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Quantitative Inhibition of *Haemophilus influenzae* by Trimethoprim/Sulfamethoxazole

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We evaluated the inhibitory effect of trimethoprim (TMP) and sulfamethoxazole (SMZ), alone and in combination, against 34 strains of *Haemophilus influenzae*. Growth inhibition was determined after incubation for 18 h by comparing viable counts of cultures in drug-containing medium with corresponding counts of control cultures in drug-free medium. In a modified, thymidine-deficient Levinthal broth, the numbers of colony-forming units of all the isolates tested were reduced 100-fold or more by TMP/SMZ (1.25/25 μg/ml) as compared with growth without drug. Inhibition was significantly greater with TMP/SMZ than with either TMP or SMZ alone. Ampicillin-susceptible and ampicillin-resistant strains were equally susceptible to TMP/SMZ. Growth of nontypable strains was inhibited more than growth of type b organisms.

Some investigators have reported that all the isolates of *Haemophilus influenzae* they tested were susceptible to trimethoprim-sulfamethoxazole (TMP/SMZ) (1, 4, 8, 10, 14), whereas others have found varying numbers of resistant strains (3, 13). Tests for susceptibility of *H. influenzae* to TMP/SMZ have also yielded variable results in different media (10, 12) and ambiguity of visually determined end points (1, 13). Koch and Burchall (9) demonstrated that activity of TMP against *Escherichia coli* varied inversely with the thymidine content of the media used. Using sulfadiazine and *Staphylococcus aureus*, Yourassowsky and Schoutens (18) found that end points for the minimum inhibiting concentration (MIC) are difficult to define in agar dilution tests because of a gradual diminution in size of colonies with increasing concentrations of drug. We have confirmed this phenomenon with both *S. aureus* and *H. influenzae* by using TMP and SMZ, separately and combined. In this report we present the results of more detailed studies on the inhibition of *H. influenzae* by TMP and SMZ, separately and combined.

MATERIALS AND METHODS

Organisms. Thirty-four strains of *H. influenzae* were tested; 12 were type b and 22 were not typable. Twenty-four of the isolates were obtained from the Laboratory of Medical Microbiology at Boston City Hospital, six from C. Thornsberry (Center for Disease Control, Atlanta, Ga.), and four from S. R. M. Bushby (Burroughs Wellcome Co., Research Triangle Park, N.C.). The organisms were identified as *H. influenzae* by their colonial morphology and requirement for hemin and nicotinamide adenine dinucleotide. They were serotyped by the Quellung reaction with antisera for types a through f obtained from the Center for Disease Control. The isolates from Boston City Hospital included one type b and 17 nontypable from middle ear effusions in children, three type b from blood, one type b from cerebrospinal fluid, and one type b and one nontypable from nasopharyngeal cultures of children with acute respiratory disease.

Drugs. TMP lactate (Burroughs Wellcome Co.) was dissolved in water to a final concentration of 20 μg per ml of the base. SMZ (Hoffmann-LaRoche, Inc.) was suspended in water, to which was added the minimum volume of 0.1 N NaOH required for solution, to yield a final concentration of 400 μg/ml. These stock solutions were made up monthly, and portions for individual tests were stored at −20°C. Twofold dilutions of the drugs were made in liquid media. Each strain of *H. influenzae* was tested for beta-lactamase production by the method of Kirven and Thornsberry (8). The MICs of ampicillin for strains that produced beta-lactamase were ≥12.5 μg/ml by tube dilution assay and were considered ampicillin resistant.

Media. Levinthal broth (LB) was prepared by using horse blood, as described by McLinn et al. (11). Modified LB (MLB) was prepared by substituting thymidine-poor, modified Mueller-Hinton broth (MMHB) (Difco, batch 50287) for brain-heart infusion broth. Thymidine (J. T. Baker Chemical Corp., Phillipsburg, N.J.), 0.8 μg/ml, and/or 32,000 U of thymidine phosphorylase per ml (kindly supplied

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by S. R. M. Bushby) were added to the MLB or LB for some tests in these media.

Tests of susceptibility. Susceptibility tests were done by a twofold tube dilution technique. The range of final concentrations of TMP/SMZ (wt/wt) was from 10/200 to 0.16/3.1 μg/ml for strains of *H. influenzae*. Equal volumes (0.5 ml) of a 10^{-6} dilution of an overnight growth in MLB and of drug were added, and the tubes were incubated at 37°C overnight. Visual determination of turbidity and quantitative determination of colony-forming units (CFU) in each tube were made at 18 h. The MIC was recorded as the lowest concentration that yielded no visible growth with the aid of a hand lens (×3). The minimum bactericidal concentration (MBC) was defined as that which gave no growth after transfer to solid, drug-free medium and incubation for 18 h. Controls on the antibacterial activity of TMP/SMZ in the media used were carried out with strains of *S. aureus* (ATCC 25922) and *E. coli* (ATCC 25923), using twofold dilutions of TMP/SMZ in a fixed ratio of 1:20 (wt/wt) at decreasing final concentrations from 10/200 to 0.01/0.2 μg/ml. The inoculum of these organisms in all tests was a dilution of overnight broth growth containing 10^2 to 10^3 CFU/ml.

Quantitative determination of growth inhibition by TMP and SMZ, separately and combined, was performed with each of the isolates of *H. influenzae*. After overnight incubation, the number of CFU per milliliter in MLB with drug was compared with that of a control culture in drug-free broth. A 10^{-6} dilution of an overnight MLB culture of *H. influenzae* was added to each of four wells in one row of a microtiter plate (Cooke Laboratories). The median inoculum for these investigations was 5.4×10^2 (range, 6×10^1 to 2.2×10^3) CFU/ml. TMP, SMZ, and TMP/SMZ, in a volume of 0.05 ml, were added to three of these wells to give final concentrations of 1.25, 25, and 1.25/25 μg/ml, respectively. These concentrations were chosen because they are within the range attainable in serum with the usually recommended doses. Drug-free MLB was added to the fourth well. The microtiter plates were incubated at 37°C overnight on a shaker. After incubation, the contents of each well were stirred, and 0.025-ml samples of duplicate dilutions of the control and drug-containing cultures were placed on the surface of chocolate agar plates, spread with a glass rod, and incubated for 18 h.

The degree of inhibition (DI) for each strain was defined as $\log_{10}$ reduction in CFU in the drug-containing culture compared with that in the drug-free control. Thus, a DI of 2 represents 99% fewer CFU per milliliter, and a DI of 5 indicates 99.999% fewer CFU per milliliter than the in the control culture. The drugs were considered bactericidal for strains of *H. influenzae* when no viable colonies developed after incubation for 18 h. The inoculum for growth curves ranged from 2.1×10^2 to 7.7×10^2 CFU/ml. Viable counts were determined at 0, 4, 7, 12, and 24 h in the presence of TMP/SMZ (1.25/25 μg/ml) and in control cultures.

Three strains of *H. influenzae* were tested for the combined action of TMP and SMZ. The strains selected were the most SMZ-resistant isolate encountered (16395), one of intermediate susceptibility (49711), and a highly susceptible strain (32425). Inhibition was assayed with concentrations of the two drugs in checkerboard fashion in microtiter plates, using twofold dilutions of the drugs with concentrations of TMP ranging from 10 to 0.039 μg/ml and those of SMZ ranging from 200 to 3.1 μg/ml. The DI for each drug and for the combination was determined by comparing colony counts of the growth in each drug-containing well with those in the corresponding drug-free control after incubation for 18 h.

Statistical methods. The results for TMP, SMZ, and TMP/SMZ were compared by two-way analysis of variance (15). Isolates for which the drug was bactericidal (DI > 8) were assigned a DI of 8 for the analysis. The mean DI for ampicillin-resistant and ampicillin-susceptible and for nontypable and type b strains were compared by Student's *t* test. Cochran's *Q* test (5) was used to compare the bactericidal activity of TMP/SMZ with that of TMP or SMZ alone. The Spearman rank-order test (15) was used in correlating the initial inoculum and DI.

RESULTS

Effect of the medium. The antibacterial activity of TMP/SMZ against *S. aureus* or *E. coli* was essentially the same in MMHB or MLB but was less in LB (Table 1). Thymidine phosphorylase substantially reduced the MIC and MBC for *S. aureus* in LB. The antibacterial activity of TMP/SMZ in MLB was diminished by addition of thymidine and restored by addition of thymidine phosphorylase. MLB was chosen for testing *H. influenzae* because it supported growth of this organism better than MMH alone.

Determination of MIC. Visual estimation of end points with *H. influenzae* were ambiguous, and reproducibility between investigators was unreliable. Table 2 illustrates the gradual increase in CFU per milliliter and decrease in DI observed with two strains over the range of concentrations of TMP/SMZ evaluated. All tubes yielding any growth on subculture showed slight turbidity with mild agitation; only tubes that gave no growth were clear.

Quantitative tests of inhibition of *H. influenzae*. When tested in MLB, growth of all 34 strains of *H. influenzae* was reduced by 99% or more (DI ≥2) by TMP/SMZ, 1.25/25 μg/ml, as compared with drug-free controls. The mean DI was 6.0, with a range of 2.8 to >8.O. The Spearman rank correlation between initial inoculum and DI was 0.3. This correlation was not significant over the narrow range of inoculum used and does not explain this variability. The individual drugs, TMP (1.25 μg/ml) and SMZ (25 μg/ml), gave significantly less inhibition, the mean DI being 4.4 and 3.4, respectively ($P < 0.01$ by two-way analysis of vari-

TABLE 1. *Susceptibility of Staphylococcus aureus and Escherichia coli to TMP/SMZ in various liquid media*

Medium	*S. aureus*[a]		*E. coli*[b]	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
MMHB	0.04/0.8	0.16/3.1	0.08/1.6	0.16/3.1
LB	0.63/12.5	10/200	0.63/12.5	10/200
LB + TP[c]	0.08/1.6	0.08/1.6		
MLB	0.08/1.6	0.08/1.6	0.04/0.8	0.16/3.1
MLB + TP	0.08/1.6	0.08/1.6	0.04/0.8	0.16/3.1
MLB + thymidine[d]	10/200	10/200		
MLB + thymidine + TP	0.04/0.8	0.08/1.6		

[a] ATTC 25922.
[b] ATTC 25923.
[c] Thymidine phosphorylase (TP), 32,000 U/ml.
[d] Thymidine, 0.8 μg/ml.

TABLE 2. *Quantitative effect of TMP/SMZ on growth of two strains of Haemophilus influenzae after incubation for 18 h*

Conc of TMP/SMZ (μg/ml)	Strain 16395			Strain 49711		
	CFU/ml	Log_{10}	DI	CFU/ml	Log_{10}	DI
10/200	0		>8	3.7×10^3	3.6	6.1
5/100	$5.2 \times 10.^2$	2.7	5.8	9.6×10^4	5.0	4.7
2.5/50	6.7×10^3	3.8	4.7	3×10^6	6.5	3.2
1.25/25	8.8×10^4	4.9	3.6	1.4×10^7	7.2	2.5
0.62/12.5	4.8×10^4	4.7	3.8	3×10^7	7.5	2.2
0.31/6.2	4.1×10^5	4.6	2.9	4.3×10^7	7.6	2.1
0.16/3.1	1×10^6	6.0	2.5	1.6×10^8	8.2	1.5
0	3.2×10^8	8.5		5.0×10^9	9.7	

ance) (Fig. 1). Twenty-four of the 34 strains were inhibited by a lower concentration of TMP when combined with SMZ than when TMP was used alone; nine gave equivalent inhibition by TMP in the presence or absence of SMZ, and one strain was inhibited by TMP alone (DI = 4.9) more than by TMP/SMZ (DI = 3.8). Twenty-nine of the 34 strains were inhibited more by TMP/SMZ than by SMZ alone, and five gave equivalent inhibition. Bactericidal activity (DI ≥ 8) was demonstrated against 14 strains with TMP/SMZ (1.25/25 μg/ml), against eight strains with TMP alone (1.25 μg/ml), and against five of those eight also with SMZ alone (25 μg/ml) ($Q = 12.5; P > 0.001$).

The 12 ampicillin-resistant strains did not differ from the 22 ampicillin-susceptible strains in their susceptibility to TMP/SMZ ($t = 0.14; P > 0.5$) (Table 3). The drug combination was bactericidal against 12 (54%) of the 22 nontypable strains and against two (17%) of the 12 type b strains (χ^2 with Yates correction, 3.3; $0.05 < P < 0.1$). The mean DI was also greater for nontypable strains than for type b strains ($t = 2.43; P < 0.05$) (Table 2). This difference could not be attributed to variation in size of the initial inoculum.

Studies with three strains of *H. influenzae* demonstrated that the assay used gives results that are affected by inoculum size, yielding greater inhibition with low inoculum (Fig. 2). Growth curves showed that MLB supported good growth of *H. influenzae* and that TMP/SMZ inhibited growth, when compared with controls, throughout the entire period studied (Fig. 3), the first observation being made after 4 h.

Studies of the combined action of TMP and SMZ against *H. influenzae* strains 49711, 16395, and 32425 illustrate the variability in the effects. Addition of TMP (0.156 μg/ml) to broth containing SMZ (12.5 μg/ml) gave inhibition consistently exceeding that obtained in broth containing 50 μg of SMZ per ml only for strain 49711 (Table 4). Strain 32425 was so highly susceptible that interactions could not be evaluated at the concentrations used. Results with 16394 suggest an additive effect when TMP (0.156 μg/ml) is combined wth the low concentrations of SMZ (12.5 μg/ml). Drug antagonism was not observed in any of the tests with the possible exception of one strain with a DI of 4.9 for TMP alone and 3.8 for TMP/SMZ.

Isobolograms constructed from results of tests for combined action of TMP/SMZ showed apparent synergy only when the end points

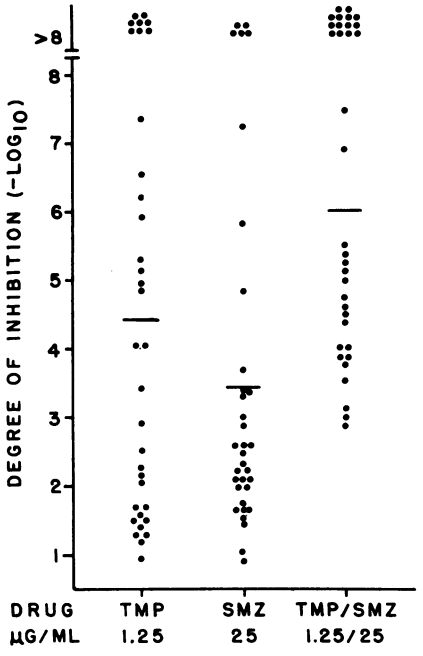

FIG. 1. *DI (defined as $\log_{10}$ reduction in viable count after incubation for 18 h in drug-containing medium compared with count of control, drug-free medium) of 34 strains of H. influenzae by TMP, 1.25 μg/ml, and SMZ, 25 μg/ml, separately and combined (TMP/SMZ, 1.25/25 μg/ml). The horizontal lines indicate the means of DI; these are, respectively, 4.4 ($P > 0.02 < 0.05$), 3.5 ($P > 0.05 < 0.1$), and 6.0 ($P > 0.01 < 0.02$).*

TABLE 3. *Susceptibility of 34 strains of Haemophilus influenzae to TMP/SMZ (1.25/25 μg/ml) in MLB*

Strains of *H. influenzae*	No. tested	No. killed[a] (%)	DI[b]	
			Mean	Range
All	34	14 (41)	6.0	2.8–8
Nontypable	22	12 (55)	6.5	2.8–8
Type b	12	2 (17)	5.0	3.1–8
Ampicillin resistant	12	5 (42)	5.9	3.1–8
Ampicillin susceptible	22	9 (41)	6.0	2.8–8

[a] No growth after transfer to solid, drug-free medium and incubation for 18 h.

[b] Defined as $\log_{10}$ reduction in CFU at 18 h as compared with growth in control (drug-free medium).

were charted for relatively low degree of inhibition (DI ~ 2); using DI ~ 4, synergy was not clearly evident. Data for strain 16395 are shown in Fig. 4.

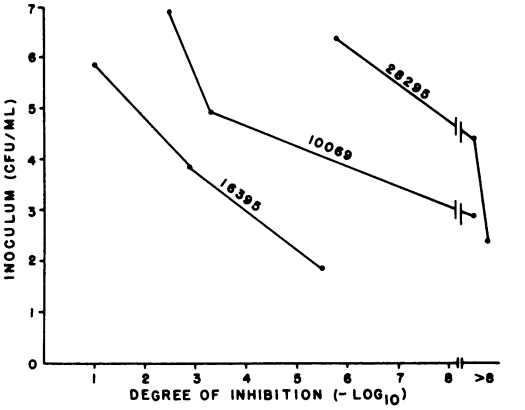

FIG. 2. *Effect of size of inoculum on DI of three strains of H. influenzae by TMP (1.25 μg/ml) + SMZ (25 μg/ml). For definition of DI, see legend to Fig. 1.*

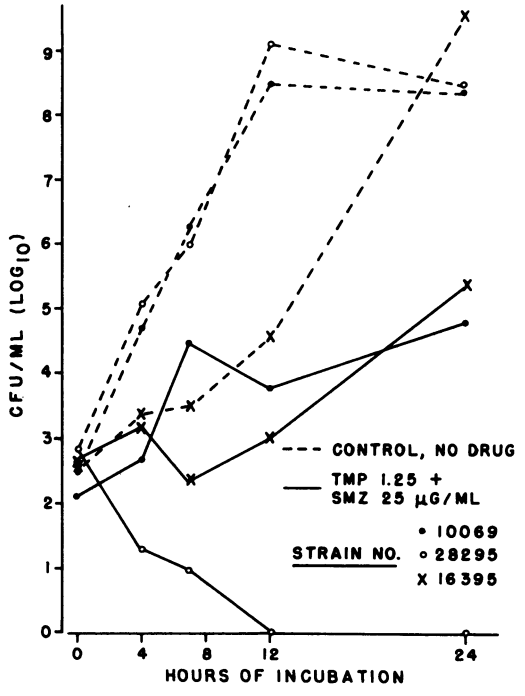

FIG. 3. *Growth of three strains of H. influenzae in MLB with and without TMP/SMZ, 1.25/25 μg/ml.*

DISCUSSION

Our results indicate that all the isolates of *H. influenzae* tested were inhibited by TMP/SMZ and that quantitatively the strains varied widely in the degree of their susceptibility. The combination TMP/SMZ produced greater inhibition of bacterial growth than either TMP or SMZ alone, and was bactericidal against

TABLE 4. *DI of three strains of Haemophilus influenzae produced by some combinations of TMP and SMZ*

Strain of *H. influenzae*	SMZ (μg/ml)	DI[a]					
		TMP (μg/ml)					
		0	0.16	0.31	0.63	1.25	2.5
16395	0		1.1	1.3	1.4	1.7	2.0
	12.5	1.5	2.2	2.1	2.2	2.4	2.4
	50	2.3	2.5	2.3	2.4	2.4	3.2
49711	0		0.5	1.0	1.0	2.3	3.0
	12.5	0.5	3.3	3.3	3.8	3.8	4.0
	50	1.5	3.3	3.4	4.0	4.3	4.7
32425	0		0.7	0.8	5.2	8	8
	12.5	8	8	8	8	8	8
	50	8	8	8	8	8	8

[a] Defined as in Table 3.

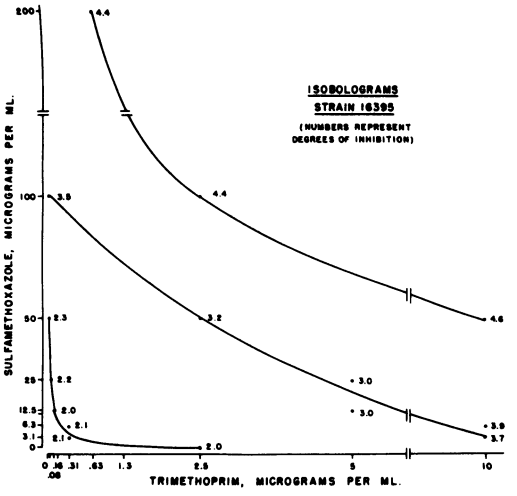

FIG. 4. *Isobolograms based on inhibition of H. influenzae 16395 by combinations of TMP and SMZ. The isobols represent minimum concentrations of the two drugs which together produced DI values (see Fig. 1) of approximately 2 (range, 2.0 to 2.3) (bottom curve), 3 (range, 3.0 to 3.9) (middle curve), and >4 (range, 4.4 to 4.6) (top curve). The greatest synergistic effect was produced when DI was ~2 (lowest curve).*

significantly more strains. The technique used permitted quantitation of susceptibility to these drugs. However, the relation of the quantitative inhibition to clinical response will require further investigation.

Synergy has been variously defined on the basis of the greater antibacterial activity of a combination of drugs as compared with the action of each component individually. The MIC, MBC, and rate of bactericidal action have been used as the quantitative measures of determining synergy. On the basis of the findings presented in this paper, synergy may be defined as a greater degree of inhibition of the test organism by the combination of TMP/ SMZ as compared with that by TMP or SMZ alone. By adding the requirement that the inhibition of the combination be at least 1 log greater than that of the more active of the components, TMP/SMZ could be considered as acting synergistically against 19 of the strains of *H. influenzae* tested. For the strain (16395) shown in Fig. 4, synergy as interpreted from the shape of the isobolograms was clearly shown only when the DI was about 2 and is probably not of any importance.

TMP/SMZ may represent an alternative to current regimens for therapy of infections due to *H. influenzae*. Frazen and Brandberg (6) reported successful treatment of a patient with bacteremia and pneumonia due to *H. influenzae* type b. Sabel and Brandberg (14) sterilized the cerebrospinal fluid in an infant with *H. influenzae* type b meningitis by adding TMP/SMZ to therapy with ampicillin after the latter alone had failed. TMP/SMZ may be effective therapy also for localized infections, such as otitis media and sinusitis, which are frequently caused by *H. influenzae*, and this combination may prove to be useful against infection with strains of *H. influenzae* that are resistant to ampicillin or other antibiotics.

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