Differentiation of Metronidazole-Sensitive and -Resistant Clinical Isolates of *Helicobacter pylori* by Immunoblotting with Antisera to the RdxA Protein

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Antimicrobial resistance in *Helicobacter pylori* is a serious and increasing problem, and the development of rapid, reliable methods for detecting resistance would greatly improve the selection of antibiotics used to treat gastric infection with this organism. We assessed whether detection of the RdxA protein could provide the basis for determining the susceptibility of *H. pylori* to metronidazole. In order to raise polyclonal antisera to RdxA, we cloned the *rdxA* gene from *H. pylori* strain 26695 into the commercial expression vector pMAL-c2, purified the resultant fusion protein by affinity chromatography, and used this recombinant RdxA preparation to immunize rabbits. We then used this specific anti-RdxA antibody to perform immunoblotting on whole bacterial cell lysates of 17 metronidazole-sensitive and 27 metronidazole-resistant clinical isolates of *H. pylori*. While a 24-kDa immunoreactive band corresponding to the RdxA protein was observed in all metronidazole-sensitive strains, this band was absent in 25 of 27 resistant isolates. Our results indicate that testing for the absence of the RdxA protein would identify the majority of clinical isolates that will respond poorly to metronidazole-containing eradication regimens and have implications for the development of assays capable of detecting metronidazole resistance in *H. pylori*.

Helicobacter pylori is a gram-negative, microaerobic, spiral bacterium that colonizes the stomachs of approximately half the world's population (7). 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 (2, 25, 26). Modern triple-drug regimens are highly effective for treating *H. pylori* infection, but bacterial resistance to the two most effective antibiotics, metronidazole and clarithromycin, is a serious and increasing problem. It has been estimated that 11 to 70% of clinical strains isolated in western Europe and the United States are resistant to the 5-nitroimidazoles, and this prevalence is far higher in developing countries and in certain immigrant populations (7). Although there have been conflicting reports concerning the clinical impact of metronidazole resistance in *H. pylori*, many studies have now demonstrated that resistance to this class of antibiotics does reduce the efficacy of metronidazole-containing eradication regimens and is therefore an important predictor of treatment failure (3, 6, 11, 12, 15, 16). 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 (23, 34).

Because *H. pylori* is slow growing, susceptibility testing by culture-based methods is cumbersome and in practice rarely

performed before empirical antibiotic treatment is commenced (24). However, many centers are reassessing the importance of routine susceptibility testing, appreciating that this will provide a far more rational approach to the use of antibiotics. However, cost implications, ease of access to noninvasive tests, and practical problems, such as exist for the determination of metronidazole resistance, mean that it is unlikely that routine testing for antimicrobial susceptibility will be universally adopted. A rapid and useful alternative is to identify resistance markers directly in gastric biopsy specimens, and several tests have been developed to detect the limited number of the point mutations within the peptidyltransferase region of 23S rRNA that are associated with macrolide resistance in this organism (27, 30, 33). However, it has not been possible to develop similar genotype-based tests for metronidazole, since resistance is associated with many different alterations of the *rdxA* gene (which encodes an oxygen-insensitive NADPH nitroreductase), including missense and frameshift mutations and deletions and insertion of transposable elements (5, 10, 14, 20, 29, 31). Furthermore, recent reports have demonstrated that inactivation of other reductase-encoding genes, including *fdxB* (which encodes ferredoxin-like protein) and *frxA* (which encodes NADPH flavin oxidoreductase), are also associated with resistance to metronidazole (17–19). While the precise contribution of other mechanisms to the resistant phenotype remains unclear, current evidence suggests that secondary mutations in these genes result in transition to high-level resistance once inactivation of *rdxA* has occurred. Although the development of a simple assay capable of detecting metronidazole resistance does not appear straightforward, it would represent a major advance in the antibiotic management of patients with *H. pylori*

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infection. We hypothesized that such a system could be developed based on the detection of the RdxA protein of *H. pylori*. Our strategy for detection of the RdxA protein was to use immunoblotting with specific anti-RdxA antibody.

MATERIALS AND METHODS

Bacteria and growth conditions. *Escherichia coli* strain TG1 (9) 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. The antibiotic carbenicillin (100 µg/ml) was added as required.

In the first part of the study we used the metronidazole-sensitive *H. pylori* strains SS1, G27, and HAS-141 (4, 13, 22) and isogenic strains in which the *rdxA* gene had been disrupted and which were resistant to metronidazole (15). For the investigation of production of RdxA in clinical isolates, we used 47 strains that had been isolated from patients who had undergone upper gastrointestinal endoscopy for duodenal ulceration and nonulcer dyspepsia. These included 30 strains (10 metronidazole sensitive and 20 metronidazole resistant) isolated from patients in the United Kingdom and 14 strains (7 metronidazole sensitive and 7 metronidazole resistant) from French and North African patients (31). *H. pylori* strains were routinely cultured on a blood agar medium (blood agar base no. 2 [Oxoid, Basingstoke, United Kingdom]) supplemented with 10% horse blood (TCS Microbiology, Biotolph Claydon, United Kingdom) and the following antibiotics: 10 µg of vancomycin (Sigma Chemicals, Poole, United Kingdom)/ml, 2.5 IU of polymyxin (Sigma Chemicals)/liter, 5 µg of trimethoprim (Sigma Chemicals)/ml, and 4 µg of amphotericin B (Sigma Chemicals)/ml. *H. pylori* metronidazole-resistant isolates and isogenic *rdxA* deletion mutants were grown on medium additionally supplemented with 8 µg of metronidazole (Sigma Chemicals) and 25 µg of kanamycin (Sigma Chemicals)/ml, respectively. 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.

Susceptibility testing. Susceptibility to metronidazole of the isogenic strains and French and North African isolates was assessed by agar dilution determination of the MIC. Susceptibility to metronidazole of the United Kingdom strains was assessed by the E-test (AB Biodisk, Solna, Sweden). For agar dilution, inoculates yielding 10⁴ CFU/spot were inoculated onto plates of IsoSensitest agar (Oxoid) enriched with 10% horse blood containing doubling dilutions of metronidazole. The MIC was defined as the lowest concentration of antibiotic inhibiting growth when the plates were read after 72 h incubation under microaerobic conditions (generated as described above) at 37°C. The E-test was performed according to the manufacturer's instructions. Isolates were considered resistant to metronidazole if the MIC was ≥8 µg/ml (36).

General molecular biology techniques and cloning of the *rdxA* gene. We used the alkaline lysis procedure (28) for small-scale and MIDI Qiagen (Crawley, United Kingdom) columns 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. All DNA manipulations and analyses were performed using standard protocols (28).

The *rdxA* gene (HP0954) of strain 26695 (32) was amplified by PCR using the oligonucleotide primers HP0954-5 (GGAATCTTTTGGATCAAGAAAAAGAAGACAA) and HP0954-6 (AAAAGTGCAGTTTAAACAAATGCCAC TCCTTGA). The 695-bp PCR product was then electroeluted, purified, and restricted with *EcoRI* and *PstI*. Finally, the restricted fragment was ligated into *EcoRI*-*PstI*-restricted pMAL-c2 (a commercial expression vector available from New England Biolabs, Beverly, Mass.) and transformed into *E. coli* TG1.

Expression and purification of recombinant RdxA fusion protein. Recombinant *H. pylori* RdxA protein was expressed as a MalE-RdxA fusion (of 66 kDa) and purified as described previously (8). Briefly, fresh 500-ml volumes of L broth, containing carbenicillin (100 µg/ml) and 30% (wt/vol) glucose, were inoculated with overnight cultures (5 ml) of strain TG1 harboring the recombinant plasmid and incubated with shaking at 37°C. When the optical density at 600 nm of the culture reached 0.5, isopropyl β-D-thiogalactopyranoside (IPTG) (final concentration, 1 mmol/liter) was added, and the cells were incubated for a further 4 h. Following induction with IPTG, the cells were harvested by centrifugation (10,000 × g for 30 min at 4°C) and resuspended in 25 ml of column buffer (200 mmol of NaCl, 1 mmol EDTA in 10 mmol Tris-HCl/liter [pH 7.4]) containing (per liter) the following protease inhibitors (supplied by Boehringer, Mannheim, Germany): 2 µmol of leupeptin, 2 µmol of pepstatin, and 1 µmol of phenylmethylsulfonyl fluoride. The recombinant protein was purified from *E. coli* cell extracts by affinity chromatography on amylose columns. First, intact cells were lysed by passage through a French pressure cell (16,000 lb/in²). Cell debris was removed by centrifugation (20,000 × g for 20 min at 4°C), and the lysate was

diluted in column buffer to give a final concentration of 25 mg of protein per ml prior to loading on a column (20 by 2.6 cm) of amylose resin (New England Biolabs). The resin was washed with column buffer at 0.5 ml/min until the A₂₈₀ returned to baseline levels. Finally, the MalE-fused recombinant protein was eluted from the column by washing with column buffer containing 10 mmol of maltose solution/liter. Fractions containing the fusion proteins were pooled, dialyzed against distilled water, and lyophilized. The fusion protein was resuspended in distilled water at the final concentration of 1 mg of lyophilized material/ml and stored at -20°C. The purity of the recombinant protein preparation was controlled by the Bradford assay (Sigma Chemicals) and SDS-PAGE analyses.

Generation of polyclonal rabbit antisera against RdxA. Polyclonal antisera against recombinant MalE-RdxA was produced by immunizing rabbits with 100 µg of purified recombinant protein in Freund's complete adjuvant. Four weeks later, the rabbits were booster immunized with 100 µg of protein in Freund's incomplete adjuvant. At week 6, the animals were terminally bled and the sera were stored at -20°C.

Protein analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. Two-day cultures of *H. pylori* were harvested, washed in sterile distilled water, and suspended in double-strength sample buffer (62.5 mmol of Tris base/liter [pH 6.8], 4.0% sodium dodecyl sulfate, 5.0% mercaptoethanol, 30% glycerol, 0.025% bromophenol blue), prior to solubilization by boiling for 5 min. Protein concentrations were estimated using a commercial version of the Bradford assay (Sigma Chemicals). Solubilized bacterial cell extracts, containing 20 µg of protein, were analyzed on slab gels, comprising a 4.5% acrylamide stacking gel and a 17.5% resolving gel, according to the procedure of Laemmli (21). Electrophoresis was performed at 200 V in a Mini-PROTEAN II electrophoresis cell (Bio-Rad Laboratories, Hemel Hempstead, United Kingdom). Molecular weight standards from Bio-Rad were run on each gel.

Proteins were transferred to nitrocellulose paper in a Mini Trans-Blot transfer cell (Bio-Rad) set at 0.8 mA/cm for 1 h, with cooling. Nitrocellulose membranes were blocked with 5% milk powder (Sigma Chemicals) and 1% Tween prepared in phosphate-buffered saline, with gentle shaking at room temperature for 2 h. Membranes were reacted at 4°C overnight with antisera that had been diluted 1:100 in 50% *E. coli* TG1 extract, 5% milk powder, and 0.2% Tween in phosphate-buffered saline and incubated for 4 h at room temperature to remove nonspecific antibodies to *E. coli*. Immunoreactants were detected with anti-rabbit peroxidase-linked immunoglobulin (Amersham, Little Chalfont, United Kingdom) diluted 1:10,000 and reaction products were visualized on autoradiographic film by chemiluminescence using the ECL Western blotting detection system (Amersham).

RESULTS

Generation of polyclonal rabbit antisera against RdxA. To assess the specificity of the antisera we performed immunoblot analysis of the MalE-RdxA fusion protein as well as whole-cell extracts of the well-characterized, metronidazole-sensitive *H. pylori* strains SS1, G27, and HAS-141. The antisera reacted strongly with the MalE-RdxA fusion protein, and in *H. pylori* strains SS1, G27, and HAS-141 a band of approximately 24 kDa (equivalent to the predicted molecular mass of the RdxA protein of *H. pylori* [10]) was visualized (Fig. 1). In contrast, this 24-kDa band was absent from solubilized protein preparations prepared from isogenic *H. pylori* strains in which the *rdxA* gene had been disrupted by mutagenesis (15) and which were resistant to metronidazole (Fig. 1).

Immunoblotting of metronidazole-sensitive and -resistant strains of *H. pylori* with anti-RdxA antibody. To assess whether detection of the RdxA protein could provide the basis for determining the susceptibility of *H. pylori* to metronidazole, we used the specific anti-RdxA antibody to perform immunoblotting on whole bacterial cell lysates of 17 metronidazole-sensitive and 27 metronidazole-resistant clinical isolates of *H. pylori*. While a 24-kDa immunoreactive band corresponding to the RdxA protein was observed in all metronidazole-sensitive strains, this band was absent in 25 of 27 resistant isolates (Fig.

FIG. 1. Immunoblot analysis of metronidazole-sensitive and -resistant clinical isolates of *H. pylori* using polyclonal antisera to *H. pylori* RdxA. Lane 1, *H. pylori* strain SS1; lane 2, isogenic *rdxA* mutant in strain SS1; lanes 3, 5, and 7, metronidazole-sensitive *H. pylori* strains; lanes 4 and 6, metronidazole-resistant *H. pylori* strains.

1). In some metronidazole-resistant strains a faint parasite band was observed at approximately 22 kDa. We were able to remove this in the majority of strains by preadsorption of the antisera with *E. coli* TG1 cells prior to immunoblotting.

DISCUSSION

The development of rapid, genotype-based tests to detect resistance to metronidazole in *H. pylori* has been hindered by the fact that resistance is associated with many different mutations within *rdxA* and possibly other reductase-encoding genes. Our results demonstrate a high correlation between production of the RdxA protein and susceptibility of *H. pylori* to metronidazole, confirming that the *rdxA* gene is inactivated in the vast majority of resistant isolates and that mutations in other genes are either rare or involved in transition to high-level resistance. We, and other groups, have demonstrated that resistant strains frequently contain frameshift mutations within their *rdxA* gene that result in the creation of a translational stop codon in the region immediately downstream of the mutation, and such strains would be predicted to produce a truncated RdxA protein. (10, 14, 20, 29, 31). A particularly important observation of this study was that production of the RdxA protein was completely abrogated in all but one of the resistant strains; none of the examined strains produced a truncated protein. We therefore conclude that in the majority of resistant strains, mutational inactivation of the *rdxA* gene prevents production of the protein or results in production of an abnormal polypeptide which is subsequently degraded. This suggests that testing for the absence of the RdxA protein would identify the majority of clinical isolates that will respond poorly to metronidazole-containing eradication regimens and has important im-

plications for the development of assays capable of detecting metronidazole resistance in *H. pylori*. The advantage of using this approach is that it will identify all resistant strains that carry mutations affecting expression of the *rdxA* gene, including those that have not yet been identified by sequence analysis.

Although a small number of metronidazole-resistant strains of *H. pylori* have been reported in which the nucleotide sequence of the *rdxA* gene has been unchanged there has been no further analysis of these mutants to confirm whether they have decreased RdxA activity or synthesis (1, 14, 31, 35). We are now examining whether the RdxA enzymes produced by the two resistant strains are functionally inactive or whether other mechanisms are responsible for their resistant phenotype. We are also using a larger collection of strains to assess how common such isolates are in clinical practice. In addition, we plan to refine our approach to develop a rapid and simple test that will allow the detection of metronidazole resistance in *H. pylori*. Such an assay would represent an important advance in the clinical management of *H. pylori* infection, allowing a more rational approach to the use of this antibiotic.

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