COMBINED ANTIBIOTIC THERAPY FOR THE SUPPRESSION OF RESISTANT VARIANTS IN URINARY INFECTION

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One of the most important problems arising from antibiotic therapy is the emergence of resistant variants. While this is most serious in chronic diseases requiring prolonged courses of treatment, the treatment of acute infections can also result in the appearance of resistant organisms. Treatment will then be ineffective, and if the organism causes infection in others it will be resistant from the start. This phenomenon is best known in connexion with the staphylococci, a large proportion of which, in hospital, are now resistant to penicillin and already show evidence of becoming resistant to the newer antibiotics.

The use of pairs of antibiotics has been investigated by most workers primarily from the point of view of their synergistic or antagonistic action against the infecting organism, but it is becoming more widely recognized that of equal if not greater importance is the ability of combined treatment to suppress the emergence of resistant variants, which, if a single antibiotic was used, would continue the infection after the elimination of the parent strain (Carpenter *et al.*, 1945; Dowling, 1951; Klein and Schorr, 1953). Such combined treatment has been particularly successful in tuberculosis, and this problem has been reviewed by Mitchison (1954).

Antibiotics are now being used extensively for the treatment of urinary infections, some 80% of which are caused by the coliform group of organisms. With these organisms it is not uncommon for treatment with a single antibiotic to fail or for a recrudescence to occur owing to the emergence of a resistant variant. This is particularly so with streptomycin; indeed, with as many as 50% of urinary infections resistant organisms may appear and render further treatment with this antibiotic futile (Garrod, 1950). The use of the tetracyclines for urinary infections is also not uncommonly followed by the development of resistant variants (Herrold, 1951). That combined treatment might overcome these difficulties was suggested by Kaipainen (1952), who showed by means of repeated subcultures in tubes that when streptomycin was combined with either chloramphenicol or tetracycline strains of *E. coli* showed little or no increase in resistance to the antibiotics.

In urinary infections it is particularly important that effective methods of treatment should be available. Prolonged or repeated attacks are apt to lead to the development of chronic pyelonephritis with all its attendant dangers. The present work is concerned with an *in vitro* investigation of the value of combinations of two antibiotics in suppressing the emergence of resistant variants against strains of coliform bacilli isolated from cases of urinary infection. The simple technique of the gradient plate (Szybalski, 1952) was used for the investigation.

Methods

Gradient plates were prepared as illustrated in Fig. 1. The lower layer of nutrient agar was poured into a Petri dish, which was kept on a slant so as to form a layer of diminishing thickness from one side of the plate to the other. The upper layer contained antibiotic. The concentrations were 12 μ g. of streptomycin or chloramphenicol per ml., 6 μ g. of tetracycline per ml., or combinations of these antibiotics in the same concentrations. Streptomycin+tetracycline, streptomycin+chloramphenicol, and tetracycline+chloramphenicol were the combinations tested. The antibiotic diffuses downwards into the lower layer and within a short time there is established an even gradient of antibiotic concentration which remains relatively stable during the course of the experiment; from 12 μ g. of streptomycin or chloramphenicol per ml. or 6 μ g. of tetracycline per ml. at one end of the plate to practically zero at the other end. This range of concentration was found to be the most suitable for the majority of coliforms tested, and, moreover, encompassed the levels which commonly occur in the tissues of a patient between doses of the drugs.

FIG. 1.—Gradient plate. The lower layer of agar is poured into a Petri dish which is kept on a slant. When the lower layer has set an equal volume of agar containing antibiotic is poured on top.

Forty-two strains of coliform bacilli obtained from urinary infections were tested; 39 *E. coli*, 2 *A. aerogenes*, and 1 paracolon bacillus. These were grown for four to six hours in nutrient broth and the concentrations adjusted so that 0.5 ml. contained 10^8 organisms. This population was flooded over the surface of each plate. The plates were dried and incubated for 24 hours. The number of resistant colonies on each plate was counted and a replica of the plate was then made on to a nutrient agar surface by means of the velvet-pad method (Lederberg and Lederberg, 1952), in order to assess the bactericidal effect of the antibiotics (Hilson and Elek, 1954).

Experimental Findings

Incidence of Resistant Variants

A typical experiment is shown in Fig. 2. When the antibiotics were used singly the lower third of the gradient plate, where the antibiotic concentration was lowest, showed diffuse growth of the organism. This area ended fairly abruptly, and above there were varying numbers of discrete resistant colonies. With streptomycin there were usually

FIG. 2.—Illustrations of results after 24 hours' growth of a coliform bacillus on gradient plates. A is a streptomycin plate, B is either a chloramphenicol or a tetracycline plate, and C is a plate containing two of these antibiotics. With the single antibiotics there is diffuse growth in the lower part of the plate and, above, varying numbers of discrete resistant colonies. With the pairs of antibiotics resistant colonies are absent.

about 100 to 200 colonies in a wide zone, often extending into the uppermost segment of the plate. Only one out of 32 streptomycin-sensitive organisms showed a narrow band of colonies lying just above the line of inhibition of the parent strain. Both tetracycline and chloramphenicol showed a much smaller number of discrete colonies, usually lying just above the area of diffuse growth. With the three drugs the colonies varied in size from 1 to 2 mm. in diameter

to pin-point size. All the colonies, however, had a similar degree of resistance to the antibiotics, about three to four times that of the parent organism. Table I shows the numbers of resistant colonies formed by 32 coliform strains sensitive to each of the three antibiotics.

TABLE I.—Incidence of Resistant Variants in 32 Strains of *Coliform Bacilli* on Gradient Plates

No. of variants (per 10^8 orgs.) No. of strains	Streptomycin			Chloramphenicol			Tetracycline		
	25-50	50-150	150-200	5-10	10-25	25-50	0-5	5-10	10-25
	4	20	8	8	18	6	6	22	4

Fig. 2 (C) illustrates the results obtained with the pairs of antibiotics. The results were identical with the three combinations tested. With every one of the 32 strains that were sensitive to both antibiotics the zone of diffuse growth occurred lower in the plate, and, in fact, in most cases the plates showed no growth at all. Moreover, above the zones of diffuse growth resistant colonies were absent. Combinations of two antibiotics therefore not only had a synergistic action against the growth of the organism but also prevented the emergence of resistant variants. On the other hand, 10 coliform strains that were resistant to one or other of the two antibiotics in the combinations behaved exactly as if exposed only to the effective antibiotic.

Bactericidal Effects

The chances of the emergence of resistant variants are closely related to the living bacterial population. It is therefore important to distinguish between bactericidal and bacteriostatic effects, and for this purpose the replica-plate method has been used. The organisms on the surface of the gradient plate, including viable but inhibited organisms, were

transferred on to an antibiotic-free plate, where the viable cells could grow and form colonies. Typical results are shown in Fig. 3. Streptomycin was essentially bactericidal, the colonies present on the plate arising from organisms transferred from the already resistant colonies on the original gradient plate. Chloramphenicol and tetracycline, on the other hand, were mainly bacteriostatic, the replica plates showing diffuse growth.

Fig. 3.—Illustrations of replica plates made from gradient plates by the velvet-pad method. The replicas show the survival of the population inoculated as against the inhibition of the population on the gradient plates. A is a replica from a streptomycin plate, B is from either a chloramphenicol or a tetracycline plate. C and D are the two kinds of result obtained with streptomycin plus either chloramphenicol or tetracycline.

were obtained. With streptomycin+tetracycline and with streptomycin+chloramphenicol about half the organisms tested showed no growth on the replica plates. The remaining organisms showed an appearance similar to that obtained on a gradient plate containing streptomycin only. In the first case, therefore, the resistant variants had been killed by the combined antibiotics. In the second case the resistant variants had only been inhibited; the organisms resistant to streptomycin but inhibited by tetracycline or chloramphenicol multiplied to form colonies when the antibiotics

were removed. The former result would seem to indicate that tetracycline and chloramphenicol may occasionally possess some bactericidal activity, having killed the streptomycin-resistant variants. Indeed, with three of our strains it was possible to demonstrate that both tetracycline and chloramphenicol, when used alone, had a bactericidal effect exceeding 99%.

Replica plates from the gradient plates containing tetracycline+chloramphenicol showed, in the majority of cases, diffuse growth, indicating that, although the resistant variants were effectively inhibited, the combination of these drugs had little or no bactericidal activity. However, with the three coliform strains against which tetracycline and chloramphenicol possessed striking bactericidal activity the replica plates showed only a scanty growth of discrete colonies. These findings indicate that generalizations about the bactericidal action of a given antibiotic are often not valid for a particular strain of organism.

Chances Against Emergence of Resistant Variants

An attempt was made to confirm the value of antibiotic combinations for the suppression of resistant variants by observing the incidence of variants resistant to each antibiotic used separately. Populations of 10^8 organisms were inoculated on to plates containing the antibiotics in concentrations just above the inhibitory levels and the number of resistant colonies present after 24 hours' incubation was counted. Each experiment was repeated six times from separate cultures in order to obtain the extent of the fluctuation in the numbers of resistant colonies. Table II shows

TABLE II.—Fluctuation in Numbers of Resistant Variants Obtained with 6 Cultures of a Single Strain of *Coliform Bacillus* Following Exposure to Minimum Inhibitory Levels of Antibiotics

	1	2	3	4	5	6	Average
Streptomycin 5 μ g./ml.	27	44	47	106	62	17	50
Chloramphenicol 5 μ g./ml.	15	25	25	32	17	19	22
Tetracycline 2 μ g./ml.	7	12	5	0	7	6	6

the results with one of the strains of coliform bacilli. The organism was inoculated on six separate occasions on to plates containing 5 μ g. of streptomycin per ml., 5 μ g. of chloramphenicol per ml., and 2 μ g. of tetracycline per ml. Fluctuation in numbers did occur, as expected, but the figures were not widely different. Streptomycin showed the highest number of resistant variants, averaging 50 resistant cells per 10^8 organisms. Chloramphenicol was next with 22, and tetracycline had the lowest number with only about 6 per 10^8 organisms.

Two further strains tested in this way gave numerically similar results. These findings are likely to apply to all the strains used in the investigation, since all produced approximately the same number of resistant colonies on the gradient plates (Table I). In fact, although not strictly quantitative, the gradient plate gives a fair indication of the mutation rate. The majority of the strains formed between 50 and 150 resistant colonies on the streptomycin plates, between 10 and 25 on the chloramphenicol plates, and between 5 and 10 on the tetracycline plates, with a few strains forming somewhat higher or lower numbers. It is interesting that two strains which formed an unusually large number of resistant colonies on streptomycin also produced unusually large numbers of variants on both tetracycline and chloramphenicol.

The average incidence of variants calculated on the above figures for streptomycin is 1 in 2×10^8 , for chloramphenicol 1 in 5×10^8 , and for tetracycline 1 in 15×10^8 . By simple multiplication it is possible to calculate the chance of a variant arising which is simultaneously resistant to two antibiotics. Thus, for streptomycin+chloramphenicol it is 1 in 10^{13} , for streptomycin+tetracycline it is 1 in 3×10^{13} , and for chloramphenicol+tetracycline it is 1 in 7.5×10^{13} . It is

unlikely that a population of 10^{13} organisms (which would represent about 10 litres of an overnight broth culture) could be obtained in the human body during the course of an acute infection. The longer the infection persists, however, the more likely is it that sufficiently large numbers of bacteria will be concerned.

These calculations are based on the assumption that the mutation rate of an organism which has become resistant to one antibiotic does not at the same time alter towards the second antibiotic. To test this point variants resistant to one antibiotic were subcultured and the incidence of variants resistant to a second antibiotic was determined in the manner already described. It was found that in the case of all three antibiotics no significant change in mutation rate occurred.

Discussion

Our results agree with those of Kaipainen (1952) that combinations of antibiotics are effective in suppressing the emergence of resistant variants of the coliform group of organisms, provided the organism is sensitive to both the antibiotics in the combination. The calculated chances of a doubly resistant variant arising provide theoretical support for the value of combined therapy.

There is good evidence that a bactericidal effect should be aimed for in treating urinary infections: otherwise inhibited organisms, lying protected in devitalized tissue, may cause a relapse after withdrawal of the drug (Nesbitt *et al.*, 1951; Stansfeld and Webb, 1954). Streptomycin is the only effective bactericidal drug at present available for urinary infections, but its use is notoriously apt to result in the appearance of resistant variants. Our results show, however, that when it is combined with tetracycline or chloramphenicol the bactericidal action is retained and the resistant variants are effectively suppressed. In about half the cases this suppression is complete in so far that the resistant variants have actually been killed. In the other half the resistant variants are only inhibited, and treatment must be sufficiently prolonged to allow the body defences to destroy these few remaining organisms; otherwise relapses are possible. Chloramphenicol+tetracycline is the most effective combination for suppressing the resistant variants, but with only a few strains of coliform bacilli is there adequate bactericidal action.

We have not yet carried out a clinical trial of the value of antibiotic combinations in preventing relapses, but the *in vitro* tests indicate that good results are to be expected. While this work has been concerned only with urinary coliform infections it seems likely that the results would also apply to the treatment of other infections with organisms which are capable of becoming resistant to a single antibiotic. The wider use of antibiotic combinations could perhaps avert the threat of the increasing numbers of antibiotic-resistant bacteria.

Summary

Combinations of streptomycin, tetracycline, and chloramphenicol have been tested for their value in suppressing the emergence of resistant variants of coliform bacilli isolated from cases of urinary infection.

Streptomycin+tetracycline and streptomycin+chloramphenicol are the most effective combinations. Not only are resistant variants suppressed, but these combinations possess bactericidal activity which is probably also an important factor in preventing relapses in urinary infections. Chloramphenicol+tetracycline prevent the emergence of resistant variants but have little or no bactericidal activity against most strains.

Calculations of the chances of a variant arising which is simultaneously resistant to two antibiotics show that the efficacy of combined treatment is explained by the large size of the bacterial population required for such

doubly resistant variants to arise. It is unlikely that during the course of an acute urinary infection sufficiently large bacterial populations can be attained.

It is suggested that these findings have an application in clinical practice.

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THE "MULTIFIDUS TRIANGLE" SYNDROME AS A CAUSE OF RECURRENT LOW-BACK PAIN

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Among the many patients who have attended the department of physical medicine at St. Thomas's Hospital complaining of recurrent attacks of acute low lumbar pain, it has been possible to identify a small group with a substantially similar pattern of history, signs, and symptoms. These suggested that the cause was a lesion in the soft tissues occupying a triangular zone on either side of the posterior surface of the sacrum. Livingston (1941) described such a syndrome, and referred to the area involved as the "multifidus triangle" because in it lies the lowermost portions of the multifidus muscle, which acts to stabilize the spine in its relation to the sacrum (see Diagram). He stressed that attacks are often brought on by physical exercise involving bending and twisting of the lumbar spine. A sharp localized pain may occur, followed by a persistent discomfort ascribed to one spot just below the posterior superior iliac spine. This ache may gradually decrease during succeeding days, so that the patient may not be particularly concerned. However, a feature of such cases is the tendency for attacks to recur after intervals of relative freedom. Steindler and Luck (1938), in a series of 451 patients suffering from low-back pain, observed that in more than 20% of these cases it was possible to demonstrate a local point of tenderness situated in the deeper tissues. They were of the opinion that the lesions represented either a deep ligamentous injury or a myofascial lesion. The work of Lewis and Kellgren (1939) suggests that such a focus may result in