bacterial counts obtained varied from 2.9×10^3 to 9.1×10^5 CFU/g of tissue. Although the bacterial loads recovered from mice infected with the *rdxA* mutant (group 2) were approximately 10-fold lower than those recovered from mice infected with SS1, this difference was not statistically significant.

A recommended triple-therapy regimen (omeprazole, clarithromycin, and metronidazole) eradicated infection from 100% of mice inoculated with *H. pylori* SS1 (group 3) and from one of eight (12.5%) mice inoculated with the *rdxA* mutant (group 4; $P < 0.001$; Table 4). The bacterial counts in the six mice still infected with the *rdxA* mutant after treatment were similar to those observed in nontreated, *rdxA* mutant-inoculated mice (between 3.5×10^3 and 6.7×10^5 CFU/g of tissue).

In contrast, when metronidazole was replaced by nitrofurantoin, the regimen failed to eradicate infection from any of the SS1-inoculated mice in group 5 ($P < 0.001$; Table 4). In group 6, the regimen with nitrofurantoin eradicated infection in one of seven (14%) mice inoculated with the *rdxA* mutant (Table 4). The bacterial counts in the mice in the groups still infected with either *H. pylori* SS1 or the *rdxA* mutant after treatment were similar to those observed in nontreated mice (between 5.0×10^3 and 5.9×10^6 CFU/g of tissue and between 4.0×10^4 and 6.3×10^5 CFU/g of tissue, respectively).

Metronidazole-resistant *H. pylori* cells were isolated from all *rdxA* mutant-inoculated mice still infected 1 month after the completion of treatment (groups 2, 4, and 6; Table 4). In contrast, none of the SS1-inoculated mice in groups 1, 3, and 5 were infected with metronidazole-resistant isolates (Table 4). None of the mice still infected 1 month after the completion of treatment harbored nitrofurantoin-resistant isolates (Table 4). At the time of sacrifice (1 month), serological testing was not predictive of successful eradication of *H. pylori* (results not shown).

DISCUSSION

Currently the most effective regimens for the eradication of *H. pylori* combine a proton pump inhibitor with two of the following antibiotics: metronidazole, clarithromycin, and amoxicillin (11, 12). It is, however, increasingly recognized that the rising prevalence of resistant *H. pylori* strains, particularly those resistant to metronidazole, threatens to compromise the efficacy of these regimens. Although there has been controversy regarding the clinical relevance of metronidazole resistance, it is now generally accepted that there is a global decrease in the efficacies of treatment regimens involving metronidazole when strains are resistant to this agent (37). This problem has led to the evaluation of a number of compounds with properties similar to those of metronidazole but without the problems of resistance (7, 33, 38). Like that of metronidazole, the bactericidal mechanism of action of the nitrofurans involves enzymatic reduction of the parent compound to generate electrophilic radicals (2, 36). These compounds have good in vitro activity against *H. pylori* (16, 40, 47), and antimicrobial combinations that included nitrofurantoin have been shown to have a greater in vitro bactericidal effect against a metronidazole-sensitive and a metronidazole-resistant strain of *H. pylori* than those with metronidazole (7). In addition, *H. pylori* does not appear to readily acquire resistance to this group of antimicrobial agents (22).

In *E. coli*, resistance to the nitrofuran derivatives occurs in a stepwise manner and results from mutations in genes encoding oxygen-insensitive nitroreductases (*nfsA* and *nfsB*) (35). First-step resistance results from an *nfsA* mutation, while the increased resistance associated with second-step mutants is due to mutation of *nfsB* (55). While there is no homolog of NfsA in the genome sequences of *H. pylori*, NfsB has 22.9% amino acid sequence identity with RdxA, the nitroreductase responsible for reductive activation of metronidazole in *H. pylori* (1, 18, 49, 55). The identity between NfsB and RdxA is particularly high

(71%) in a conserved 14-residue region corresponding to Ser-37 to Val-50 (1, 49, 55). As well as being responsible for susceptibility to metronidazole, the activity of the oxygen-insensitive NADPH nitroreductase encoded by the *rdxA* gene might also be associated with reduction of, and hence susceptibility to, nitrofurantoin in *H. pylori*. In order to test this hypothesis, we constructed an isogenic *rdxA* deletion mutant from three different strains of *H. pylori*. Although the metronidazole MICs for strains SS1, G27, and HAS-141 carrying the mutant *rdxA* were significantly higher than those for the respective parental strains, the MIC for mutant SS1-*rdxA* was not sufficiently raised for this strain to be considered resistant by standard criteria (56). This observation is unlikely to result from residual RdxA activity and provides indirect evidence for additional mechanisms of metronidazole resistance in *H. pylori* which may increase the degree of resistance in an additive, stepwise fashion. The nitrofurantoin MICs for the *rdxA* mutants and respective parental strains were identical, suggesting that this enzyme is not responsible for the reductive activation of nitrofurantoin in *H. pylori* and that inactivation of *rdxA* does not result in resistance to this antimicrobial agent. Whether nitrofurantoin is reduced by one of the other putative nitroreductases identified in *H. pylori* remains to be determined. Alternatively, the mechanism of action in this organism may not require production of reactive nitrofurantoin metabolites by a bacterial reductase.

To confirm these observations and to determine whether other mechanisms of cross-resistance to metronidazole and nitrofurantoin might exist in *H. pylori*, we examined the MICs of metronidazole and nitrofurantoin for a series of well-characterized strains of *H. pylori*. These included a group of 10 metronidazole-sensitive and 20 metronidazole-resistant isolates generated in vivo by treating mice infected with the metronidazole-sensitive SS1 *H. pylori* strain with various regimens involving metronidazole (27). Of the 20 resistant isolates, 9 were known to contain mutations within the *rdxA* gene. In one, the *rdxA* gene was intact, suggesting that other mechanisms were responsible for the resistant phenotype of this isolate (26). In addition a series of 10 paired metronidazole-sensitive and -resistant clinical strains (48) were also tested for susceptibility to nitrofurantoin. The nitrofurantoin MICs for all strains tested were within a range (0.5 to 4 μg/ml) that would be considered susceptible for a comparative antimicrobial, such as metronidazole. There were no significant differences between the nitrofurantoin MICs for the metronidazole-sensitive and -resistant SS1 isolates, regardless of whether the *rdxA* gene was intact or not, or between MICs for the sensitive and resistant isolates of each individual pair of clinical strains. These data suggest that nitrofurantoin has comparable in vitro activities against metronidazole-sensitive and -resistant strains of *H. pylori* and that there is no cross-resistance between metronidazole and nitrofurantoin in this organism.

Clinical trials have demonstrated that triple therapies involving furazolidone (including omeprazole, clarithromycin, and furazolidone regimens) are able to achieve a high cure rate of *H. pylori*, and such regimens may prove particularly useful in areas where the prevalence of metronidazole-resistant strains is high (10, 33, 46, 57). The clinical evaluation of nitrofurantoin has been limited to studies in which the agent was given either as monotherapy or in combination with bismuth subsalicylate and not as part of a triple-agent regimen (4, 21, 24, 39, 44). Similarly, an assessment of the ability of various antimicrobial agents to eradicate *H. pylori*-infected gnotobiotic piglets only examined nitrofurantoin monotherapy (29). We therefore wanted to compare the abilities of two regimens to eradicate metronidazole-sensitive and -resistant strains from mice: the first regimen was a standard triple therapy involving metronidazole (12), and the second was the same regimen with nitrofurantoin substituted for metronidazole. To establish the infections, mice were inoculated with *H. pylori* SS1 and an SS1-derived *rdxA* mutant (27). Although the bacterial loads recovered from mice infected with the *rdxA* mutant were approximately 10-fold lower than those from mice infected with SS1, this difference was not statistically significant. The apparent ability of the *rdxA* mutant to colonize mice at levels similar to those for the parental strain was observed despite a reported decreased fitness of the mutant in the stationary phase of in vitro growth (J. Y. Jeong, W. W. Su, P. S. Hoffmann, and D. E. Berg, Abstr. 99th Gen. Meet. Am. Soc. Microbiol., abstr. D/B-182, 1999).

In previous work, we have demonstrated that prior exposure of *H. pylori* to metronidazole had a considerable negative influence on eradication of the organism by a regimen involving metronidazole (27). In this study, the efficacy of the regimen with metronidazole was significantly reduced in mice infected with the metronidazole-resistant *rdxA* mutant (eradicated in one of eight mice) compared to its efficacy in mice infected with the susceptible SS1 strain (eradicated in all mice). The magnitude of this effect is likely to reflect the fact that the stomachs of the *rdxA* mutant-infected mice contained a population of bacteria entirely made up of resistant isolates rather than the mixed population of sensitive and resistant strains that is frequently observed in clinical practice (20, 28). This observation provides compelling evidence for the role of metronidazole resistance in determining the successful outcome of regimens with metronidazole. When metronidazole was replaced by nitrofurantoin, the regimen failed to eradicate infection from any of the SS1-inoculated mice and eradicated infection in only one of seven mice inoculated with the *rdxA* mutant. Nitrofurantoin therefore appears to be similar to a number of antimicrobial agents that have been found to be ineffective in eradicating *H. pylori* in clinical practice despite good in vitro activity (19). The failure of the regimen with nitrofurantoin to eradicate *H. pylori* may be due to poor delivery of the drug, resulting in an insufficient concentration of the antimicrobial to exert an effective antibacterial activity in vivo (17). It is also possible that therapy was unsuccessful because of intrinsic differences between the activity of nitrofurantoin in mice and in humans. However, the limited data available suggest that nitrofurans are capable of therapeutic activity in murine models of infection (9, 15). Alternatively, it is possible that *H. pylori* has a low level of metabolic activity within the stomach and is thus relatively resistant to certain bactericidal agents.

These data demonstrate that, despite good in vitro activity and lack of induction of resistance (and particularly cross-resistance to metronidazole), nitrofurantoin is unable to eradicate *H. pylori* from mice when included as a component of a triple-therapy regimen. The *H. pylori* SS1 mouse model appears to be a suitable system for assessing novel anti-*H. pylori* agents and for determining the ability of new regimens to eradicate resistant strains.

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