TIMED BACTERIOSTATIC AND BACTERICIDAL ACTIVITIES OF SELECTED ANTIMICROBIAL AGENTS AGAINST *BACTEROIDES FRAGILIS* ISOLATED FROM CLINICAL SPECIMENS

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The activities of selected antimicrobial agents were evaluated for bacteriostatic and bactericidal activities for a large number of clinically obtained strains of *Bacteroides fragilis*, with special reference to the incubation time of the microbes with the drugs. If the mode of action of a drug is categorized as bactericidal when the ratio of bactericidal concentration/bacteriostatic concentration is low ($\leqq 4$), and as bacteriostatic when high ($\geqq 8$), during given periods of incubation, then clindamycin, minocycline and chloramphenicol appeared to be bacteriostatic, and cefoxitin, cefmetazole, latamoxef (moxalactam) and metronidazole bactericidal, when the incubation time was brief (6 hours). All these drugs acted bactericidally on most of the test strains, if the time of incubation was prolonged to 24 hours.

Serious infections due to *Bacteroides fragilis* are reported to be increasing. A variety of antimicrobial agents are currently in use for such infections. Many reports concerning microbial susceptibility to these drugs have appeared to date. However, there is very little information which deals with the timed bacteriostatic and bactericidal activities of these agents against a large number of test strains. In this work it is shown that the activities of antimicrobial agents depend not only on the drug concentrations, but also significantly on how long the various strains are incubated with these drugs.

Materials and Methods

Bacterial Strains

Recent clinical isolates of 52 strains of *B. fragilis* obtained at Tokyo Metropolitan Komagome Hospital were used.

Antimicrobial Agents

Cefoxitin (Daiichi Seiyaku Co., Ltd., Tokyo), latamoxef (moxalactam) and metronidazole (Shionogi & Co., Ltd., Osaka), clindamycin (Japan Upjohn Ltd., Tokyo), minocycline (Lederle (Japan) Ltd., Tokyo), cefmetazole and chloramphenicol (Sankyo Co., Ltd., Tokyo) were obtained as gifts.

Media

GAM broth (Nissui Seiyaku Co., Ltd., Tokyo) and GAM agar (Nissui Seiyaku Co., Ltd., Tokyo) were used. The GasPak system (BBL, Cockeysville, Maryland) was used to generate and maintain anaerobic conditions. Incubation was performed in a 35°C incubator.

Determination of Agar Dilution MIC

The inoculum size of test strains used for assessment of agar dilution MIC was 10^6 colony-forming units (cfu) per ml. These inocula were transferred onto the drug-containing agar plates by means of a STEERS' type inocula replicator[1,2] which provided 0.001 ml amounts. After incubating anaerobically for 24 hours, the lowest drug concentration yielding no visible colony formation on the agar plates was designated as the MIC.

Definition of Antibacterial Activities by the Broth Dilution Method

Fig. 1 depicts the antibacterial activities of a drug with incubation periods of 6 hours (a) and

Fig. 1. Schematic presentation of the potentially maximal number of cfu surviving after incubation of microbes for 6 hours (a), and 24 hours (b), with various antimicrobial activities of a drug.

The broken line represents the theoretical microbiostatic concentration. Conventional MIC is also illustrated.

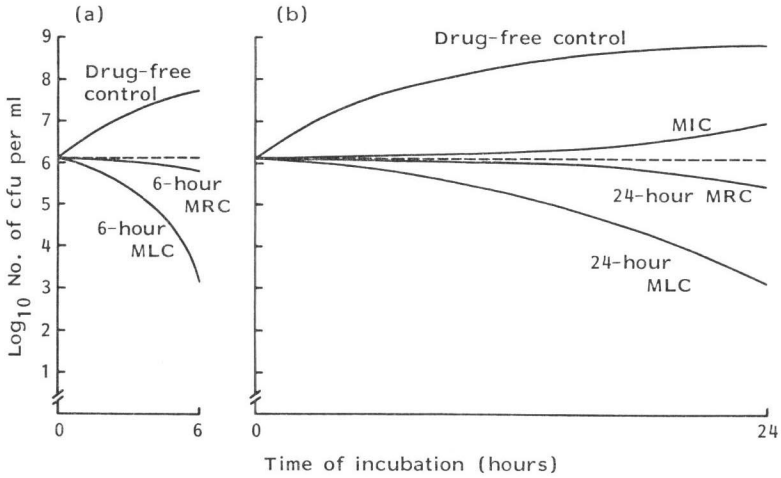


Table 1. Number of cfu in overnight cultures of GAM broth in 7 consecutive studies.

No.	Antimicrobial agent used	No. of strains	No. of cfu	
			Range ($\times 10^9$/ml)	Median ($\times 10^{10}$/ml)
1	Cefoxitin	27	3~32	2.0
2	Cefmetazole	27	5~35	2.4
3	Latamoxef	27	8~48	2.9
4	Clindamycin	27	2~26	1.2
5	Minocycline	27	8~30	1.9
6	Chloramphenicol	27	2~32	1.4
7	Metronidazole	27	15~42	2.4

24 hours (b). Minimally reducing concentration (MRC) was used, as detailed previously[8], to represent an approximation of the theoretical bacteriostatic concentrations of each drug. The 6-hour and 24-hour MRCs represent the lowest concentrations of the drug yielding no increase in the cfu number after incubation periods of 6 hours and 24 hours, respectively. Conventional MIC, another approximation to the theoretical bacteriostatic concentration with incubation times of 24 hours, is also shown. For the assessment of bactericidal activity, the term "minimal lethal concentration (MLC)" which customarly represents the concentrations killing 99.9% of the bacteria[4] was used. In this experiment, the minimal drug concentration which produced a 99.9% kill of cfu after an incubation time of 6 hours, was designated as 6-hour MLC, and the concentration which produced the same magnitude of kill after a 24-hour incubation was designated as 24-hour MLC, as detailed previously[8].

Assessment of Timed Bacteriostatic and Bactericidal Activities

Table 1 represents the cfu numbers of *B. fragilis* in overnight cultures of GAM broth in 7 consecutive studies. It is apparent that the variation of cfu numbers in each culture was relatively small; ranging from the order of 10^9 to 10^{10} per ml. The median value ranged from 1.2×10^{10} to 2.9×10^{10} per ml. Based on this result, a simplification was made in the determination of the MRC end-point; the end-point was judged to be the lowest concentration of the drug producing a 50% decrease in the cfu number from the median value in each study, rather than from the cfu numbers of individual strains, as had been done in a previous paper[8]. With the exception of the above modification, the methodology was essentially the same as previously detailed[8].

Results

Selection of Bacterial Strains for Use in the Assessment of Timed Antibacterial Activities

Agar dilution MICs of 7 antimicrobial agents, were assessed for 52 strains of *B. fragilis* (Table 2). The MIC results for most of these strains were: 6.25~12.5 μg/ml of cefoxitin, 3.13~6.25 μg/ml of cefmetazole, 0.39~0.78 μg/ml of latamoxef and metronidazole, 0.0125~0.05 μg/ml of clindamycin, and 0.78~1.56 μg/ml of minocycline and chloramphenicol. Out of these 52, a total of 27 strains in the range of above MICs were selected and used in the following study.

Comparative Agar and Broth Dilution MICs and 24-hour MLCs

The agar and broth dilution MICs and 24-hour MLCs are compared in Table 3. The MICs of

Table 2. Distribution of agar dilution MICs of 7 antimicrobial agents for 52 strains of *Bacteroides fragilis*.

Agent	MIC (μg/ml)															
	0.0063	0.0125	0.025	0.05	0.1	0.2	0.39	0.78	1.56	3.13	6.25	12.5	25	50	100	>100
Cefoxitin										6	34	10	1	1		
Cefmetazole									2	20	23	3	3		1	
Latamoxef					1	3	19	15	2	5	3	1	3			
Clindamycin	2	9	11	7	4	4						1				14
Minocycline			15	1	2		4	16	12	2						
Chloramphenicol							2	36	14							
Metronidazole					2	5	31	10	3		1					

Table 3. Comparison of agar and broth dilution MICs and 24-hour MLCs of 7 antimicrobial agents for 27 strains of *B. fragilis*.

Agent	Medium	Activity	No. of strains showing MIC or 24-hour MLC (μg/ml) of												
			0.0125	0.025	0.05	0.1	0.2	0.39	0.78	1.56	3.13	6.25	12.5	25	50
Cefoxitin	Agar	MIC									4	22	1		
	Broth	MIC									1	24	2		
		24-hour MLC										10	15	2	
Cefmetazole	Agar	MIC									2	21	4		
	Broth	MIC										12	13	2	
		24-hour MLC										5	15	6	1
Latamoxef	Agar	MIC						1	8	17			1		
	Broth	MIC							23	3			1		
		24-hour MLC							16	6	1	3	1		
Clindamycin	Agar	MIC		2	3	11	10	1							
	Broth	MIC		1	1	5	15	5							
		24-hour MLC			2	2	2	10	11						
Minocycline	Agar	MIC									9	13	5		
	Broth	MIC									11	13	3		
		24-hour MLC									1	8	16	2	
Chloramphenicol	Agar	MIC								5	22				
	Broth	MIC									4	23			
		24-hour MLC										17	9	1	
Metronidazole	Agar	MIC					1	4	21	1					
	Broth	MIC						1	15	10	1				
		24-hour MLC								18	7	1	1		

cefoxitin, cefmetazole, clindamycin, chloramphenicol and metronidazole were slightly lower in agar than in broth. In contrast, the agar dilution MICs of latamoxef and minocycline were almost the same or slightly higher than the broth dilution MICs. The 24-hour MLCs were almost the same or 2~4 times higher than the broth dilution MICs for these drugs. In summary, the difference between the agar dilution MICs and 24-hour MLCs was relatively small.

Correlation of Timed Antibacterial Activities and the Conventional Killing-curve Result

The cumulative per cents of strains inhibited (MRCs and MIC) and killed (MLCs) by increasing cefoxitin concentrations, with the indicated incubation periods, are presented in Fig. 2. It is apparent that the 6-hour MRC for most of the strains was 6.25 μg of this compound per ml, which approximately agreed with the MIC. The 24-hour MRC was slightly higher than this concentration. However, the 6-hour MLC was 2~16 times higher than the 6-hour MRC. The 24-hour MLC was lower and approached the 24-hour MRC and MIC levels. The ratios of 24-hour MLC/24-hour MRC and 24-hour MLC/MIC then became smaller, suggesting that the apparent mode of action of this drug is bactericidal with prolonged periods of incubation. Fig. 3 gives the result of 27 strains of *B. fragilis* with clindamycin. In contrast to cefoxitin, the 6-hour MLC was 8 fold or larger than the 6-hour MRC for most of the strains. This result indicates that the action of this drug was bacteriostatic on the test strains, if the incubation period was as short as 6 hours. The 24-

Fig. 2. Activities of cefoxitin against 27 strains of *B. fragilis*.

The result shows the cumulative percent of strains inhibited (MRCs) and killed (MLCs) after indicated periods of incubation (6 hours and 24 hours) with increasing concentrations of the drug. Conventional MIC is also illustrated.

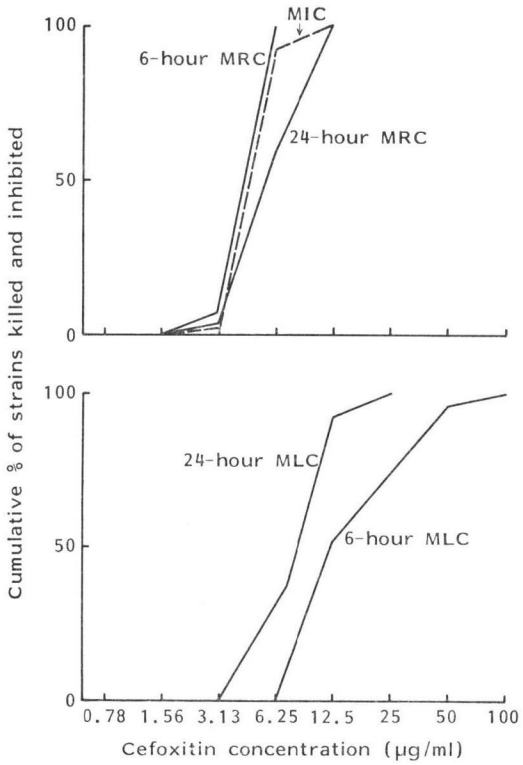
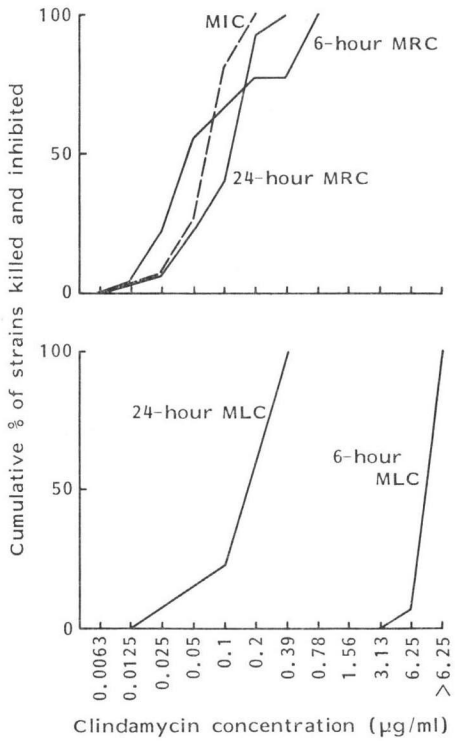


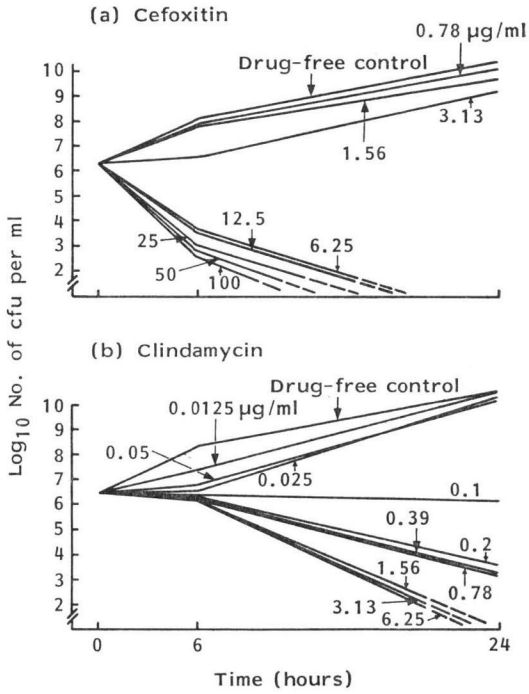
Fig. 3. Activities of clindamycin against 27 strains of *B. fragilis*.

Abbreviations are the same as in Fig. 2.



hour MLC was lower than the 6-hour MLC. This low value of 24-hour MLC produced small ratios of bactericidal/bacteriostatic concentrations, indicating that this drug is bactericidal if a prolonged period of incubation is used. That cefoxitin acts bactericidally, and clindamycin bacteriostatically, during the first 6-hour period of incubation, and that the latter then becomes bactericidal by 24 hours of incubation, was also confirmed by the conventional killing-curve study. Cefoxitin (Fig. 4, a) produced a rapid decrease of cfu, during the 6-hour incubation period, while with clindamycin (Fig. 4, b), there was no significant reduction during such a brief period of incubation. Higher concentration of clindamycin resulted in a significant reduction of cfu, if the incubation was prolonged to 24 hours.

Fig. 4. Effect of (a) cefoxitin and (b) clindamycin on a strain of *B. fragilis*.



Assessment of the Apparent Mode of Action of Antimicrobial Agents in Relation to Time of Incubation

The comparative data on timed bacteriostatic with bactericidal concentrations of the 7 antimicrobial agents is summarized in Table 4. With cefoxitin, the ratio of 6-hour MLC/6-hour MRC was 2 for 12,

Table 4. Comparison of timed bacteriostatic concentrations with timed bactericidal concentrations of 7 antimicrobial agents for 27 strains of *B. fragilis*.

Agent	Type of comparison	Ratio			
		1	2	4	≧8
Cefoxitin	6-hour MLC/ 6-hour MRC		12	8	7
	24-hour MLC/24-hour MRC	18	9		
	24-hour MLC/MIC	10	16	1	
Cefmetazole	6-hour MLC/ 6-hour MRC	2	9	8	8
	24-hour MLC/24-hour MRC	17	10		
	24-hour MLC/MIC	14	13		
Latamoxef	6-hour MLC/ 6-hour MRC		8	11	8
	24-hour MLC/24-hour MRC	19	4	3	1
	24-hour MLC/MIC	18	5	3	1
Clindamycin	6-hour MLC/ 6-hour MRC				27
	24-hour MLC/24-hour MRC	10	17		
	24-hour MLC/MIC	3	18	6	
Minocycline	6-hour MLC/ 6-hour MRC				27
	24-hour MLC/24-hour MRC	12	15		
	24-hour MLC/MIC	2	23	2	
Chloramphenicol	6-hour MLC/ 6-hour MRC		2	2	23
	24-hour MLC/24-hour MRC	16	9	2	
	24-hour MLC/MIC	15	9	3	
Metronidazole	6-hour MLC/ 6-hour MRC		8	11	8
	24-hour MLC/24-hour MRC	5	20	2	
	24-hour MLC/MIC	4	19	3	1

4 for 8, and $\geqq 8$ for 7, of the 27 strains. If the apparent mode of action of a drug is categorized as bactericidal when the ratio is low ($\leqq 4$), and bacteriostatic when high ($\geqq 8$)[5], then cefoxitin can be considered to be bactericidal with 20 (12 plus 8) and bacteriostatic with 7 of the 27 strains, when the exposure time is brief (6 hours). This drug acted bactericidally on all the 27 strains if a prolonged (24 hours) period of exposure was used (bactericidal action being defined by the ratios of 24-hour MLC/24-hour MRC and 24-hour MLC/MIC). With the above system of categorization, clindamycin, minocycline and chloramphenicol tended to be bacteriostatic, and cefoxitin, cefmetazole, latamoxef and metronidazole bactericidal, when the exposure time was 6 hours. All these drugs acted bactericidally, on most of the strains, if the exposure was prolonged to 24 hours.

Discussion

Elimination of microbes from infected sites is usually a prerequisite in the management of acute bacterial infections. Phagocytic cells such as neutrophiles and macrophages, which ingest and kill microbes, are generally accepted to constitute the first internal defense mechanism of the human body against bacterial invasion. In patients whose defense mechanisms are seriously impaired, antimicrobial agents often have to substitute for the role of these phagocytic cells. Therefore drugs with more potent activity, such as bactericidal agents, are generally used especially in treatment of infections in seriously affected patients. There are many patients whose defense mechanisms are impaired to varying degrees[6]. The assessment of not only the bacteriostatic but also the bactericidal action of antimicrobial agents is therefore of importance for use in the analysis of *in vitro* data, and in the assessment of clinical effectiveness.

Assessment of effective drug concentrations in relation to the time of incubation is also essential, since the duration of effective concentrations of most drugs needed to inhibit or kill the microbes at infected foci is short and rarely exceeds 24 hours. The data obtained in the present study appear, however, to give the actual antimicrobic concentrations at shorter incubation periods than indicated (6 hours and 24 hours), since the GasPak system requires more than 1 hour for generation of anaerobic conditions[7].

It was evident that the apparent mode of action of the β-lactams, cefoxitin, cefmetazole and latamoxef and metronidazole was bactericidal while for clindamycin, minocycline and chloramphenicol it was bacteriostatic, if the incubation period was 6 hours. This result is comparable to that where aerobes were used as the test organism[8]. All these seven drugs became bactericidal when the incubation was prolonged to 24 hours. Such time-related variation in mode of action should be taken into account in the scheduling of multiple dosing regimens for drug administration[9,10]. In addition, the possible presence of L-form variants[11] induced by high antibiotic concentrations should also be considered in the use of antibiotics which inhibit cell wall synthesis.

As in the present study, the "lethal" concentration of an antimicrobial agent is usually seen as the minimal concentration which produces a 99.9% kill. However, the bactericidal or "lethal" concentration varies according to the definitions used by various authors; *e.g.*, that producing a 99% kill[12], 99.9% kill[1,3~5,13], 99.99% kill[8], or complete kill[13,14].

Further work will be necessary before a more complete understanding of this area can be attained.

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