STUDIES ON ANTIBIOTIC SYNERGISM AND ANTAGONISM

SYNERGISM AMONG SEVEN ANTIBIOTICS AGAINST VARIOUS BACTERIA
IN VITRO¹

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In an increasing number of microbial infections of man, treatment with single antibiotics fails to cure. Some of these infections, however, respond favorably to combined treatment with two antibiotic drugs. Certain pairs of these drugs have proved successful in a limited number of clinical situations. An outstanding example is the frequent cure of bacterial endocarditis due to enterococci by the simultaneous administration of penicillin and streptomycin, whereas neither of these drugs alone cures more than a small number of cases (Hunter, 1950; Robbins and Tompsett, 1951; Cates, Christie, and Garrod, 1951). The synergism of penicillin and streptomycin in vitro is manifested by a marked increase in the bactericidal rate beyond that obtainable with any concentration of either drug alone and also by the killing of the entire exposed bacterial population, a feat which neither of the two drugs can achieve alone (Jawetz, Gunnison, and Coleman, 1950; Gunnison, Jawetz, and Coleman, 1950).

As more microorganisms which are resistant to certain antibiotics are encountered in medical practice, there is an increased temptation to employ more than one antimicrobial drug routinely. Such indiscriminate use of two drugs is probably inadvisable inasmuch as antagonism between certain antibiotics has been demonstrated both in vitro and in experimental animals (Jawetz, Gunnison, Speck, and Coleman, 1951; Speck, Jawetz, and Gunnison, 1951; Jawetz, Gunnison, and Speck, 1951). To obtain a rational basis for the selection of antibiotics for combined therapy it is essential that the relationships among these drugs be studied.

This report deals with some quantitative relationships in several synergistic combinations of antibiotics active against a number of different bacteria. The results may contribute clues to the mechanism of combined antibiotic action. An attempt has been made to answer some of the questions which arise as to the combined effect of two antibiotics in vitro.

MATERIALS AND METHODS

Bacteria. The following organisms were studied: Streptococcus faecalis (enterococcus), strains no. 16 and no. 17, from cases of bacterial endocarditis; Streptococcus pyogenes, strain C203; Micrococcus pyogenes var. aureus, strain D, from a case of bacterial endocarditis and the Heatley strain (no. 4); Klebsiella pneumoniae.

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moniae, strain A-D; Proteus vulgaris, strain C from an infection of the urinary tract, strains Ro and Mi from wound infections, and strain Mo from a patient's intestinal flora.

Media. Proteose no. 3 agar (Difco) and broth of similar composition were used. For S. pyogenes, one per cent blood was added to the agar. For P. vulgaris, 0.125 per cent chloral hydrate was added to the agar to prevent swarming.

Antibiotics. Stock solutions in sterile buffered 0.85 per cent salt solution were stored in the refrigerator and final dilutions were prepared in broth immediately before use. Commercial crystalline preparations of potassium penicillin G and streptomycin sulfate were used. Aureomycin hydrochloride was supplied by Dr. S. Hardy; terramycin hydrochloride by Dr. H. Anderson; chloramphenicol by Dr. G. Rieveschl; bacitracin by Dr. L. Smith; and neomycin sulfate by Dr. E. L. Burbridge.

Methods. Broth containing the diluted antibiotics was inoculated with an 18 hour culture of the test bacteria to give a concentration of $10^7$ to $10^8$ organisms per ml in a total volume of 15 ml. Samples of 0.5 ml were removed at intervals during incubation at 37 C, and the number of viable bacteria was estimated by plate counts. Details of the methods and criteria for the significance of observed results have been published previously (Gunnison, Jawetz, and Coleman, 1950).

RESULTS

In this series of studies synergism in vitro between two antibiotics is defined as a marked increase in the rate of bactericidal action within the first 24 hours of exposure as compared to the rate with either drug alone, and killing of greater numbers of bacteria than could be expected from simple summation of single drug effects. Antagonism between two antibiotics is defined as a decrease in the bactericidal rate as compared to the more active drug. Ultimately, after extended incubation, both synergistic and antagonistic pairs of drugs are more likely to kill all exposed bacteria in vitro than are single drugs.

Synergism was shown by at least one pair of antibiotics against each of the microorganisms studied (table 1). Antagonism was exhibited by some pairs of drugs against certain of the test organisms (table 1). As every combination of drugs was not tested against every organism, table 1 is illustrative rather than complete. It emphasizes, however, that each bacterial strain reacted differently to the antibiotic mixtures so that no generalization can be made about which pairs of drugs exhibit synergism even against a given bacterial species. For example, although one strain of Micrococcus pyogenes var. aureus was highly susceptible to a combination of streptomycin and terramycin, another strain of this species was not affected as strikingly by this pair of drugs. Furthermore, the synergistic action of penicillin and streptomycin was more marked against one strain of enterococcus than against the other. Of four strains of P. vulgaris two displayed synergism between penicillin and chloramphenicol while the other two were unaffected by this combination. Hence, the response of bacteria to drug mixtures cannot be predicted but is characteristic of the individual strain.

What is the relationship between the antimicrobial activity of a drug when
used alone and its participation in synergistic action with a second drug? As illustrated in figure 1, an antibiotic employed in a concentration having no apparent effect on the rate of bacterial multiplication may give synergism when added to another drug exhibiting only slight inhibitory action; i.e., an ineffect-

**TABLE 1**

*Synergistic and antagonistic pairs of antibiotics in vitro*

<table>
<thead>
<tr>
<th>ANTIBIOTICS</th>
<th>STREPTOCOCCUS PARACASIS</th>
<th>STREPTOCOCCUS PYOGENES</th>
<th>MICROCOCCUS PYOGENES VAR. AUREUS</th>
<th>KLEBSIELLA PNEUMONIAE</th>
<th>PROTEUS VULGARIS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strain no. 16</td>
<td>Strain C203</td>
<td>Strain H</td>
<td>Strain D*</td>
<td>Strain A-D</td>
</tr>
<tr>
<td>Penicillin + streptomycin</td>
<td>+++ +</td>
<td>+++ +</td>
<td>0</td>
<td>+++ +</td>
<td>+</td>
</tr>
<tr>
<td>Penicillin + bacitracin</td>
<td>+++ +</td>
<td>+++ +</td>
<td>0</td>
<td>+++ +</td>
<td>+</td>
</tr>
<tr>
<td>Penicillin + neomycin</td>
<td>- - -</td>
<td>- - -</td>
<td>0</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Penicillin + chloramphenicol</td>
<td>- - -</td>
<td>- - -</td>
<td>0</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Penicillin + aureomycin</td>
<td>- - -</td>
<td>- - -</td>
<td>0</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Penicillin + terramycin</td>
<td>- - -</td>
<td>- - -</td>
<td>0</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Streptomycin + bacitracin</td>
<td>+++ +</td>
<td>+++ +</td>
<td>+++ +</td>
<td>+++ +</td>
<td>++</td>
</tr>
<tr>
<td>Streptomycin + neomycin</td>
<td>- - -</td>
<td>- - -</td>
<td>0</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Streptomycin + chloramphenicol</td>
<td>0</td>
<td>0</td>
<td>+ +</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Streptomycin + aureomycin</td>
<td>0</td>
<td>0</td>
<td>+ +</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Streptomycin + terramycin</td>
<td>0</td>
<td>0</td>
<td>+ +</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bacitracin + neomycin</td>
<td>- - -</td>
<td>- - -</td>
<td>+++ +</td>
<td>++ +</td>
<td>+</td>
</tr>
<tr>
<td>Bacitracin + chloramphenicol</td>
<td>- - -</td>
<td>- - -</td>
<td>+ +</td>
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<td>-</td>
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<tr>
<td>Bacitracin + aureomycin</td>
<td>- - -</td>
<td>- - -</td>
<td>+ +</td>
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<td>-</td>
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<tr>
<td>Bacitracin + terramycin</td>
<td>- - -</td>
<td>- - -</td>
<td>+ +</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Neomycin + chloramphenicol</td>
<td>- - -</td>
<td>- - -</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Neomycin + aureomycin</td>
<td>- - -</td>
<td>- - -</td>
<td>+ +</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Neomycin + terramycin</td>
<td>- - -</td>
<td>- - -</td>
<td>+ +</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

0 = No synergism nor antagonism of concentrations tested, effect = that of more active drug.

- = Antagonism = decrease in bactericidal rate below that of the more active drug.

+ = Synergism = slight increase in bactericidal rate, some organisms survive.

++ = Synergism = increase in bactericidal rate within 24 hours, all organisms usually killed.

+++ = Synergism = increase in bactericidal rate within 8 hours, all organisms usually killed.

Blank = not tested.

*Completely penicillin resistant (500 units not bacteriostatic).*

Maximal synergism was...
usually shown when both drugs were employed in static or slowly bactericidal concentrations; if either drug was rapidly bactericidal alone, the synergistic effect, of course, was less readily observed (table 2).

Is antibiotic synergism a mutual, reciprocal phenomenon between two drugs or is it a unilateral one with one drug always augmenting the action of the other in a fixed relationship? Previous experiments dealing with antibiotic antagonism suggest that the phenomenon of interference of one drug with the action of a second drug is unilateral (Jawetz, Gunnison, and Speck, 1951; Speck, Jawetz, and Gunnison, 1951; Jawetz, Gunnison, and Speck, 1951). Thus, aureomycin, chloramphenicol, and terramycin can interfere with the action of either penicillin or streptomycin, but not vice versa. Antibiotic synergism, on the other hand, seems to be a reciprocal phenomenon in those combinations examined thus far. As illustrated in table 2, synergism was observed between bacitracin and terramycin acting on a micrococcus strain (no. 4), provided that either drug was present in a bacteriostatic concentration. This is clearly a mutual effect, not the unilateral augmentation of one drug by another. Similar mutual effects were seen with mixtures of penicillin and streptomycin acting upon Klebsiella and various streptococci (figures 1 and 2). However, in all instances thus far studied, penicillin must be used in a concentration at least slightly bacteriostatic in order to show a synergistic effect. For example, although an apparently in-

Figure 1. The effect of varying concentrations of penicillin and streptomycin on the viable count of an enterococcus. Synergism is demonstrable when either drug is present in a concentration which is active alone, while the other drug is employed in a concentration having little effect alone.
effective concentration of streptomycin plus a bacteriostatic amount of penicillin may result in marked synergism, the reverse may not be true. As long as the penicillin was at least able to inhibit growth, however, it made no difference whether the penicillin or the streptomycin was present in the more active concentration (figure 1). Hence, although the phenomenon is a mutual one, the detailed quantitative aspects of synergistic relationships vary with different drugs and organisms.

Might certain drug combinations exhibiting synergism against a given microorganism be antagonistic in other concentrations? The data in table 2 and figure 1 and in similar surveys with other systems have failed to reveal any instance of both synergistic and antagonistic effects occurring with a given pair of drugs

<table>
<thead>
<tr>
<th>TERRAMYCIN</th>
<th>BACTRACIN UNITS/ML</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>µg/ml</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>Ineffective</td>
</tr>
<tr>
<td>0.25-0.5</td>
<td>Bacteriostatic</td>
</tr>
<tr>
<td>2.0</td>
<td>Slowly bactericidal</td>
</tr>
<tr>
<td>10.0</td>
<td>Rapidly bactericidal</td>
</tr>
</tbody>
</table>

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- = No synergism, effect equals that of more active drug alone.
+ = Slight acceleration of bactericidal rate.
+++ = Moderate acceleration of bactericidal rate.
++++ = Marked acceleration of bactericidal rate.
Blank = Not tested.

Ineffective, bacteriostatic, bactericidal = effect of each concentration of drug when acting alone.

acting on a given bacterial strain. With changes in concentration of the two drugs resulting in a shift in their relative activity, a synergistic pair may cease to be synergistic and merely show the same effect as that of the more active drug, but it has never become antagonistic (table 2). Likewise, antagonism between two drugs may be overcome by changing their relative concentrations, but synergism in such a pair against the same organism has never resulted. When a given pair of drugs acts on two different bacterial species, however, it may act synergistically against one organism and antagonistically against the other (table 1). This is illustrated by a combination of terramycin and bacitracin in which terramycin is synergistic with bacitracin when acting on a strain (no. 4) micrococcus and yet shows interference with the bactericidal action of bacitracin on a strain (C203) of hemolytic streptococcus (table 1).

What might account for such differences in the reactions of a pair of drugs against two different organisms? The main observable difference between the synergistic bacitracin-terramycin versus micrococcus strain (no. 4) system and
the antagonistic bacitracin-terramycin versus streptococcus (strain C203) system is the great susceptibility of the streptococcus to bacitracin (inhibition by 0.01 unit/ml) and the relative resistance of the micrococcus (inhibition by 1 unit/ml). Similar observations have been made in other systems. For example, terramycin interferes with the bactericidal effect of streptomycin when both act on a streptomycin-sensitive strain of Klebsiella, but the same two drugs are synergistic against a streptomycin-resistant micrococcus (Jawetz, Gunnison, and Speck, 1951).

If drug A is synergistic with drug B against a given organism and drug B is synergistic with drug C against that organism, will drugs A and C also be synergistic? This question has not been answered unequivocally. As pointed out below the drugs in certain groups have been interchangeable in combinations synergistic against a variety of organisms. On the other hand, against micrococcus (strain no. 4), for example, penicillin plus streptomycin and streptomycin plus terramycin are synergistic, but penicillin plus terramycin are antagonistic (table 1).

Is it possible to designate certain pairs of drugs as "synergistic", others as "antagonistic"? It has been established earlier that synergism and antagonism with any given drug pair depend on the response of an individual strain of bacteria, and cannot be stated summarily even for a bacterial species. It has been
possible, however, to integrate our past experience with drug combinations into a hypothesis that may permit better planned experimentation in the future and that has already lent itself to a useful approach in clinical problems. An outline of this proposed hypothesis of combined antibiotic action is presented here.

The more common antibiotics appear to fall into two distinct groups: 1, penicillin, streptomycin, bacitracin, and neomycin; 2, aureomycin, chloramphenicol, and terramycin. Drugs of group 1 often have acted synergistically with one another, provided the test organism was somewhat susceptible at least to a high concentration of each member of the pair when used alone (Jawetz, Gunnison, and Coleman, 1950; Bachman, 1949). Antibiotics of group 1 have never exhibited antagonism to one another. Drugs of group 2 have shown neither synergism nor antagonism toward each other but sometimes gave simple additive effects.

If an organism was very highly sensitive to a member of group 1, then the addition of a drug from group 2 usually resulted in antagonism (Jawetz, Gunnison, Speck, and Coleman, 1951; Speck, Jawetz, and Gunnison, 1951; Jawetz, Gunnison, and Speck, 1951). Conversely, if the organism was relatively resistant to a member of group 1, and somewhat susceptible to group 2 agents, then addition of 1 and 2 occasionally yielded synergistic effects (Jawetz, Gunnison, and Speck, 1952; Spies et al., 1951). Drugs of group 1 have never interfered with drugs of group 2. Thus far synergism between penicillin and a drug of group 2 has been demonstrated in this laboratory in only two instances; namely, penicillin plus chloramphenicol was moderately synergistic against two strains (Ro and Mo) of P. vulgaris. Synergistic action of penicillin with other drugs of group 2 has been reported from other laboratories (Spies et al., 1951).

Drugs of a given group acted rather uniformly in a qualitative sense and sometimes could be substituted for one another. The concentrations entering into synergistic relationships, however, were a function of the sensitivity of individual bacterial strains. Whenever an organism was insensitive to a very large excess of any drug, that agent was incapable of participating in a synergistic pair. Supporting evidence for the preceding hypothesis will be presented elsewhere.

Polymyxin B has been tested in a number of instances in combination with drugs of both groups against representative organisms. No definite synergism nor antagonism could be established with any combination. This may be associated with the extremely sharp end point encountered in polymyxin titrations, i.e., the narrow range between concentrations devoid of any inhibitory effect and those rapidly lethal to exposed bacteria.

**DISCUSSION**

Synergism is defined for the purpose of this series of papers as the ability of a pair of drugs to produce a more rapid rate of bactericidal action within the first 24 hours of exposure than either member of the pair alone, and killing of greater numbers of bacteria than could be expected from simple summation of single drug effects. This definition is used because of its correlation with the therapeutic effects of combinations of antibiotics in infections in experimental animals and in man (Hunter, 1950; Cates, Christie, and Garrod, 1951; Jawetz,
Gunnison, Speck, and Coleman, 1951; Speck, Jawetz, and Gunnison, 1951; Jawetz, Gunnison, and Speck, 1951; Spies et al., 1951; Lepper and Dowling, 1951; Jawetz, Gunnison, and Speck, 1951). It is not implied that this is the sole, nor even the most generally applicable, form of synergism among antimicrobial agents.

The data presented here may throw some light upon underlying problems in the practical applications of this type of antibiotic synergism. In contrast to the unilateral antibiotic antagonism described previously (Jawetz, Gunnison, Speck, and Coleman, 1951; Speck, Jawetz, and Gunnison, 1951; Jawetz, Gunnison, and Speck, 1951), antibiotic synergism seems to be a reciprocal, mutual phenomenon. Ordinarily, only one member of a synergistic pair of drugs need exhibit inhibitory action in the concentration used. A drug concentration which alone lacks any apparent effect upon the microbial population may enter into striking synergism with another agent. Consequently, it is not logical to search for synergistic effects only among drugs which in low concentrations inhibit the microorganisms in the test tube. It seems true, however, that those drugs which are ineffective even in very high concentration against a given organism probably will not enter into synergistic combinations against that organism. These facts emphasize the difficulty in the empirical search for synergistic combinations to be used against strains resistant to single antibiotics.

It is impossible to state that any given pair of antibiotics is generally "synergistic" or "antagonistic". It would be completely misleading to claim that drug A should not be administered with drug B because of interference, or that drugs C and D should be given together because of synergism unless reference is made to a specific strain of bacteria. The evidence presented proves that a given pair of drugs may be synergistic when acting on one organism and antagonistic when acting on another. This applies not only to different species, but even to different strains of the same species of bacteria. The fundamental reasons for these differences in behavior are probably linked with basic features of bacterial physiology and will not be understood until the mode of action of the individual antibiotics is known.

Is synergism due to a simultaneous blocking effect of the two agents or does one drug so modify the bacterial cells as to make them more susceptible to the other? In experiments reported in detail elsewhere, the synergistic effects of two antibiotics added simultaneously were compared with the action of these drugs when added in sequence (Miles, Coleman, Gunnison, and Jawetz, 1952). In repeated tests with bacitracin plus terramycin versus micrococcus and with penicillin plus streptomycin versus enterococcus no synergism could be observed unless both drugs were present simultaneously.

The question likewise arises whether synergism might be the result of one drug reducing the number of bacteria in the exposed population to a low level at which the other antibiotic might be markedly active. Repeated tests have ruled out this possibility for a bacitracin-terracycin versus micrococcus system and for a penicillin-streptomycin versus enterococcus system (Gunnison, Jawetz, and Coleman, 1950). In the latter system, it has been established that
synergism cannot be explained by the ability of one drug to kill all but the most resistant members of the bacterial population which in turn are affected by the other drug (Gunnison, Jawetz, and Coleman, 1950). Although the rate of bactericidal action is a function of both the drug concentration and of the number and kind of bacteria acted upon, the rate with synergistic combinations is uniformly greater than that with either member of the combination at any population level. This suggests that synergism is not a sequential phenomenon and that it differs qualitatively in some respects from the dynamics of action of a single antibiotic.

It remains to be established whether the type of synergistic effect described here bears any relation to the desired therapeutic action of combinations of antibiotics in vivo. Antibiotic synergism and antagonism have been demonstrated in both acute and subacute experimental infections of mice, paralleling closely the results obtained in vitro. These experimental infections have a high, uniform mortality if untreated. From past experience it appears likely that results of treatment of these mouse infections have some bearing on the therapy of certain human diseases in which killing of infecting microorganisms rather than their inhibition is of paramount importance. Such diseases are subacute bacterial endocarditis and bacterial meningitis (Hunter, 1950; Lepper and Dowling, 1951). Observations in these diseases suggest that in vitro determination of synergism and antagonism aids in the selection of combinations of antibiotics for successful therapy. For example, a strain (M) of micrococcus was found highly susceptible to a mixture of streptomycin and terramycin while not inhibited in vitro by 50 µg per ml of any single antibiotic. The patient suffering from bacterial endocarditis caused by this organism was promptly cured by that combination after having failed to respond to other drugs (Jawetz, Gunnison, and Speck, 1951). In another instance, a strain of enterococcus (no. 16) was isolated from the blood stream of a patient subsequently cured by combined therapy with penicillin and streptomycin; this combination proved highly synergistic in vitro. Another strain (no. 17) of enterococcus, cultured from a patient with bacterial endocarditis who subsequently failed to respond to massive combined therapy with penicillin and streptomycin, did not exhibit marked synergism, and the two drugs did not destroy all exposed enterococci in vitro (Gunnison, Jawetz, and Coleman, 1950). Lepper and Dowling (1951) have demonstrated striking antibiotic antagonism in pneumococccic meningitis in man, predictable on the basis of experimental data (Speck, Jawetz, and Gunnison, 1951).

It must be remembered, on the other hand, that in many human infections prolonged inhibition of microorganisms by drugs will permit recovery of the host, and killing of the bacteria may not be essential. There is no evidence as yet to indicate what, if any, relationship may exist between the type of synergism expressed here as an increase in bactericidal rate and that manifested as augmented bacteriostasis in vitro. Work is in progress to attempt an understanding of this problem. It should be reemphasized that statements and conclusions made in this paper regarding synergistic action refer solely to synergism evi-
denced by a marked increase in rate of bactericidal action in vitro and corresponding results in experimental animal infections.

SUMMARY

Synergism between two antibiotics, manifested by an increase in the rate of death of exposed bacteria as compared to the rate with either drug alone, was demonstrated for various combinations of drugs acting on five species of pathogenic bacteria.

A given pair of drugs might act synergistically toward one species of bacteria, and yet one member of the pair might interfere with the action of the other against another species of bacteria. Furthermore, a given pair of drugs might be synergistic against one strain of a given species and antagonistic when acting on another strain of the same species. In a synergistic pair, at least one drug must exhibit at least bacteriostatic activity when acting alone. The synergism is a reciprocal, mutual phenomenon; either drug may be used in an active concentration in combination with a less effective or sometimes even an ineffective dose of the other drug. In no instance did a given pair of drugs show both synergism and antagonism when varying concentrations were tested against a given strain of bacteria.

The possible mechanism of synergism is discussed, and a hypothetical scheme of combined antibiotic action is presented.

REFERENCES


