Technique for Determining the Bactericidal Effect of Drug Combinations

VICTOR LORIAN AND GEORGETA FODOR

Division of Microbiology and Epidemiology, Department of Pathology, The Bronx-Lebanon Hospital Center, Bronx, New York 10456, and Department of Microbiology, Mount Sinai School of Medicine, New York, New York 10029

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Two paper strips, each containing different antimicrobial agents, were placed on plates on Mueller-Hinton agar to permit antibiotic to enter the agar. A filter membrane was placed on this plate, and the microorganisms were planted on the membrane. After 6 h of incubation at 37 C, the membrane was transferred to antibiotic-free Mueller-Hinton agar containing triphenyltetrazolium hydrochloride and incubated for 18 h at 37 C. Specific growth patterns were indicative of additive (indifferent), synergistic, or antagonistic effects of the drug combination used. Trimethoprim and sulfamethoxazole proved to act synergistically against 85% of *Escherichia coli*, 86% of *Klebsiella*, and 89% of *Proteus mirabilis* strains tested. A few strains resistant to either drug were susceptible to their combination. The technique was useful against organisms with widely differing susceptibilities to the two antimicrobial agents tested.

A number of methods have been devised to establish the bactericidal effect of antibacterial agents against pathogenic organisms (8, 9, 11, 13). The replica transfer method was introduced by Lederberg and Lederberg (10). Elek et al. (6, 7) and Streifeld (14) applied this method using a velvet replicator for the study of antibiotic combinations. Chabbert and Waterworth (4) applied the same principle using a cellophane membrane for the study of synergistic effects of antibiotics. They used filter paper strips impregnated with antibiotics placed at right angles to diffuse the drugs into the agar.

In this paper we report the usefulness of a modified cellophane technique in which the cellophane has been replaced by a filter membrane. The technique was tested with the association of trimethoprim plus sulfamethoxazole.

MATERIALS AND METHODS

One hundred strains each of *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella*, isolated from specimens submitted to this laboratory, were tested for susceptibility to trimethoprim and sulfamethoxazole singly or in combination.

Media and reagents. Media used were as follows: (i) Mueller-Hinton agar (MHA) (BBL) with 0.25% dextrose and 0.04% triphenyltetrazolium hydrochloride (TTC) (Biological Products, N.J.) (after the agar was autoclaved and cooled at 52 C [in a 52 C water bath], 1 ml of a 4% solution of TTC in 20% alcohol was added to 100 ml of MHA with 0.25% dextrose); Trypticase soy broth (TSB) (BBL). Filter membranes

#PHWPO9025, 9 cm in diameter (Millipore Corp., Bedford, Mass.), and filter paper grade #740E (Schleicher and Schuell, Inc. Keene, N.H.) were used. Trimethoprim and sulfamethoxazole were from Hoffman-La Roche, Inc., N.J. Trimethoprim (10 mg) was dissolved in 100 ml of boiling water containing 0.2 ml of hydrochloric acid p.a., and sulfamethoxazole (200 mg) was dissolved in 98 ml of boiling water containing 2 ml of a 4% sodium hydroxide solution.

Antibiotic-impregnated plates were prepared in the following manner. Strips 4 cm long and 0.9 cm wide were cut from the filter paper (water absorption per strip was 0.29 ml). One strip was soaked in the trimethoprim solution (producing a charge of 29 µg per strip) and another strip was soaked in the sulfamethoxazole solution (producing a charge of 580 µg per strip). The strips were then placed at a 90° angle, with ends touching, on MHA plates and were incubated at 37 C for 18 h to diffuse the drugs into the agar. Then the strips were removed and discarded. Because no additive or antagonistic effects have been reported with the combination of trimethoprim and sulfamethoxazole, gentamicin (60 µg/ml) and streptomycin (50 µg/ml) were used as possible controls for an additive effect, and nalidixic acid (500 µg/ml) with nitrofurantoin (600 µg/ml) were used as possible controls for an antagonistic effect.

Technique. A filter membrane was placed on the surface of the antibiotic-impregnated plate, and 0.2 ml of inoculum (a 10⁻² broth dilution of an 18-h culture in TSB) was spread on the surface of the membrane with a swab by cross-striking four times. The plates with the inoculated membrane were incubated at 37 C for 6 h. The membrane was then removed and placed on MHA containing TTC and incubated for 18 h, after which time the results were

read. Where growth of organisms had occurred, the TTC was reduced to red formazan, making the growth area more obvious. No growth (bactericidal effect) was indicated by the membrane remaining white.

To determine if this procedure could identify synergism when applied to bacterial isolates with widely differing susceptibilities to trimethoprim and sulfamethoxazole, 20 strains of *E. coli* were tested by the broth dilution technique. These strains were chosen at random from those tested on filter membranes.

Tubes with 2 ml of TSB containing the drugs

separately and in combination (see Table 2) were inoculated with 0.05 ml of a 10^{-8} dilution of an 18-h TBS culture and incubated for 20 h at 37 C. One loop (0.003 ml) from each clear tube was planted on blood agar and incubated at 37 C for 20 h.

The drug concentration in the first tube, showing no visible turbidity, was considered as the minimal inhibitory concentration (MIC), and the concentration in the first tube producing less than 30 colonies (more than 99.9% killing) was considered as the minimal bactericidal concentration (MBC).

FIG. 1. Various inhibition patterns produced with two drug combinations on four different strains of *E. coli*. (a) Trimethoprim (T) and sulfamethoxazole (S) show a synergistic effect only for trimethoprim, and indifference of the combinations. (b) Streptomycin (ST) and gentamicin (G) show an additive (indifferent) effect. (c) T and S show synergistic effect. (d) Nitrofurantoin (N) and nalidixic acid (NA) show an antagonistic effect.

TABLE 1. Effect of trimethoprim-sulfamethoxazole (T&S) on some *Enterobacteriaceae* (filter membrane technique)

Strains tested	Resistant to both T & S separately	No. of strains		
		Susceptible to trimethoprim only	Susceptible to sulfamethoxazole only	Showing synergistic effect
<i>E. coli</i> , 100	3 ^a	14	2	85 ^b
<i>Klebsiella</i> , 100	0	13	1	86
<i>Proteus mirabilis</i> , 100	0	11	0	89
Total, 300	3	38	3	260

^a All three strains were inhibited by the combination.

^b One strain resistant to sulfamethoxazole showed synergy with the combination.

RESULTS

The growth patterns, after membrane transfer and incubation with four different strains of *E. coli*, are shown in Fig. 1. Trimethoprim and sulfamethazole, streptomycin and gentamicin, nitrofurantoin and nalidixic acid were the pairs tested. A bactericidal effect of a single drug (Fig. 1a) was indicated by the white oval area

FIG. 2. The combination of trimethoprim (T) with sulfamethoxazole (S) produced a bactericidal effect on a strain of *E. coli* resistant to either drug separately.

surrounding only the zone over the strip impregnated with trimethoprim. An additive effect was shown by two oval areas joining at a right angle (Fig. 1b). A synergistic effect was shown by a zone of no growth inside the angle formed by the two strips (Fig. 1c). An antagonistic effect was shown by an indentation of growth inside the angle (Fig. 1d). The reading was easy and no gradations were recorded.

Results with trimethoprim and sulfamethoxazole are shown in Table 1. This combination had a synergistic effect on 85% of *E. coli*, 86% of *Klebsiella*, and 89% of strains of *P. mirabilis*. Forty-one strains resistant to either sulfamethoxazole or trimethoprim were observed. Trimethoprim was more active than sulfamethoxazole in that 13% of the strains were susceptible only to trimethoprim and 1% of the strains were susceptible only to sulfamethoxazole. Usually when synergistic effects were observed, the strains were susceptible to both drugs. However, there were three instances in which strains of *E. coli* were resistant to both trimethoprim ($>5 \mu\text{g/ml}$) and to sulfamethoxazole separately ($>100 \mu\text{g/ml}$), but were susceptible to their combination (Fig. 2).

In Table 2 the results of the broth dilution tests are shown. There is more than a 100-fold difference between the MBC of the most susceptible and the MBC of the most resistant strain tested. Strains with an MBC over $100 \mu\text{g}$ of sulfamethoxazole per ml and over $5 \mu\text{g}$ of trimethoprim per ml were considered resistant.

TABLE 2. Number of strains of *E. coli* (20 strains tested) susceptible to various concentrations of sulfamethoxazole, trimethoprim, and their combination (broth dilution method)^a

Determination	No. of <i>E. coli</i> strains susceptible										
Concn of sulfamethoxazole ($\mu\text{g/ml}$)	>100	100	50	25	12.5	6.25	3.12	1.5	0.75	0.38	0.18
MIC to sulfamethoxazole	3	0	3	2	4	5	3	0	0	0	0
MBC to sulfamethoxazole	3	5	4	3	5	0	0	0	0	0	0
Concn of trimethoprim ($\mu\text{g/ml}$)	>5	5	2.5	1.25	0.6	0.3	0.15	0.075	0.04	0.02	0.01
MIC to trimethoprim	0	3	4	5	4	1	1	0	2	0	0
MBC to trimethoprim	4	5	6	1	1	0	1	1	1	0	0
Concn of combination:											
Sulfamethoxazole ($\mu\text{g/ml}$)	>100	100	50	25	12.5	6.25	3.12	1.5	0.75	0.38	0.18
Trimethoprim ($\mu\text{g/ml}$)	>5	5	2.5	1.25	0.6	0.3	0.15	0.075	0.04	0.02	0.01
MIC to sulfamethoxazole + trimethoprim	0	0	0	0	3	3	3	3	5	3	0
MBC to sulfamethoxazole + trimethoprim	0	1	4	3	4	3	3	1	1	0	0

^a The MBCs for trimethoprim or sulfamethoxazole were 2 to 4 times higher than the corresponding MICs and the MBCs for trimethoprim plus sulfamethoxazole were 8 to 16 times higher than the corresponding MICs.

There were three strains resistant to sulfamethoxazole and four strains resistant to trimethoprim, but none was resistant to the combination. The results shown with the broth dilution for each strain were comparable to the data obtained from the filter membrane technique.

DISCUSSION

The membrane technique described determines whether two drugs act together to produce additive (indifference), synergistic, or antagonistic effects in regard to bactericidal effect. In this investigation the technique used has the advantage over the cellophane method in that the filter membrane is commercially available. Thus, the laborious procedure for the preparation of cellophane membrane can be avoided. The technique with filter membranes proved easy enough to be used in routine work, producing accurate results with strains of widely differing susceptibilities to the drugs tested.

Although sulfonamides are classically bacteriostatic (1), under appropriate conditions they have been shown to exert a bactericidal effect (5, 12). Thus, the observation here of a bactericidal effect by sulfamethoxazole alone is neither a new finding nor at variance with some previous observations.

The bactericidal effect of the combination of trimethoprim and sulfamethoxazole described here also confirms previous investigations (2, 3). In addition, it was observed with both techniques that some organisms which were resistant to either of the two drugs separately were susceptible to the synergistic effect of their combination.

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