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Interaction of Metronidazole with Resistant and Susceptible *Bacteroides fragilis*

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The kinetics of the lethal action of metronidazole and the formation of acetamide have been studied in a strain of *Bacteroides fragilis* which is relatively resistant to metronidazole. As with a susceptible strain of *B. fragilis*, the data are consistent with a model in which a labile intermediate in metronidazole metabolism interacts either with water to form acetamide or with a bacterium to cause its death. Although the relatively resistant strain grows more slowly than the susceptible one and is killed less rapidly by metronidazole, the resistant strain displays the same relationship between the lethal action of metronidazole and metronidazole metabolism to acetamide. The relatively resistant strain, like the susceptible one, has an enhanced lethal response to metronidazole in the presence of a strain of *Escherichia coli*. The results suggest that the proposed labile reactive intermediate of metronidazole forms more slowly in the resistant strain.

Since both the bactericidal action of metronidazole (6) and the formation of some of its metabolites (4, 5) depend on conditions which favor nitro-group reduction, it seemed logical to explore the possibility that the two phenomena might be related. One basis for this relationship could be a common intermediate that interacts either with microbial macromolecules to exert a bactericidal effect or with other molecules, such as water, to form stable metabolites. Chrystal et al. (2) proposed several possible models of a relationship between the formation of metronidazole metabolites and the lethal effect of metronidazole. They found the model shown in Fig. 1 to be the most parsimonious one for explaining their data.

The possibility that the reactive intermediate (M*) forms in one bacterium and yet is stable enough to be detected by its effect on another bacterium is favored by the finding that *Escherichia coli* potentiates the effect of metronidazole on a susceptible strain of *Bacteroides fragilis* (2). This observation suggests that *E. coli*, although quite insusceptible to metronidazole, might nevertheless generate M* which could be detected by its lethal effect on the more susceptible *B. fragilis*. The possibility that the hypothetical intermediate M* forms in *E. coli* as well as in such susceptible bacteria as *B. fragilis* is supported by the similarity of the metabolism of metronidazole in the two bacteria (2).

The model shown in Fig. 1 was tested by studying the relationship between the bactericidal activity and metabolism of metronidazole in a relatively resistant strain of *B. fragilis*. By comparing these data with those obtained with a susceptible strain, we were able to gain further evidence that the bactericidal activity of metronidazole occurs in a manner consistent with the proposed model.

MATERIALS AND METHODS

Materials. Crystalline metronidazole (melting point, 158 to 160°C) was a gift from G. D. Searle and Co. (Chicago, Ill.). [2-^{14}C]metronidazole (18.6 mCi/mmol) was a gift from May and Baker Ltd. (Dagenham, England). *p*-Nitrobenzoic acid (PNBA) and *p*-amino-

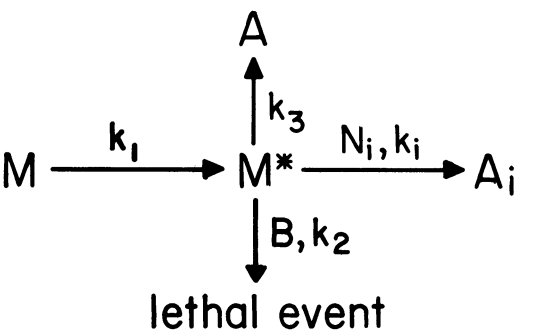

FIG. 1. A model relating the metabolism and the lethal effect on bacteria of metronidazole. Metronidazole (M) is metabolized with rate constant k_1 to a labile intermediate (M*) which can react with a bacterium (B) with rate constant k_2 to kill it. Alternatively, M* may react with water to form acetamide (A), or with other compounds (N_i) in the medium to yield other metabolites (A_i).

benzoic acid (PABA) were purchased from Eastman Organic Chemicals (Rochester, N.Y.). All other chemicals were purchased from Fisher Scientific Co. (Boston, Mass.) unless otherwise specified.

Bacterial incubation conditions and growth analysis. All bacterial incubations were carried out at 37°C in prereduced, anaerobically sterilized brucella broth (Difco Laboratories, Detroit, Mich.) supplemented with 5 μg of hemin per ml. The susceptible strain of *B. fragilis* was that described previously (2). The relatively resistant strain of *B. fragilis* was that originally isolated by Ingham et al. (3), which was kindly provided by B. Goldin and F. Tally. Cultures incubated overnight reached stationary phase and were used at a concentration of 10^9 cells per ml. Metronidazole was added to these cultures to obtain a final concentration of 100 μg/ml. Incubations were carried out in an oxygen-free atmosphere provided by a V.P.I. anaerobic culture system (Bellco Glass, Inc., Vineland, N.J.), with a gas mixture containing 5% carbon dioxide, 10% hydrogen, and 85% argon. Samples were removed from the incubation mixtures at intervals and analyzed for both acetamide content and the number of viable bacteria as described previously (2).

Analysis of bacterial growth rates. Stationary-phase culture (1 ml) was added to 9.0 ml of brucella broth, and the mixture was incubated in a water bath at 37°C in test tubes (Pyrex no. 9860) suited for spectrophotometric analysis. At intervals, the absorbancy of the culture medium was measured at 420 nm by means of a spectrophotometer (Coleman Jr. II, model 6/20).

Statistical analysis. All linear regressions and comparisons of slopes were performed with a Hewlett-Packard 9845B computer.

Nitroreductase activity. The nitroreductase activity of the two strains was measured by the formation of PABA from PNBA. The method of Bratton and Marshall was used (1). PNBA was added to cultures of *B. fragilis* to obtain a final concentration of 100 μg/ml. Samples of the culture medium were removed at intervals and analyzed both for PABA concentration as determined from a standard curve and for the number of viable bacteria (2).

RESULTS

The lethal action of metronidazole was slower in the resistant strain of *B. fragilis* than in the more susceptible strain (Fig. 2). Two explanations that accord with the proposed model (Fig. 1) are possible; either the relatively resistant strain may have a diminished capacity to carry out the conversion of M to M* (decreased k_1), or it may be less susceptible to the lethal effect of M* (decreased k_2).

The two possibilities may be distinguished by examining the rate of the formation of acetamide in the two strains. If the formation of acetamide from metronidazole is slower in the resistant strain, the resistant strain probably has a diminished capacity to catalyze the conversion of M to M*. Acetamide formed more slowly in the resistant strain (Fig. 3), a result which, incidentally, is consistent with the slower destruction of metronidazole that has previously been reported

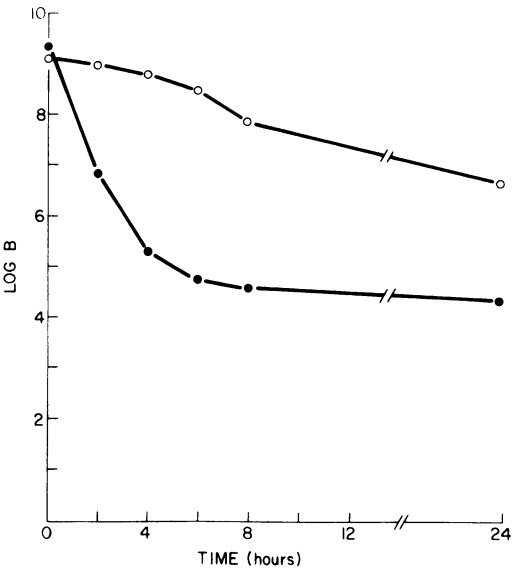

FIG. 2. Time-dependent loss of viable *B. fragilis* in the presence of metronidazole. The medium contained metronidazole at a concentration of 12.5 μg/ml. The number of surviving bacteria (B) is shown for the resistant (○) and susceptible (●) strains of *B. fragilis*.

for this strain (7). In terms of the model of Fig. 1, the diminished rate of formation of acetamide is compatible with a decreased k_1 for the resistant strain.

The alternative explanation for increased resistance namely, that the resistant strain is less susceptible to the lethal effect of M* was also considered by examining the possible influence

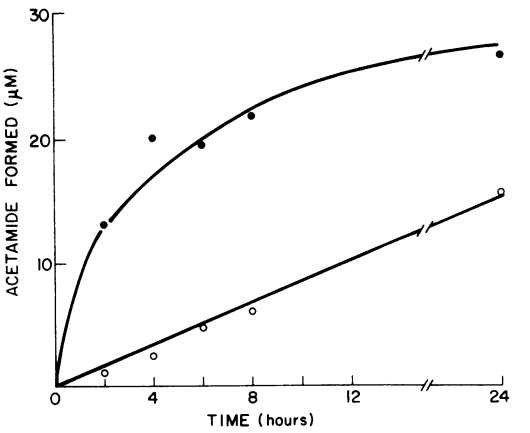

FIG. 3. Time course of the formation of acetamide from metronidazole during incubation with *B. fragilis*. The concentration of metronidazole was 12.5 μg/ml. Incubation mixtures contained either the resistant (○) or susceptible (●) strain of *B. fragilis*. This experiment was done three times, and the results shown are of a typical experiment.

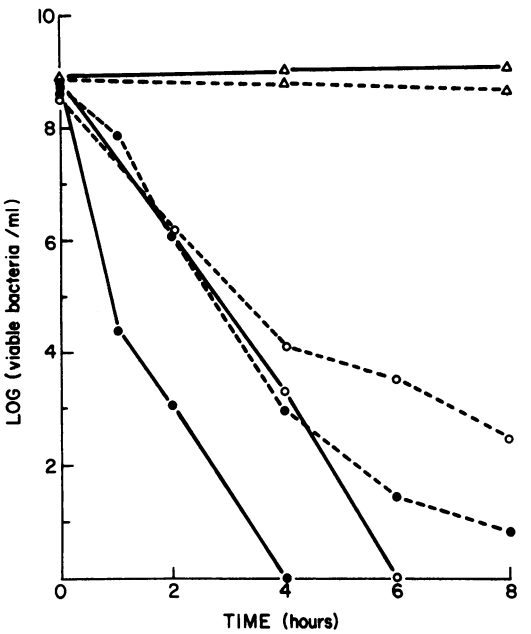

FIG. 4. Effect of metronidazole on the survival of resistant and susceptible *B. fragilis* incubated in the presence and absence of *E. coli*. The survival curves are shown for the susceptible (solid lines) and resistant (dotted lines) strains of *B. fragilis* incubated with 100 μg of metronidazole (circles) per ml and in the presence (●) or absence (○) of *E. coli*. Control incubations containing only *B. fragilis* are also shown (Δ).

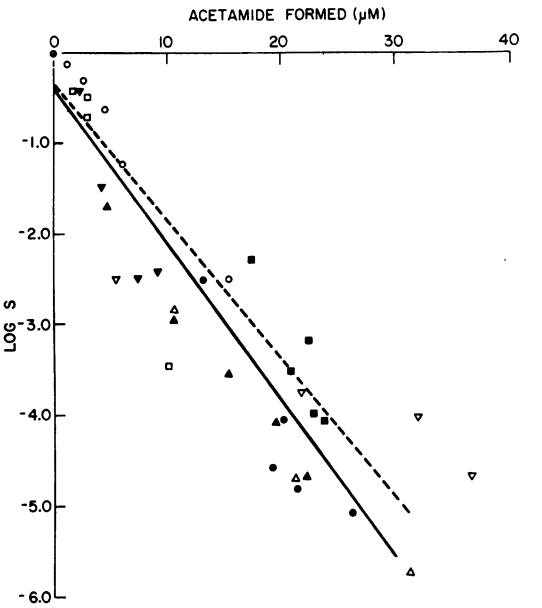

FIG. 5. Relationship between bacterial survival (log S) and acetamide formation. The results of a single experiment are shown by the same symbol where open symbols (and the dotted line) refer to the resistant strain while closed symbols (and the solid line) refer to the susceptible strain. The lines are the best fit of their respective data points.

of *E. coli* on the lethal effect of metronidazole on *B. fragilis*. We previously suggested that the enhanced effect of metronidazole on *B. fragilis* in the presence of *E. coli* is the result of the formation of M* by *E. coli* in the medium (2). If both strains of *B. fragilis* are equally susceptible to M*, the addition of *E. coli* should increase the susceptibilities of both strains to the lethal effect of metronidazole. The addition of *E. coli* enhanced the lethal effect of metronidazole on a culture of the resistant *B. fragilis* and on the susceptible culture (Fig. 4). Unfortunately, methods for enumerating the viable *B. fragilis* are not precise enough to determine whether the enhancement by *E. coli* is of the same magnitude in the two strains. Nevertheless, the results seem to exclude a large difference in the susceptibilities of the two strains to the lethal effect of the proposed intermediate, M*.

Additional evidence suggesting that the two strains are equally susceptible to M* comes from an examination of the relationship between bactericidal activity and acetamide formation for each strain in the presence of metronidazole. The model in Fig. 1 predicts a linear relationship between the log of bacterial survival and the formation of acetamide (2), and the data of Fig. 5

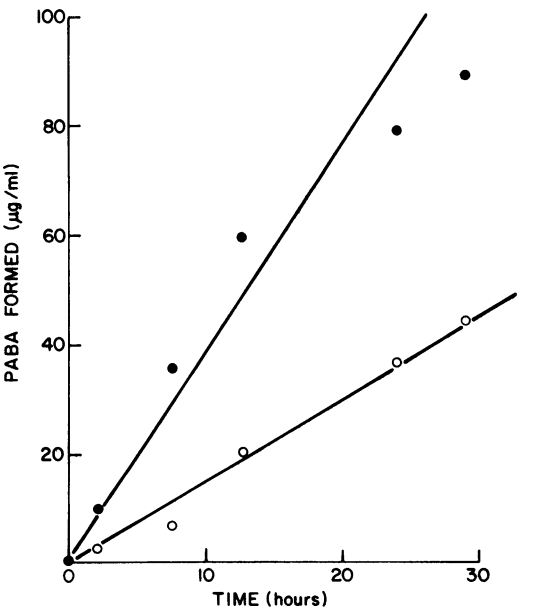

FIG. 6. Time dependent appearance of PABA. Cultures of susceptible (●) and resistant (○) *B. fragilis* containing 10^8 bacteria per ml were incubated with 100 μg PNBA per ml, and the formation of PABA was measured.

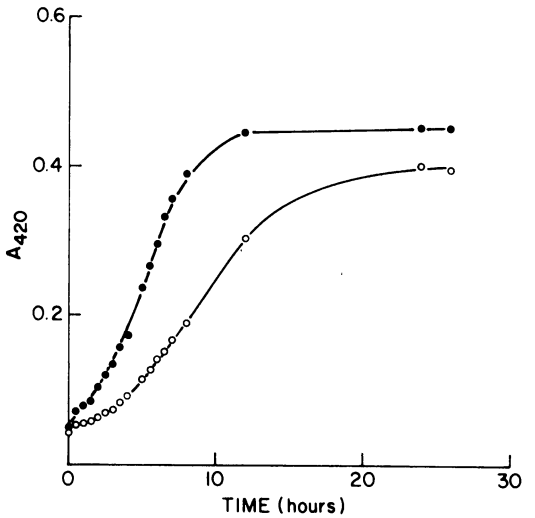

FIG. 7. Growth curves of resistant and susceptible *B. fragilis*. Symbols: ●, susceptible *B. fragilis*; ○, resistant *B. fragilis*.

indicate that both strains conform to this relationship. If, however, the two strains have different susceptibilities to M*, a difference in the slopes of the two lines might be expected. The slopes of the resistant strain (0.15) and of the susceptible strain (0.17) were not different ($P > 0.2$) (Fig. 5).

Since reduction of the nitro group is believed to convert metronidazole into a biologically active form, M*, we examined the nitroreductase activity of the two strains in more detail. Tally et al. (7) have shown that one measure of nitroreductase activity, the rate of reduction of *m*-nitrobenzoic acid, occurs at approximately one-sixth the rate in cell-free extracts of the resistant strain of *B. fragilis* than in the susceptible strain that they used. A similar measure of nitro group reduction, the relative rates of reduction of PNBA, is consistent with this observation. Resting cells of the susceptible strain reduced PNBA at twice the rate of the resistant strain (Fig. 6).

One possible explanation for the differences found in the two strains is that they grow at different rates; the susceptible strain grows at a faster rate than the resistant one (Fig. 7). During log growth, the doubling time of the resistant strain is 1.6 times that of the susceptible strain. Thus, there appears to be a correlation between growth rate and the rate of reduction of PNBA in the two cultures.

DISCUSSION

The results reported above indicate that there is a correlation between rate of nitro-group reduction, growth rate, and susceptibility to metronidazole. It is possible that the redox potential of the two cultures is the basis for this relationship. Although the structure of M* and the mechanism by which it is formed remains unclear, our results are compatible with the model shown in Fig. 1 and suggest that diminished susceptibility to metronidazole may be related simply to a slower formation of a reactive intermediate of metronidazole in the slower-growing strain.

ACKNOWLEDGMENTS

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