SOME EFFECTS OF SUBINHIBITORY CONCENTRATIONS OF ANTIBIOTICS ON BACTERIA*

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THE medical value of an antibacterial drug is usually considered to be its contribution to the extermination of a specific microorganism from the site of infection. This is accomplished either by killing the bacteria or by interfering with their multiplication. One laboratory criterion for determining these effects is the survival of the organisms during exposure to the drug. Other criteria are changes in bacterial structure or metabolism. An "all or nothing" effect is rare. Between the extremes of no survivors or no multiplication and no effect or total survival there is a range of effects related primarily to drug concentration. Some of these effects are reviewed in this paper.

Concentrations of antibacterial agents that are greater than or equal to the minimum inhibitory concentration (MIC) or the minimum bactericidal concentration (MBC) produce dramatic changes in bacteria. At lower concentrations these effects might be expected to be proportionately milder. In reality, however, concentrations of antibiotics lower than the MIC or MBC usually show qualitatively different effects rather than milder ones. Certain morphologic changes in Gram-negative bacilli[3, 5, 6, 11, 13] and Gram-positive cocci[4, 7, 21] have been observed only with concentrations of antibiotic lower than the MIC. Both staphylococci and pneumococci produce a certain β-hemolysis only when grown in the presence of subinhibitory concentrations of penicillins.[9, 14] Only at subinhibitory concentrations does rifampin cure F^+ *Escherichia coli* from the episomes.[18]

MIC and MBC are useful terms to indicate antibacterial activity

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at high concentrations, but there has been no comparable term to define antibacterial activity at low concentration. A term was needed to indicate the lowest concentration of an antibacterial agent that would affect the rate of growth or the structure of bacteria or both. "Minimum Antibiotic Concentration" (MAC) has been suggested for this purpose.[11, 13] The significance of the MAC is perhaps best illustrated when it is expressed as a fraction of the MIC.

Penicillins and Cephalosporins

The relation of the MAC/MIC ratio to structural changes was studied in a strain of *Proteus mirabilis* exposed to several penicillins and cephalosporins. Penicillins showed a ratio of 1:19, while cephalosporins showed a ratio of 1:3.[13] At the MAC, the penicillins produced filaments while the cephalosporins produced globules.[13, 17] The cephalosporins did not produce filaments at any subinhibitory concentration studied.

Penicillins and cephalosporins are supposed to act by the same mechanism, i.e., inhibition of the D-ala-NH_2 cross-linkage between the pentapeptides of the cell wall.[2, 20] It has been reported that penicillins at subinhibitory concentrations inhibit the bacteria's production of autolytic enzymes without affecting growth.[8, 19] In contrast, the cephalosporins affect growth but do not inhibit the production of autolytic enzymes.[1] It is probable that the difference in the morphological changes observed with subinhibitory concentrations of these two similar antibacterial drugs is a result of their different effects on the autolytic enzymes.[13] If there is a difference in the morphological effect there should be some difference in the mode of action.

In order to determine whether the same MAC/MIC ratio applies to sensitive as well as to highly resistant organisms, five strains of *Escherichia coli* sensitive to 3 to 6 μg./ml. ampicillin and five strains of *E. coli* approximately 3,000 times more resistant to ampicillin (MIC between 8,000 and 10,000 μg./ml.) were exposed to subinhibitory concentrations of ampicillin and examined microscopically for structural changes. It was observed that the MAC/MIC ratio was similar for the sensitive and the resistant strains (Table I).

The bactericidal effect of subinhibitory concentrations of ampicillin and cephaloridine was determined for two strains of *E. coli* and two strains of *P. mirabilis*. After six hours, at the MIC and at one half the MIC both drugs had killed 99.99% of the organisms as compared to

TABLE I. EFFECT OF SUBINHIBITORY CONCENTRATIONS OF AMPICILLIN ON SENSITIVE AND RESISTANT STRAINS OF *E. COLI*.

E. coli Strain	*Ampicillin (μg./ml.)*		
	MIC	*MAC*	*MAC/MIC*
1	3.12	0.39	1/8
2	3.12	0.39	1/8
3	6.25	0.39	1/16
4	3.12	0.18	1/16
5	3.12	0.39	1/8
6R	10,000	1,250	1/8
7R	8,000	1,250	1/6
8R	10,000	625	1/16
9R	10,000	312	1/32
10R	8,000	625	1/13

the control. At one quarter the MIC ampicillin still produced more than 99% killing; however, cephaloridine produced a decrease of only one log in the growth of *P. mirabilis*. At one sixth and one eighth the MIC ampicillin still produced a kill rate of more than 90%, while cephaloridine showed only a slight bacteriostatic effect for both *P. mirabilis* and *E. coli*.

The filaments, globules, and elongated bacilli resulting from the exposure of *P. mirabilis* to ampicillin or cephalothin at concentrations between the MIC and one thirty-second of the MIC were examined by electron microscopy and the number of ribosomes per square micron was counted. Both ampicillin and cephalothin caused a significant reduction (up to 50%) in the number of ribosomes in cells exposed to one eighth, one quarter, or one half of the MIC.[15] The filaments did not show any significant change in the cell wall or the cytoplasmic membrane at or below one half the MIC. The finding of reduced ribosomal density at lower concentrations than those required to stop growth or cause serious defects in the cell wall is in contrast to the current view that the initial lesion produced by penicillins is a defect in the synthesis of murein.[20]

Experiments with subinhibitory concentrations of penicillin and *Staphylococcus arueus* (strain 209 P) revealed another site of action of penicillin.[10]

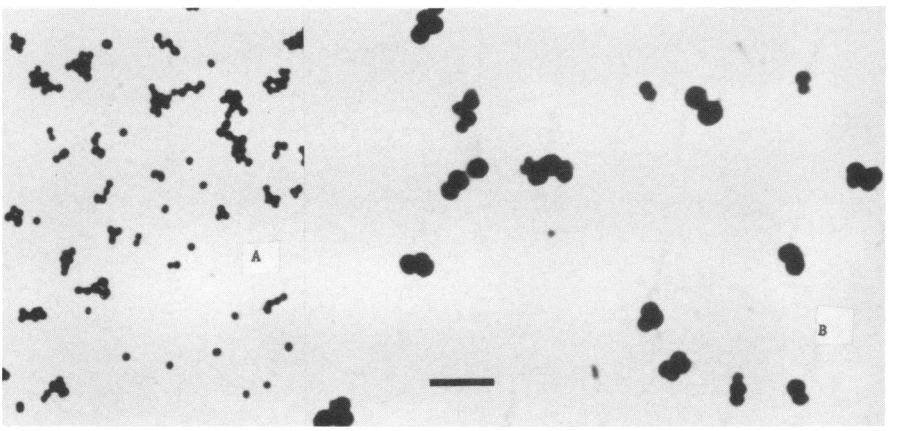

Fig. 1. *Staphylococcus aureus* stained by gram (1,400×). Bar indicates 10 μm. A—Control grown on drug-free media. B—Grown on media containing penicillin at ⅓ the minimum inhibitory concentration (MIC).

The study suggested that subinhibitory concentrations of penicillin did not kill staphylococci and did not produce significant alterations in the peripheral cell wall. Subinhibitory concentrations of penicillin seem to inhibit lysis of the cross wall without inhibiting cell division. This resulted in the formation of what appeared to be abnormally large cells but which were in fact clusters of staphylococci, prevented from separating by the presence of many wide cross walls (Figures 1 and 2). The inhibition of cross-wall separation was reversible. When incubated on drug-free media, the clusters of unseparated staphylococci separated into smaller clusters and cells of normal appearance and growth rate.

Growth Rate

The effects of subinhibitory concentrations of penicillin, ampicillin, cephaloridine, tetracycline, chloramphenicol, and gentamicin on the growth rate of *Staphylococcus aureus*, *Salmonella typhimurium*, and *E. coli* were investigated. The results are shown in Table II. The number of colony-forming units of the organisms grown in broth with antibiotics was smaller after six hours than in the control. A reduction of one log was observed for *S. aureus* at concentrations equal to one

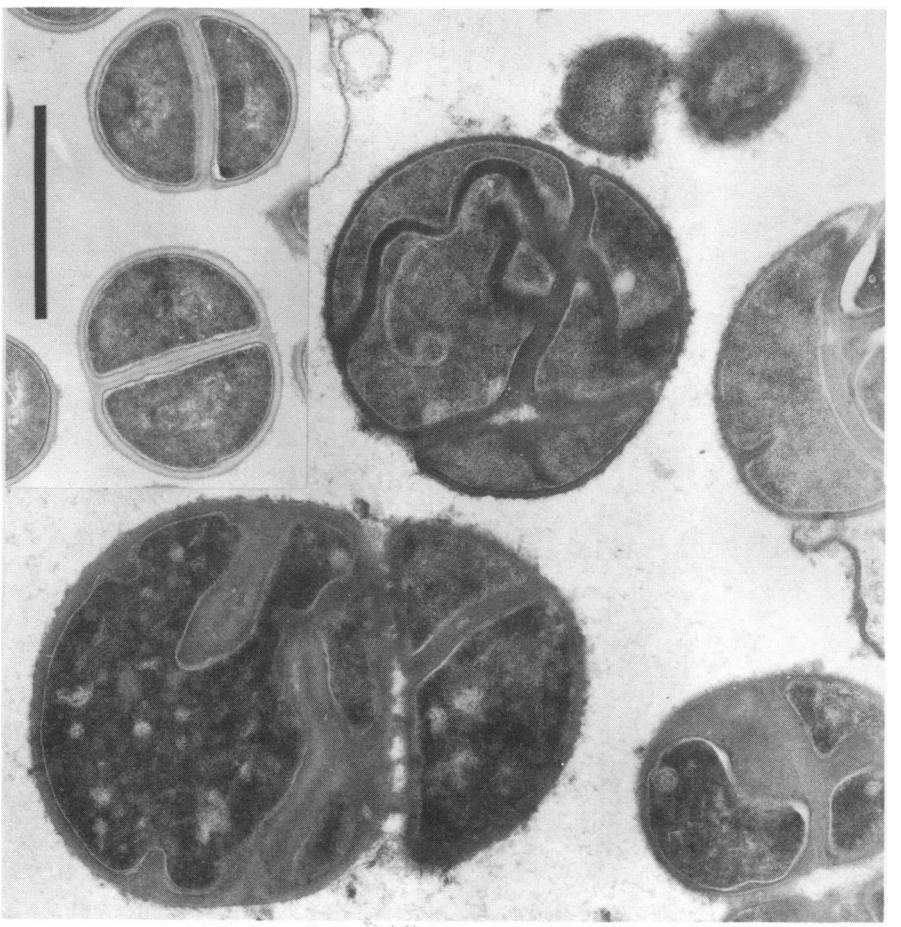

Fig. 2. *Staphylococcus aureus* grown on agar containing ⅓ MIC of penicillin. Insert (upper left) shows control cells. Electron micrograph (30,000×). Bar indicates 1 μm.

third the MIC of cephaloridine, one eighth the MIC of penicillin, and one twenty-fourth the MIC of gentamicin. S. *typhimurium* was inhibited at the same rate by tetracycline at one third the MIC and gentamicin at one twentieth the MIC.

Structure

Subinhibitory concentrations of drugs can produce characteristic lesions in bacteria. *S. aureus* exposed to one third the MIC of penicillin grew three to five times larger than the control and contained numerous

TABLE II. SUBINHIBITORY CONCENTRATIONS OF VARIOUS ANTIBIOTICS EXPRESSED AS FRACTIONS OF THE MIC (MAC/MIC RATIOS) PRODUCING A DECREASE OF AT LEAST ONE LOG IN THE BACTERIAL POPULATION IN COMPARISON TO THE CONTROL AFTER SIX HOURS.

	Antibiotics						
Organism	*Penicillin-G*	*Ampicillin*	*Cephaloridine*	*Tetracycline*	*Chloramphenicol*	*Gentamicin*	*Lincomycin*
S. aureus							
MIC*	0.06		0.05	5.0	5.0	2.0	1.3
MAC/MIC†	1/8		1/3	1/8	1/6	1/24	1/8
Salmonella							
MIC*		2.5	12.0	45.0	7.5	10.0	
MAC/MIC†		1/10	1/8	1/3	1/6	1/20	
E. coli							
MIC*		5.0	6.0	20.0	10.0	8.0	
MAC/MIC†		1/12	1/4	1/4	1/8	1/12	

*μg/ml.
†MAC/MIC producing at least a one log decrease in the bacterial population in comparison to the control.

thick septa. In contrast, *S. typhimurium*, *E. coli*, and *P. mirabilis* exposed to one quarter the MIC of ampicillin grew into filaments and showed no signs of division at all. *S. typhimurium* exposed to one half the MIC of gentamicin showed a generally lower density of ribosomes, except at the ends of the cell. This might have caused a relative transparency in the middle of the cell that could account for the bipolar appearance observed on the Gram stain. In laboratory practice such abnormal forms of bacteria produced by subinhibitory concentrations of antibiotics can simulate the appearance of different species and can on occasion indicate the presence of antibiotics at the site of infection.[12]

AGGLUTINATION

Since the antigenic constituents of the cell wall of Gram-negative bacteria for the most part attached by covalent linkages to the peptidoglycan,[19] it is conceivable that antibacterial agents that affect the peptidoglycan or produce morphologic and biochemical changes in the cell wall could cause changes in the antigenic structure of Gram-negative bacilli. The fluorescent antibody fixation and the agglutinability

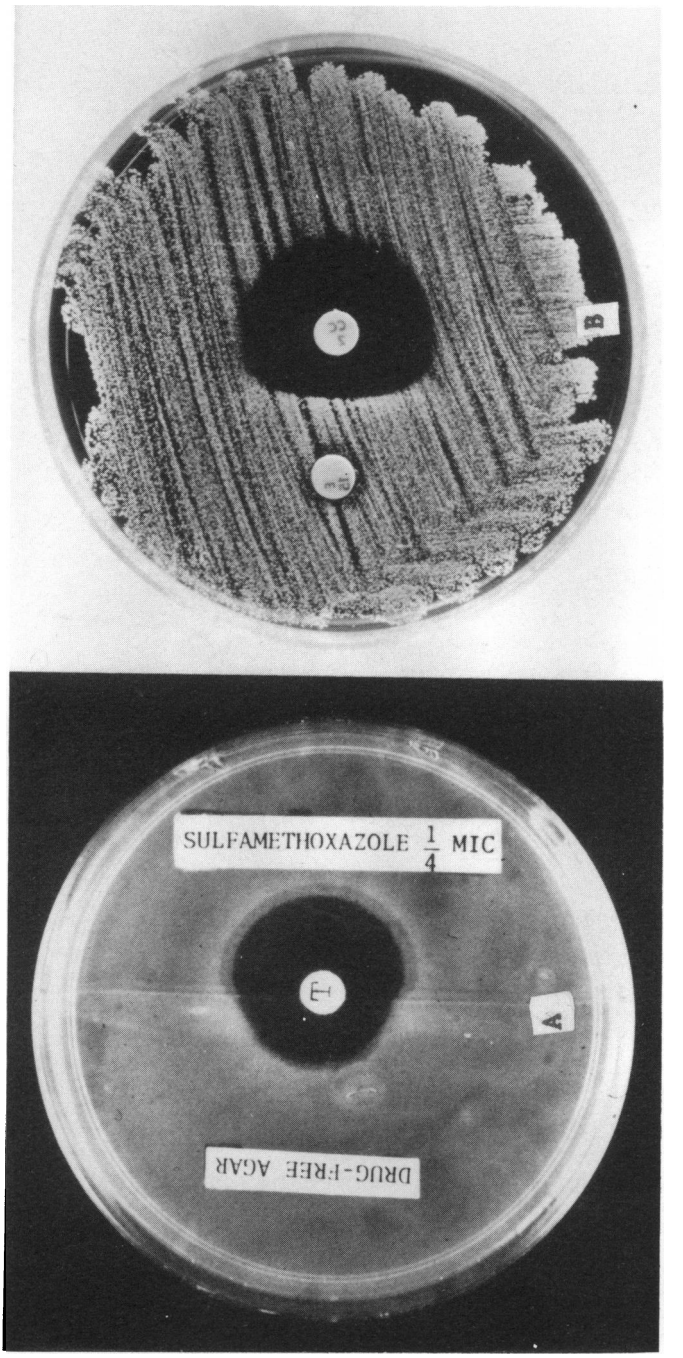

Fig. 3. Effect of subinhibitory concentrations of antibacterial agents in combination. A—Synergism, sulfamethoxazole, although not active alone, enhances the effect of trimethoprim. B—Antagonism. Erythromycin, not active alone, impairs the activity of clindamycin.

with somatic O antisera were tested on five strains of *Salmonella* grown in the presence of ampicillin, gentamicin, tetracycline, or chloramphenicol. There was practically no difference between the fluorescence of the control cells and that of the cells grown on agar containing the subinhibitory concentrations of antibiotics. The filaments of *Salmonella* produced with ampicillin showed the same fluorescence as the control bacilli. This should indicate that there are no significant qualitative changes in morphology. The agglutinability of the bacteria grown on agar with antibacterial agents, however, was different from that of the control. The agglutination titer of bacilli exposed to tetracycline, gentamicin, and chloramphenicol was lower than the titer in the control. The agglutination titer of filaments was higher than that of the control.[16]

Synergy and Antagonism

It is the current view that synergy between two antibacterial agents can occur only when the organism in question is susceptible to each of the two drugs separately.

Subinhibitory concentrations of one antibiotic can, however, act synergistically or antagonistically in combination with other drugs. Sulfamethoxazole significantly enhanced the antibacterial effect of trimethoprim against a strain of *E. coli* that was not sensitive to sulfamethoxazole at the concentrations used (Figure 3A).

Erythromycin significantly inhibited the antibacterial effect of clindamycin against a strain of *S. epidermidis* that was resistant to erythromycin (Figure 3B).

Conclusions

Experiments with subinhibitory concentrations of antibiotics have revealed certain effects that had not been observed at higher concentrations.

1) The different morphological changes produced by penicillins and by cephalosporins in *P. mirabilis* suggest a difference in their mode of action.

Penicillins have a higher MAC/MIC ratio (a wider range of bactericidal effect) for *P. mirabilis* and *E. coli* than do cephalosporins, whether the strain is sensitive or highly resistant to the drug.

Both ampicillin and cephaloridine cause a reduction in the number

of ribosomes at lower concentrations than that which causes a visible defect in the cell wall. This suggests that an effect on ribosomes may precede the effect on the cell wall.

2) There were significant differences among the MAC/MIC ratios with regard to growth rate when *S. aureus*, *E. coli*, and *S. typhimurium* were grown in the presence of various drugs. Tetracycline produced a decrease of one log in a *S. typhimurium* population at one third the MIC, while gentamicin produced a comparable effect at one twentieth the MIC. It is possible that a drug which exhibits a large difference between the MAC and the MIC is more effective therapeutically than a drug that shows only a small difference.

3) Subinhibitory concentrations of certain antibacterial agents can produce characteristic lesions in bacteria. Penicillins at one third the MIC produced an increase in the number and thickness of cross walls in staphylococci and totally inhibited division in Gram-negative bacilli.

4) Exposure to some antibiotics produced a decrease in the agglutinability of salmonellas, but the filaments that resulted from exposure to ampicillin showed a higher agglutination titer than the control.

5) Subinhibitory concentrations of antibiotics can act synergistically or antagonistically with other drugs.

The expression Minimum Antibiotic Concentration has thus been shown to be of both theoretical and practical value. It has served to make plain the differences between the effects of various antibiotics that were not observed at the MIC, but which could be of importance when choosing a drug for the treatment of infections. The MAC defines the antibacterial activity of drugs at low concentrations just as the MIC and MBC define antibacterial activity at high concentrations.

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