

## Unique Bactericidal Action of Metronidazole Against *Bacteroides fragilis* and *Clostridium perfringens*

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The comparative bactericidal activity of penicillin G, carbenicillin, clindamycin, and metronidazole against eight susceptible strains of *Bacteroides fragilis* and four strains of *Clostridium perfringens* was determined by performing colony counts anaerobically of cultures incubated in brucella broth. With the *B. fragilis* strains, there was a lag phase of growth of approximately 8 h, during which time metronidazole did not reduce the colony counts. However, within 4 h of the onset of exponential growth, metronidazole caused an abrupt decrease in counts to less than 100 colonies per ml in all strains tested. Moreover, in two strains in which the bactericidal rate was followed hourly, a 3- to 6-log decrease occurred over 1 h or less. In contrast, penicillin G and carbenicillin caused a gradual decline in colony counts from the start of approximately 1 log for each 8-h interval and were bactericidal for all strains tested. Clindamycin demonstrated the slowest bactericidal activity and for 25% of the strains was only bacteriostatic. With the *C. perfringens* strains, after a lag phase of 4 h, an abrupt decrease in colony counts also occurred with metronidazole, whereas penicillin and clindamycin again demonstrated more gradual killing effects. These studies showed a unique, time-related bactericidal action of metronidazole as compared with the other three antimicrobial agents.

The in vitro susceptibilities of anaerobic bacteria have been extensively reported, especially in recent years, with the increasing recognition of the pathogenicity of these organisms and the resistance of certain anaerobes to commonly used antibiotics (4, 5, 12, 16). Metronidazole appears to be more consistently bactericidal against susceptible strains of *Bacterioides fragilis* than other antibiotics which are more variable in this regard (6, 15, 16). The purpose of this study was to compare its time-related bactericidal effects with those of penicillin, carbenicillin, and clindamycin against strains of two commonly isolated pathogens, *B. fragilis* and *Clostridium perfringens*. The technique used was a modification of the killing curve method in which colony counts were performed anaerobically.

### MATERIALS AND METHODS

Eight clinical isolates of *B. fragilis* (four subspecies *fragilis*, three subspecies *vulgatus*, and one subspecies *thetaiotaomicron*), as determined by criteria in the Anaerobic Bacteriology Manual, UCLA (13), and four *C. perfringens* strains from the Clinical Microbiology Laboratory, University Hospital, Seattle, were used in these studies. The organisms were maintained anaerobically on brucella agar (Difco) containing 5 µg of

hemin per ml (BA + H) at 37 C in GasPak jars (H<sub>2</sub> + CO<sub>2</sub> generator, Baltimore Biological Laboratories). After subculture in brucella broth (Difco) containing hemin (BB + H), anaerobically for 24 h in GasPak jars, colony counts of approximately 10<sup>9</sup> and 10<sup>8</sup> colony-forming units (CFUs) per ml were present with each strain of *B. fragilis* and *C. perfringens*, respectively. These cultures were diluted to obtain the inocula for the minimal inhibitory concentration (MIC) and killing curve determinations.

MIC determinations were performed by the tube dilution method in BB + H with serial twofold dilutions of the antibiotics. Inocula of 10<sup>8</sup> and 10<sup>9</sup> CFUs per ml of the *B. fragilis* and *C. perfringens* strains, respectively, were used, and the tubes were incubated anaerobically as described above and read after 24 h.

**Antimicrobials.** Standard laboratory powders of penicillin G (Pfizer), carbenicillin (Roerig), clindamycin (Upjohn), and metronidazole (Searle) were provided by the manufacturers and stock solutions of 1,000 µg/ml were prepared in sterile water and stored as recommended by them. From these solutions, the following concentrations were prepared fresh daily in BB + H and used in the killing curve studies with *B. fragilis*: penicillin G, 100 µg/ml; carbenicillin, 100 µg/ml; clindamycin, 3 µg/ml; and metronidazole, 6 µg/ml. For the *C. perfringens* strains, penicillin G, 10 µg/ml, clindamycin, 3 µg/ml, and metronidazole, 6 µg/ml, were used. Carbenicillin was not studied against this latter organism.

**Killing curve procedure. *B. fragilis* strains.** An inoculum of  $10^6$  CFUs per ml of each organism together with the appropriate antibiotic in 4 ml of BB + H (pH  $7.0 \pm 0.1$ ) were incubated anaerobically in GasPak jars at 37 C. The time-related bactericidal effect of each antibiotic was followed by colony counts of the surviving bacteria after 0, 8, 12, 16, 24, and 32 h of incubation. In addition, for two strains exposed to metronidazole, sampling was done hourly between 8 and 12 h to determine more exactly the maximum rate of bactericidal activity. The problem of maintaining constant anaerobic conditions during repeated sampling was obviated by preparing separate tubes for each sampling time and incubating them in five separate GasPak jars. Thus, each tube was sampled only once, and anaerobiosis was not interfered with before the specified sampling time. For each strain of *B. fragilis*, the killing curves of all four antibiotics together with a control (without antibiotics) were run simultaneously on the same day, to ensure uniformity of growth of the organism for each antibiotic tested.

At the specified time intervals, colony counts were performed by diluting the cultures in 0.9% saline and making pour plates (100 by 15 mm size, Falcon Plastics) with 10 to 15 ml of melted BA + H. These plates were incubated anaerobically in GasPak jars at 37 C for 48 to 72 h before counting, to ensure sufficient growth with the formation of easily visible colonies.

For clindamycin, a 1:100 dilution or more in saline was necessary before colony counting, to ensure that an inhibitory concentration was not present in the pour plates. Thus, no colony counts below 100 per ml could be accurately determined for this antibiotic. For the other three drugs, however, a 1:10 dilution in saline before colony counting was sufficient since the MICs for the various strains of *B. fragilis* were higher in relation to their concentration than for clindamycin, and therefore counts as low as 10/ml could be determined. However, for the statistical comparison of the rate of bactericidal activity of the four drugs by the Wilcoxon test for pair differences, no colony counts below 100/ml were used.

The overall mechanism of action of a drug was defined as bactericidal rather than bacteriostatic, if greater than 99.9% of the organisms in the original inoculum were killed. Therefore a reduction in the colony count to less than  $10^3$  from the starting inoculum of  $10^6$  would constitute a bactericidal effect.

All procedures apart from the periods of anaerobic incubation specifically referred to above were carried out under aerobic conditions.

**C. perfringens strains.** The method was essentially the same as described above except that colony counts were done at 0, 4, 8, 12, and 24 h and an inoculum of  $10^5$  CFUs was used. Because of the low MICs of penicillin for these strains, and the high concentration of the drug used (10  $\mu$ g/ml), penicillinase concentrate (BBL) was added before the colony counts. In addition, for two strains exposed to metronidazole, sampling was done hourly between 4 and 8 h to define more accurately the maximum bactericidal rate.

## RESULTS

***B. fragilis* strains.** The MICs of penicillin, carbenicillin, clindamycin, and metronidazole

for the eight strains of *B. fragilis* used in this study are shown in Table 1. The concentrations of each antibiotic used in the killing curve are shown in brackets below each drug. Penicillin G in general tended to have lower MICs than carbenicillin for these organisms, an observation reported previously in other studies for strains of *B. fragilis* (4, 12, 14). The MICs of clindamycin were all extremely low ( $\leq 0.1$   $\mu$ g/ml) especially when compared with the other three drugs. Metronidazole had MICs in the same range as those reported in other studies (6, 12, 15).

The average killing curve for each antibiotic against the eight strains of *B. fragilis* and the average control curve (without antibiotic) are shown in Fig. 1. Growth of these strains was fairly consistent but differed from that of most aerobic organisms in being preceded by a long lag phase of approximately 8 h, as noted by the almost flat control curve during this interval. This is due in part to the method used and can probably be attributed to two main factors: (i) the organisms were exposed to aerobic conditions when the inoculum was diluted and mixed with the drugs before the beginning of the killing curve; and (ii) anaerobic conditions were produced gradually by the GasPak system. By 12 h, however, the exponential phase of growth was well established and by 24 h greater than  $10^6$  CFUs per ml were present. The colony count then remained fairly stable from 24 to 32 h.

On examining the killing curves, some obvious differences among the four antibiotics are seen. During the first 8 h of incubation, penicillin, carbenicillin, and clindamycin reduced the colony counts on the average from  $10^6$  to  $10^5$  CFUs per ml. Metronidazole, in marked contrast, appeared to have little or no effect during this interval on the number of viable organisms, as noted by its colony count being almost

TABLE 1. MICs of penicillin G, carbenicillin, clindamycin, and metronidazole for eight strains of *B. fragilis*

Strain no.	Penicillin (100 $\mu$ g/ml) <sup>a</sup>	Carbenicillin (100 $\mu$ g/ml)	Clindamycin (3 $\mu$ g/ml)	Metronidazole (6 $\mu$ g/ml)
1	6.3	6.3	0.050	1.6
2	12.5	25	0.050	3.1
3	6.3	6.3	0.025	3.1
4	12.5	50	0.012	1.6
5	6.3	3.1	0.050	0.8
6	12.5	25	0.050	0.4
7	12.5	25	0.050	0.4
8	12.5	50	0.100	0.2

<sup>a</sup> Numbers in parentheses below each drug are the concentrations used in the killing curves.

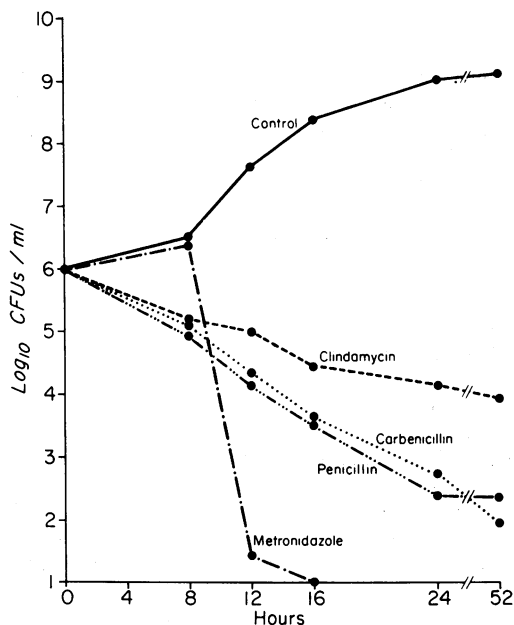


FIG. 1. Rapid bactericidal action of metronidazole against *B. fragilis* (average of eight strains) after an 8-h lag period, in contrast to the earlier, gradual decline in colony counts with the other three antimicrobials.

identical with that of the control.

During the next 4-h interval, however, as the control showed exponential growth, metronidazole caused a precipitous drop in colony counts from approximately  $10^6$  at 8 h to less than 100/ml at 12 h. The other three antibiotics, in contrast, reduced the colony counts much more gradually during this interval, and after 12 h  $10^4$  CFUs per ml on the average remained with penicillin and carbenicillin and slightly less than  $10^5$  CFUs per ml remained with clindamycin.

Longer incubation intervals for metronidazole of up to 32 h resulted in colony counts consistently less than 10 per ml, with no evidence of regrowth from surviving colonies.

The antibacterial effects of penicillin and carbenicillin continued at fairly constant but more gradual rates than metronidazole. By 32 h, both antibiotics had reduced the colony counts below  $10^3$ /ml in all strains tested and thus demonstrated an overall bactericidal effect, as defined under Materials and Methods. Moreover, there was no statistical difference between the rates of bactericidal activity of these two drugs as determined by the Wilcoxon test for pair differences ( $P > 0.05$ ).

Clindamycin demonstrated a slower bactericidal effect after 12 h as noted by its rather flat killing curve when compared with the other

three drugs. After 16 h of incubation there were on the average approximately  $10^4$  CFUs still remaining and six of eight strains had counts of this magnitude or greater. By 32 h, the average count was only slightly less than  $10^4$  CFUs per ml with this antibiotic. However, the average curve in Fig. 1 does mask the bactericidal activity of clindamycin in six of the eight strains by 32 h, since the average colony count at this time was disproportionately elevated by two strains with counts greater than  $10^4$ /ml and against which clindamycin appeared only bacteriostatic. The other six strains, in contrast, all had counts less than 100 CFUs per ml. However, clindamycin did demonstrate a slower bactericidal rate than penicillin or carbenicillin when the killing curves of the eight individual strains were compared by the Wilcoxon test for pair differences ( $P < 0.01$ ).

Figure 2 demonstrates that the maximum bactericidal effect of metronidazole occurred over 1 h or less in two strains in which colony counts were done hourly during the 8- to 12-h interval. This emphasizes the rapidity of action of this drug which coincided with the exponential growth phase noted in the control curve.

**C. perfringens strains.** The MICs of penicillin, clindamycin, and metronidazole for the *C. perfringens* strains used in this study are shown in Table 2. These organisms were generally less susceptible to clindamycin and much more susceptible to penicillin, as expected. The MICs of metronidazole were comparable to those for the *B. fragilis* strains.

The average killing and control curves for the four strains are shown in Fig. 3. The lag phase of growth for these organisms was approximately 4 h, and by 8 h greater than  $10^8$  CFUs per ml were present, indicating a more rapid generation time than for the *B. fragilis* strains. After an average peak colony count at 12 h of  $2.2 \times 10^9$ /ml, however, there was some loss of viability as noted by a slight fall in the count by 24 h.

The pattern of the killing curves with these organisms was similar to that of the *B. fragilis* strains. Metronidazole reduced the colony counts markedly between 4 and 8 h of incubation compared to the more gradual antibacterial effects of penicillin and clindamycin. After 8 h of incubation, moreover, there were on the average only slightly more than 100 CFUs per ml still present with metronidazole, whereas with the other two antibiotics approximately  $10^4$  CFUs per ml still remained.

By 24 h, penicillin had reduced the colony counts on the average to approximately 100/ml. Clindamycin, as was observed with the *B. fragilis* strains, appeared bactericidal in only three of the four strains tested, and conse-

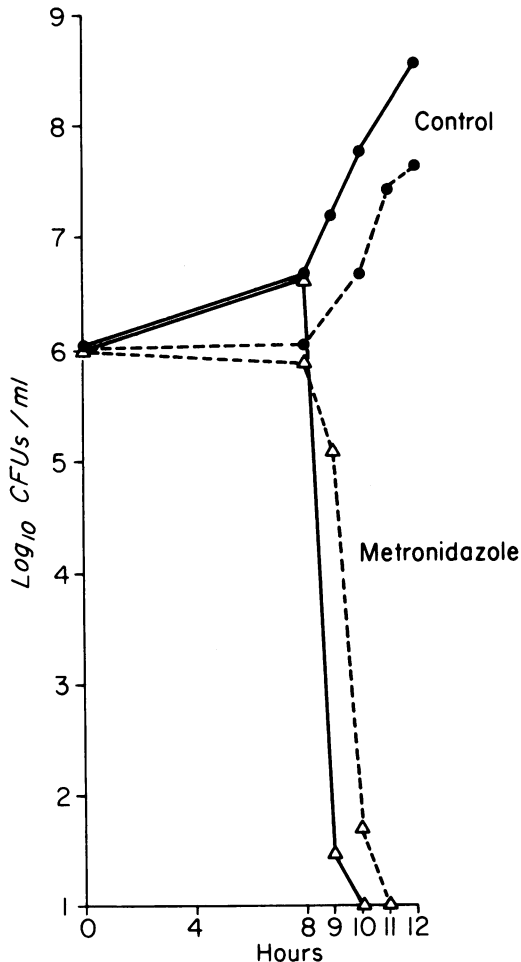


FIG. 2. Precipitous decline in colony counts in only 1 h with metronidazole for two of the eight strains of *B. fragilis* when tests were done hourly.

TABLE 2. MICs of penicillin G, clindamycin, and metronidazole for four strains of *C. perfringens*

Strain no.	Penicillin (10 µg/ml) <sup>a</sup>	Clindamycin (3 µg/ml)	Metronidazole (6 µg/ml)
1	0.05	0.40	1.60
2	0.05	0.05	0.80
3	0.40	0.10	1.60
4	0.40	0.10	1.60

<sup>a</sup> Numbers in parentheses below each drug are the concentrations used in the killing curves.

quently the high colony counts in the remaining strain ( $>10^4$ /ml) elevated the average count to greater than  $10^3$  CFUs per ml at 24 h (Fig. 3). Therefore, the average killing curve masked the bactericidal effect of clindamycin in three of the four strains by 24 h.

In two of the *C. perfringens* strains exposed to metronidazole, sampling was done hourly between 4 and 8 h. The results are presented in Fig. 4 and show that the maximum bactericidal effect occurred over a period of less than 2 h and seemed to coincide with exponential growth as was noted with the *B. fragilis* strains.

## DISCUSSION

This study demonstrates the unique time-related bactericidal effect of metronidazole as compared with penicillin, carbenicillin, and clindamycin against strains of *B. fragilis* and *C. perfringens*. Metronidazole showed little or no antibacterial activity during the initial lag phase of the two organisms, but shortly after the onset of exponential growth it caused a precipitous drop in colony counts of 3 to 6 logs within 4 h in all strains. Moreover, when this interval was examined hourly with two strains of each organism, the maximum killing effect occurred over 1 h or less in the *B. fragilis* strains and over 1 to 2 h for the *C. perfringens* strains. This unusual pattern of bactericidal activity was in marked contrast to that of penicillin, carbenicillin, and clindamycin which showed earlier but much more gradual killing effects. Whereas both penicillin and carbenicillin appeared bactericidal in all strains tested, clindamycin

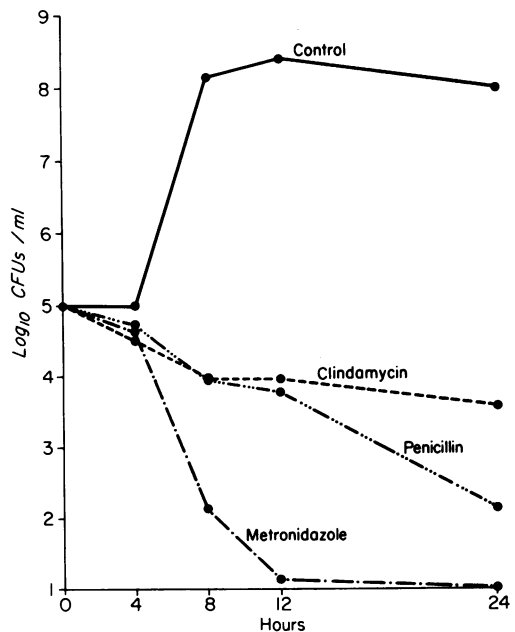


FIG. 3. Results with *C. perfringens*, which had a lag period of only 4 h, followed by much more rapid killing by metronidazole than by penicillin G or clindamycin.

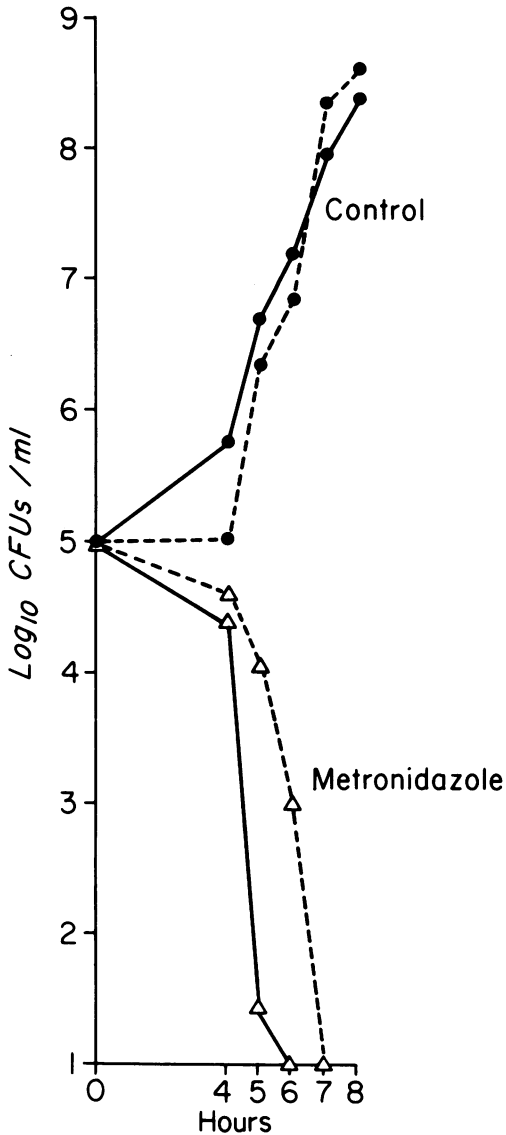


FIG. 4. Hourly colony counts with two strains of *C. perfringens*, showing a marked decline in colony counts with metronidazole in less than 2 h.

showed bacteriostatic activity only in 25%. This discrepancy in minimal bactericidal concentration versus MIC has been reported previously by other investigators (6, 16). This antibiotic also demonstrated the slowest rate of bactericidal activity against the *B. fragilis* strains, despite its extremely low MICs compared to a concentration of 3  $\mu\text{g}/\text{ml}$  used in the killing curve.

The rate of bactericidal activity of penicillin and carbenicillin against *B. fragilis* strains was essentially similar to that demonstrated by

Schoutens and Yourassowsky in a previous study (10), except that we found no statistically significant difference in the activity of these two antibiotics. These investigators used a triple-layer agar technique in which the organisms were growing logarithmically at the start of the killing curve and hence avoided the lag phase of growth present in our study. However, their method is restricted to antibiotics which can be rapidly inactivated by enzymes and was not applicable in comparing the activity of metronidazole with other drugs.

The mode of action of metronidazole has been studied by several investigators (3, 7, 8), and a hypothetical mechanism describing it has been proposed recently by Ings et al. (3). They postulated that the parent drug is reduced to a reactive intermediate within anaerobic cells which ultimately inhibits nucleic acid synthesis and leads to cell death. It is interesting to note that in their studies deoxyribonucleic acid synthesis in a strain of *C. perfringens* during exponential growth was inhibited as early as 30 min, which would explain the very early bactericidal effect of metronidazole found in our studies. In addition, they demonstrated that in strains of *Trichomonas vaginalis*, although deoxyribonucleic acid synthesis was inhibited after only 30 min also, cell death did not occur until 5 h, indicating that there is some delay between the two events in this particular organism.

A recent susceptibility study by Chow et al. (1), in which large numbers of anaerobic organisms were tested, has shown metronidazole to be most effective against bacteroides, clostridia, and fusobacteria species and generally less active against non-sporeforming gram-positive cocci.

The antibiotic concentrations used in this study were chosen to stimulate serum levels used clinically in the treatment of anaerobic infections caused by *B. fragilis* and *C. perfringens*. Penicillin and carbenicillin levels of 100  $\mu\text{g}/\text{ml}$  or more, which may be necessary for the treatment of some infections caused by strains of *B. fragilis* in particular, are more easily achieved and sustained with carbenicillin primarily because of its lower rate of renal clearance and slower inactivation by nonrenal mechanisms (11). This pharmacokinetic difference may give carbenicillin an advantage over penicillin in the therapy of these infections, since there was no significant difference in their rates of bactericidal activity in our studies, despite lower MICs for penicillin. However, in view of a recent report in which 40% of 33 strains of *B. fragilis* were resistant to 128  $\mu\text{g}$  of carbenicillin per ml (14), susceptibility testing is neces-

sary to ensure adequate therapy with this antibiotic.

Serum levels of 3  $\mu\text{g/ml}$  are easily achieved with parenteral clindamycin (2) and will exceed the MICs for most commonly isolated anaerobic pathogens (12, 16).

We have shown previously (9) that minimum serum levels of metronidazole on a dose of 500 mg orally four times daily exceed by two to three times the concentration of 6  $\mu\text{g/ml}$  used in this study and to which the majority of bacteroides, fusobacteria, and clostridia are susceptible. These favorable therapeutic levels together with its unique and rapid bactericidal action demonstrated in this study should make metronidazole a very useful addition to the therapy of certain anaerobic infections.

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