Speed of Bactericidal Action of Penicillin G, Ampicillin, and Carbenicillin on *Bacteroides fragilis*

E. SCHOUTENS AND E. YOURASSOWSKY

Hôpital Universitaire Brugmann, 1020 Bruxelles, Belgium

Received for publication 9 April 1974

In order to determine the therapeutic value of penicillins in the management of anaerobic infections caused by *Bacteroides fragilis*, the antibacterial activity of penicillin G, ampicillin, and carbenicillin against 10 strains of *B. fragilis* subsp. *fragilis*, isolated from clinical material, was studied. This was undertaken by using a triple-layer technique, enzymatic inactivation of the antibiotics, and an automatic colony counter, which allowed a quantitative and a qualitative approach to the problem. Results indicated in vitro bactericidal activity with 40 µg of penicillin G, ampicillin, and carbenicillin per ml against all strains tested. However, carbenicillin had the most rapid activity.

Bacteroides fragilis may be responsible for acute infections whose antibiotic treatment is difficult to establish. In fact, only metronidazole (9, 10) and clindamycin (9) have been shown to possess bactericidal activity in vitro against *B. fragilis*. Unfortunately, these two drugs do not act against aerobic gram-negative bacilli, frequently recovered in addition to *Bacteroides* strains in septic foci located in the gastrointestinal and genitourinary tracts (2, 5, 11). Cephalosporins and aminoglycosides are practically inactive against *B. fragilis*, which also has the reputation of being relatively resistant to ampicillin and carbenicillin. However, the minimal inhibitory concentrations (MIC) of these two antibiotics (3, 7, 17) are often lower than the blood levels that are achieved in patients treated with high doses of these substances (15).

The present study was undertaken to determine the action of penicillin G, ampicillin, and carbenicillin on *B. fragilis*, these three antibiotics being studied at a concentration (40 µg/ml) attainable in vivo with high dosage schedules of the drugs. The study was performed by using a new method which takes into account certain growth difficulties encountered with strictly anaerobic microorganisms. Further enzymatic inactivation of the antibiotics and the use of an automatic colony counter allowed measurement of the rate of antibacterial activity.

MATERIALS AND METHODS

Microbial strains. These consisted of 10 strains isolated from blood cultures, peritoneal fluids, and decubitus ulcers. All were anaerobic, nonmotile, nonsporulating, gram-negative rods whose growth was

enhanced by bile and inhibited by brilliant green. The ten strains, which produced neither indole nor hydrogen sulfide, fermented xylose and cellobiose but not arabinose, rhamnose, trehalose, melezitose, or sorbitol. Thus, they corresponded to the definition of *B. fragilis* subsp. *fragilis* given by other authors (1, 6, 16).

Determination of the speed of the bactericidal action of penicillin G, ampicillin, and carbenicillin. (i) **Preparation of the inoculum.** Some colonies from a 24-h culture were placed in 50 ml of thioglycolate medium (without indicator) to which hemin (5 µg/ml) (THCM medium) were added after autoclaving. The culture was incubated anaerobically at 37°C until turbidity corresponding to the no. 1 McFarland nephelometer standard was obtained. It was then stored without additives in liquid nitrogen in 2-ml amounts. At the time of use, a sample was removed from the liquid nitrogen and diluted 1:100 (vol/vol) in Brucella broth; 0.2 ml of this suspension constituted the inoculum for each petri dish ($\pm 6 \times 10^4$ organisms/plate).

(ii) **Triple-layer technique.** The media were poured into 9-cm diameter petri dishes and allowed to set on a horizontal support that had been verified by a spirit level. The base medium contained 1% tryptone, 0.02% beef extract, 0.05% yeast extract, 0.5% sodium chloride, 0.03% cysteine hydrochloride, 0.02% dextrose, and 1.5% agar (4); hemin (5 µg/ml) was added after autoclaving. This medium is henceforth referred to as VLH.

The bottom layer consisted of 10 ml of VLH to which 40 µg of the antibiotic to be studied per ml was added at 48°C (this medium is referred to as VLHA). The inoculum (6×10^4 organisms) was added to 10 ml of molten VLHA at 48°C to constitute the median layer, which was then poured over the bottom layer as soon as the latter had solidified. The top layer consisted of 9.5 ml of VLH to which, just before pouring, 0.5 ml of penicillinase was added (Bacto-Penase concentrate: 1 ml has a potency of at least 20,000 Levy units).

At 0 h, and for each antibiotic, 14 plates consisting

of only the bottom and median layers were incubated anaerobically (GasPak) at 37 C. Every hour, one of the plates was covered with the top layer (the first plate at 0 h, the second after 1 h, the 13th after 12 h, and the 14th after 24 h of incubation) and was then placed back in the incubator (Fig. 1).

Colony-forming units counts were performed 48 h after the zero hour (i.e., 24 h after the 14th plate was covered with its top layer).

The rapid inactivation of the antibiotic by penicillinase was shown by the absence of any antimicrobial activity in the first plate of the series. The number of colony-forming units after 48 h of incubation was identical to that found in a control plate containing neither the antibiotic nor penicillinase.

(iii) **Automated colony counting.** This measurement was obtained with a Tessorar lens coupled to a Carl Zeiss MicroVideomat. The Tessorar Zeiss Optic allowed a continued magnification from 0.4 to 12.8 $\times$. Depth of integral reproduction was 5.2 mm. Plates were orthogonally lighted by transparence. The image was captured by a Siemens Plumbicon tube, which gave a supplementary magnification of 20 $\times$ (maximal magnification co-optico-electronic of 256 $\times$) on a television screen. Electronic analysis of the image and an automated count were performed by the Micro Videomat.

In each plate, the colony-forming-unit count was effected on ten different fields whose dimensions were chosen as a function of the microbial density (optimal condition: ± 100 colonies per field). The error of counting due to the possible confluence of colonies was inferior to errors due to chance for a number of colonies per plate in the order of 10^4 (E. Yourassowsky and E. Schoutens, Symp. on Rapid Methods and Automation in Microbiol., Stockholm, Abstr. B75, 1973).

Determination of the MICs of penicillin G, ampicillin, and carbenicillin by the triple-layer technique. Each of the three layers consisted of 10 ml of VLH to which twofold dilutions of the antibiotic to be

studied had been incorporated. The median layer contained the inoculum (6×10^4 organisms). The plates were incubated for 48 h anaerobically at 37 C. The MIC was recorded as the lowest antibiotic concentration inhibiting the inoculum (containing no colony-forming units per plate).

Determination of the MICs and minimal bactericidal concentrations (MBCs) by classic procedures. (i) **Determination of MIC values by the agar dilution method (14).** Brucella agar with 5% laked horse blood, 0.5 μ g of menadione per ml, and twofold dilutions of the antibiotic to be studied was selected as medium. The plates were inoculated by means of an inocula-replicating apparatus (12) with the inoculum, freshly prepared in THCM, adjusted to the no. 1 McFarland nephelometer standard. Results were read after 48 h of anaerobic incubation at 37 C.

(ii) **Determination of MIC and MBC values by the tube dilution method (9).** Each tube contained 0.5 ml of Brucella broth with 5% laked horse blood, 0.5 μ g of menadione per ml, and twofold dilutions of the antibiotic. The inoculum freshly prepared in THCM for the determination of the MICs by the agar dilution method was diluted to 1:100 in Brucella broth; 0.5 ml of this suspension was added to 0.5 ml of the broth containing antibiotic. The tubes were incubated anaerobically at 37 C for 48 h. The first tube without macroscopic microbial growth was accepted as the MIC. To determine the MBC, 0.01 ml of the contents of each tube containing the MIC or greater of the antibiotic was subcultured onto a blood agar plate; the MBC was designated as the lowest antibiotic concentration yielding fewer than ten colonies after 48 h of anaerobic incubation at 37 C.

RESULTS

Results of MIC and MBC determinations for the ten strains of *B. fragilis* subsp. *fragilis* are listed in Table 1. Most often, the MICs for a single strain were of the same order for the three antibiotics considered. Results determined by the triple-layer technique correlated well with those obtained by the classic procedures; all strains tested by tube broth dilution were inhibited by 25 μ g or less of penicillin G, ampicillin, and carbenicillin per ml; in all but one case, the MIC figures were equivalent when determined by the triple-layer technique. The agar dilution method, however, gave slightly higher values than the other two procedures. This can be partially explained by an inoculum effect; inoculum used in the triple-layer method ($\pm 6 \times 10^4$ organisms per plate) was approximately 1,000 times lower than that delivered by a prong of the Steers replicator. The MBC levels of penicillin G, ampicillin, and carbenicillin were generally two to eight times the MIC levels; one strain, however, was not killed by antibiotic concentrations of 100 μ g per ml, though it was inhibited by 12.5 μ g of these drugs per ml.

Figure 2 illustrates the bactericidal action of

FIG. 1. Diagrammatic scheme for study of the bactericidal activity of penicillins, by using the triple-layer technique, and an enzymatic inactivation of the antibiotic. P, Top layer containing penicillinase.

TABLE 1. MICs and MBCs of penicillin G, ampicillin, and carbenicillin against ten strains of *B. fragilis* subsp. *fragilis*

Strain No.	Penicillin ($\mu\text{g/ml}$)				Ampicillin ($\mu\text{g/ml}$)				Carbenicillin ($\mu\text{g/ml}$)			
	MIC			MBC	MIC			MBC	MIC			MBC
	Triple-layer method	Agar dilution method	Tube dilution method	Tube dilution method	Triple-layer method	Agar dilution method	Tube dilution method	Tube dilution method	Triple-layer method	Agar dilution method	Tube dilution method	Tube dilution method
4	12.5	50	25	>100	12.5	25	25	50	50	50	25	50
5	12.5	50	25	50	12.5	25	12.5	50	25	100	25	50
22	12.5	12.5	12.5	25	12.5	25	6.25	50	12.5	6.25	6.25	12.5
30	12.5	12.5	12.5	12.5	12.5	25	6.25	25	25	25	12.5	25
31	6.25	25	3.12	100	12.5	12.5	1.56	12.5	12.5	12.5	3.12	25
33	3.12	12.5	12.5	25	3.12	12.5	6.25	12.5	12.5	25	6.25	12.5
41	3.12	12.5	1.56	6.25	3.12	6.25	1.56	6.25	3.12	3.12	1.56	3.12
47	12.5	25	12.5	>100	12.5	25	12.5	>100	25	25	12.5	>100
51	6.25	12.5	6.25	12.5	12.5	6.25	3.12	12.5	12.5	6.25	6.25	50
52	6.25	12.5	25	50	25	25	25	100	6.25	12.5	12.5	25

FIG. 2. Bactericidal activity of penicillin G, ampicillin, and carbenicillin against *B. fragilis*. Mean values were calculated on ten strains for each period; ranges are shown by lines.

the three antibiotics tested at a concentration of 40 $\mu\text{g/ml}$. The number of viable elements per plate is expressed as a function of the initial inoculum considered as 100%. Carbenicillin had the most rapid activity, with all strains killed after 11 h of action and some killed after 8 h. Ampicillin, a little less effective, killed the ten strains after 12 h. At that time, there were still approximately 1% of viable cells among the ten strains in the presence of 40 μg of penicillin G per ml. At 24 h, however, penicillin G killed all the organisms.

DISCUSSION

The study of the bactericidal activity of a penicillin necessitates obtaining a microbial

culture in exponential growth. In the case of *B. fragilis*, this can be difficult due to the slow start of bacterial growth, disagreement between broth turbidity and number of viable cells, and influence of the variations of media composition, or it can be disturbed because of technical manipulations such as the unfavorable role of thermic or atmospheric variations during the course of the work.

Some strains are "capricious" in liquid medium, giving heavy growth within 4 to 6 h of incubation, whereas others require overnight incubation to attain the same microbial density. A strain which grows rapidly on one day may grow slowly the next day. Planning of the work is very difficult in these conditions. The

preservation of the strains in liquid nitrogen combined with the use of the triple-layer technique allows the permanent disposition of an inoculum in exponential growth ready to be used, which seems to be only slightly influenced by environmental conditions.

The colonies that it generates form quickly, are numerous, and have the same dimensions as those produced by a fresh inoculum of the same density. Their characteristics remain constant from one sample to another.

The modifications used with the technique of measurement of the bactericidal activity of penicillins on *B. fragilis* allow a more quantitative approach to the study of the speed of action of these antibiotics. They are only valid for the study of antibiotics which can be rapidly destroyed by an enzymatic procedure. The triple-layer technique does not in itself bring a parameter that can fundamentally modify the results obtained, as the MICs so determined correlate well with those measured by the classic methods.

The ten strains used were isolated from clinical material; they can be considered representative of the strains responsible for acute infections.

In order to compare penicillin G, ampicillin, and carbenicillin, a single antibiotic concentration (40 $\mu\text{g/ml}$) was chosen, which could be readily achieved and even largely exceeded in vivo by the intravenous administration of large doses of either penicillin G (12 to 24 g/day), ampicillin (12 g/day), or carbenicillin (30 g/day). The intermittent injection of these antibiotics, rather than their continuous perfusion, determined peak blood levels capable of reaching 300 to 500 $\mu\text{g/ml}$.

The resistance of *B. fragilis* to penicillins is a widely accepted belief (2, 8, 10, 19, 20). Sutter and Finegold (13) used it as an aid in characterization of *B. fragilis*, but their criteria of resistance was the absence of growth inhibition around a disk containing 2 U of penicillin G. For a concentration of 50 μg per ml, Kislak (7) found approximately 92% of the *B. fragilis* strains susceptible to ampicillin and penicillin G and 80% of the strains susceptible to carbenicillin, figures which are close to those obtained by Bodner (3) and Werner (17).

The results of the present study attest to the in vitro bactericidal activity of penicillin G, ampicillin, and carbenicillin, at a concentration of 40 $\mu\text{g/ml}$, on *B. fragilis*. However, despite MICs similar to those of penicillin G and ampicillin, carbenicillin has a faster bactericidal activity.

Furthermore, carbenicillin is usually administered intermittently and at doses higher than those recommended for penicillin G or ampicillin; this mode of infusion could give rise to high antibiotic levels in infected foci (M. Barza et al., Prog. Abstr. Intersci. Conf. Antimicrob. Ag. Chemother., 13th, Washington, D.C., Abstr. 12, 1973).

ACKNOWLEDGMENTS

We thank M. R. Prudhomme, M. Labbe, and their colleagues for excellent technical assistance.

LITERATURE CITED

1. Beerens, H., Y. Schaffner, J. Guillaume, and M. Castel. 1963. Les bacilles anaérobies non sporulés à Gram négatif favorisés par la bile. Leur appartenance au genre *Eggerthella* (nov. gen.). Ann. Inst. Pasteur (Lille) 14:5-48.
2. Bodner, S. J., M. G. Koenig, and J. S. Goodman. 1970. Bacteremic bacteroides infections. Ann. Intern. Med. 73:537-544.
3. Bodner, S. J., M. G. Koenig, L. L. Treanor, and J. S. Goodman. 1972. Antibiotic susceptibility testing of *Bacteroides*. Antimicrob. Ag. Chemother. 2:57-60.
4. Buttiaux, R., H. Beerens, and A. Tacquet. 1969. Manuel de techniques bactériologiques, p. 514-515. Ed. Médicales Flammarion, Paris.
5. Haldane, E. V., and C. E. Van Rooyen. 1972. Treatment of severe bacteroides infections with parenteral clindamycin. Can. Med. Ass. J. 107:1177-1181.
6. Holdeman, L. V., and W. E. C. Moore. 1970. Gram-negative nonsporeforming anaerobic bacilli, p. 286-289. In J. E. Blair et al. (ed.), Manual of clinical microbiology. American Society for Microbiology, Bethesda, Md.
7. Kislak, J. W. 1972. Antibiotic susceptibility of *Bacteroides*. J. Infect. Dis. 125:295-299.
8. Martin, W. J., M. Gardner, and J. A. Washington II. 1972. In vitro antimicrobial susceptibility of anaerobic bacteria isolated from clinical specimens. Antimicrob. Ag. Chemother. 1:148-158.
9. Nastro, L. J., and S. M. Finegold. 1972. Bactericidal activity of five antimicrobial agents against *Bacteroides fragilis*. J. Infect. Dis. 126:104-107.
10. Nastro, L. J., and S. M. Finegold. 1973. Endocarditis due to anaerobic gram negative bacilli. Amer. J. Med. 54:482-496.
11. Peromet, M., M. Labbe, E. Yourassowsky, and E. Schoutens. 1973. Anaerobic bacteria isolated from decubitus ulcers. Infection 1:205-207.
12. Steers, E., E. L. Foltz, and B. S. Graves. 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. 9:307-311.
13. Sutter, V. L., and S. M. Finegold. 1971. Antibiotic disc susceptibility tests for rapid presumptive identification of gram-negative anaerobic bacilli. Appl. Microbiol. 21:13-20.
14. Sutter, V. L., Y. Y. Kwok, and S. M. Finegold. 1972. Standardized antimicrobial disc susceptibility testing of anaerobic bacteria. Appl. Microbiol. 23:268-275.
15. Walter, A. M., and L. Heilmeyer. 1969. Antibiotika-Fibel. Antibiotika und chemotherapie. 3. Auflage, p. 147-227. Georg Thieme Verlag, Stuttgart.
16. Werner, H. 1967. *Eggerthella* - Arten aus pathologischem Material und aus Stuhlproben. Arch. Hyg. 151:492-508.

17. Werner, H., H. Hussels, and F. Neuhaus. 1971. Pleuraempyem durch die anaerobe nichtsporenbildende Bakterienart *Sphaerophorus Necrophorus*. Deut. Med. Wochenschr. **96**:202-205.
18. Whelan, J. P. F., and J. H. Hale. 1973. Bactericidal activity of metronidazole against *Bacteroides fragilis*. J. Clin. Pathol. **26**:393-395.
19. Wilson, W. R., W. J. Martin, C. J. Wilkowske, and J. A. Washington II. 1972. Anaerobic bacteremia. Mayo Clin. Proc. **47**:639-646.
20. Zabransky, R. J., J. A. Johnston, and K. J. Hauser. 1973. Bacteriostatic and bactericidal activities of various antibiotics against *Bacteroides fragilis*. Antimicrob. Ag. Chemother. **3**:152-156.