

## Minimum Bactericidal Concentration of Sulfamethoxazole-Trimethoprim for *Haemophilus influenzae*: Correlation with Prophylaxis

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The inability of sulfamethoxazole-trimethoprim (SXT) to eradicate *Haemophilus influenzae* nasopharyngeal carriage in all asymptomatic patients in closed populations was examined in vitro. A broth medium was adapted for susceptibility testing of *H. influenzae* which permitted us to determine minimum inhibitory concentrations and minimum bactericidal concentrations (MBCs). The minimum inhibitory concentrations were all low, but the MBCs were bimodally distributed. Trimethoprim alone or the combination SXT either was bactericidal for *H. influenzae* isolates at low concentrations (i.e., low MBCs) similar to minimum inhibitory concentrations or showed no bactericidal activity (i.e., high MBCs). If trimethoprim was bactericidal when tested alone against *H. influenzae*, then the combination SXT was also bactericidal. *H. influenzae* carriage could not be eradicated from asymptomatic patients with SXT therapy when that combination was not bactericidal for these isolates in vitro. *H. influenzae* carriage was eradicated from patients when the activity of SXT was bactericidal in vitro. *H. influenzae* strains that are not killed by trimethoprim or SXT seem to occur at random.

Although *Haemophilus influenzae* is not usually regarded as a cause of epidemic disease, the risk of outbreaks of systemic infection caused by this organism is greater in closed populations (5, 6; M. E. Melish, A. J. Nelson, T. E. Martin, and C. W. Norden, *Pediatr. Res.* 10:348, 1976). High nasopharyngeal carriage rates for *H. influenzae* type b may be found among asymptomatic children attending day care centers (5, 6; Melish et al., *Pediatr. Res.* 10:348, 1976). Some clinicians believe that these children should receive prophylactic therapy to eradicate *H. influenzae* from the nasopharynx, because they may be a source of systemic infection which may occur in either culture-positive or culture-negative children. It has been suggested that culture-negative children may be more susceptible because they are less likely to be protected by serum anticapsular antibody (6, 7).

At one children's chronic care facility in Chicago, Ill., simultaneous carriage of ampicillin-resistant and ampicillin-susceptible strains has been reported (R. Yogeve, H. B. Lander, and A. T. Davis, *J. Pediatr.*, in press). Thus, penicillins could not be used to eradicate *H. influenzae* from the upper respiratory system. Furthermore, chloramphenicol and tetracycline, which are alternate drugs for therapy of infections,

were not considered acceptable for prophylaxis because of the potential hazards of therapy (13). Since sulfamethoxazole-trimethoprim (SXT) had been used successfully to treat *H. influenzae* infections (1) and had also been used to eliminate *H. influenzae* carriage in asymptomatic patients (Melish et al., *Pediatr. Res.* 10:348, 1976), the children in the Chicago facility were treated prophylactically with SXT. *H. influenzae* was not eradicated in some of the children. SXT therapy also failed to eradicate *H. influenzae* from the nasopharynx of children in a closed care center in California (D. Granoff, personal communication).

We obtained cultures of *H. influenzae* from the carriers in these two institutions to determine if we could demonstrate in the laboratory the reasons for the failure of SXT to eradicate these organisms. This is a report of studies with SXT, and, in addition, of in vitro data on the susceptibility of these *H. influenzae* strains to other potentially prophylactic antibiotics.

### MATERIALS AND METHODS

**Cultures.** Three groups of *H. influenzae* cultures were studied. (i) Fifty isolates (some pretreatment and some post-treatment) from 18 children in a chronic care facility in Chicago were received from A. Todd

Davis. All of the isolates were type b, and 33 were  $\beta$ -lactamase positive; the remaining 17 were  $\beta$ -lactamase negative and susceptible to ampicillin. The children from whom these strains were isolated had been treated with SXT (4 mg of trimethoprim [TMP] + 20 mg of sulfamethoxazole [SMX]/kg per day) for 5 days. This regimen failed to eradicate ampicillin-resistant *H. influenzae* from the carriers. The children were retreated with 2 $\times$  the dose for 10 days. After this treatment, *H. influenzae* were still isolated, but an unexpected result was that all isolates were  $\beta$ -lactamase positive. The  $\beta$ -lactamase-positive and -negative strains from Chicago will be referred to as the "Chicago isolates" (Yogev et al., in press). (ii) Eighteen *H. influenzae* type b cultures, isolated from children who attended a day care center in Fresno, Calif., were received from D. Granoff. Therapy with SXT had also failed to eliminate *H. influenzae* type b carriage from most of the children attending the day care center (Granoff, personal communication). All of these isolates were  $\beta$ -lactamase negative and ampicillin susceptible. These isolates will be referred to as the "California isolates." (iii) Fifty-four *H. influenzae* isolates were selected at random from routine cultures sent to the Center for Disease Control from a variety of geographic areas. Thirty-three of these strains were  $\beta$ -lactamase positive, and 21 were  $\beta$ -lactamase negative. These isolates represented both type b and nontypable *H. influenzae* strains from a variety of sources, including the upper respiratory tract, blood, and spinal fluid. This group will be referred to as the "random isolates."

**$\beta$ -Lactamase test.**  $\beta$ -Lactamase production was determined by the rapid chromogenic cephalosporin test (10).

**Antibiotics.** TMP lactate and SMX were obtained from Burroughs-Wellcome, Research Triangle Park, N.C.; tetracycline hydrochloride and doxycycline hyclate were from Pfizer, Inc., New York, N.Y.; rifampin was from Ciba Pharmaceutical Co., Summit, N.J.; and erythromycin was from Eli Lilly & Co., Indianapolis, Ind. Stock solutions of these antimicrobial agents were made in the appropriate diluents (12), and further dilutions were made in supplemented Mueller-Hinton (M-H) broth (see below). TMP was tested in concentrations ranging from 8 to 0.004  $\mu$ g/ml; SMX, from 152 to 0.15  $\mu$ g/ml; SXT, from 76/4 (34/2  $\mu$ g/ml for the California strains) to 0.08/0.004  $\mu$ g/ml; and the other antimicrobial agents, from 64 to 0.06  $\mu$ g/ml.

**Susceptibility tests.** (i) **Agar dilution test.** M-H agar (Difco) was cooled to 50°C and supplemented with 5% lysed horse blood and 2.5  $\mu$ g of reduced nicotinamide adenine dinucleotide per ml and the appropriate amount of antimicrobial agent. The inoculum was prepared by suspending some of the 24-h growth from a chocolate agar plate into M-H broth and adjusting it to contain  $10^7$  colony-forming units (CFU)/ml (11). A further 1:10 dilution of the suspension was made into M-H broth. The adjusted culture was inoculated onto the agar with a Steers replicator (9). Each inoculum "spot" contained  $10^3$  CFU. After 24 h of incubation at 35°C, the minimum inhibitory concentration (MIC) was the lowest concentration of drug at which there was a 95 to 100% reduction of growth as compared with the control.

(ii) **Broth microdilution test.** In a separate study in our laboratory, we have shown that a broth microdilution method could be used for testing the susceptibility of a variety of rapidly growing bacteria to the combination SXT (J. Swenson and C. Thornsberry, submitted for publication). M-H broth (low in thymidine) was supplemented with 5% lysed centrifuged horse blood and nicotinamide adenine dinucleotide (2.5  $\mu$ g/ml of broth) for these tests with *H. influenzae*.

Microdilution plates were prepared with the MIC 2000 dispenser (Dynatech, Inc., Alexandria, Va.). The plates were stored by freezing at -70°C. *H. influenzae* isolates were grown overnight on M-H agar with 1% hemoglobin (Difco) and 1% IsoVitalX (BBL). The inoculum was prepared by suspending some of the 24-h growth into M-H broth and adjusting it to contain  $10^7$  CFU/ml with a light-scattering photometer (11). A further 1:10 dilution of the suspension was made into 36 ml of sterile distilled water. The plates were inoculated with the MIC 2000 inoculator (Dynatech, Inc.), which delivered approximately  $10^3$  CFU to the wells of the plate. The final inoculum concentration was approximately  $10^4$  CFU/ml.

The microdilution plates were incubated for 24 h at 35°C. The MIC was read as the lowest concentration of drug inhibiting visible growth of *H. influenzae*. Minimum bactericidal concentrations (MBCs) were determined by transferring approximately 0.0015 ml from each well of the microdilution plate with the MIC 2000 inoculator to a petri dish (15 by 150 mm) containing Schaedler agar supplemented with 1% hemoglobin and 1% IsoVitalX. The plates were incubated at 35°C for 48 h. The MBC was read as the lowest concentration of drug that prevented growth of more than one colony on subculture.

## RESULTS

The MIC results for TMP and SXT for the agar and broth dilution methods are summarized in Table 1. With both methods, *H. influenzae* isolates had MICs indicating susceptibility to TMP alone and to SXT. The broth dilution method gave clear end points without trailing growth (i.e., progressive diminution of growth). The end points with the agar dilution method were more difficult to determine with all isolates, but visually there was a definite 95 to 100% reduction of growth as compared with the control. With all but three of the Chicago isolates that were  $\beta$ -lactamase positive, however, a haze persisted through all the dilutions, but with the Chicago  $\beta$ -lactamase-negative strains, there was no haze. MBCs are shown in Tables 2, 3, and 4. The Chicago *H. influenzae* isolates could be divided into two groups on the basis of their MBCs. One group was  $\beta$ -lactamase negative, and the activity of TMP or SXT was bactericidal at low concentrations. The second group of Chicago isolates was  $\beta$ -lactamase positive, but with the exception of three strains, the action of TMP or SXT was not bactericidal.

TABLE 1. MICs to TMP, SMX, and SXT for 122 *H. influenzae* isolates

Antimicrobial agent	MIC ( $\mu\text{g/ml}$ )		
	GM <sup>a</sup>	Mode	Range
TMP (agar dilution) <sup>b</sup>	0.045	0.06	0.015-0.12
TMP (broth dilution)	0.06	0.06	$\leq 0.008-0.5$
SMX (broth dilution) <sup>c</sup>	4.8	2.4	0.3->152
SXT (agar dilution)	0.6/0.03	0.6/0.03	0.3/0.015-2.4/0.12
SXT (broth dilution)	0.3/0.015	0.3/0.015	0.15/0.008-2.4/0.12

<sup>a</sup> GM, Geometric mean.

<sup>b</sup> Chicago isolates only.

<sup>c</sup> SMX MICs could not be read with the agar dilution method, because the diminution of growth was gradual and no definite end point could be determined.

TABLE 2. MBCs for 50 Chicago *H. influenzae* isolates

Antimicrobial agent	Bactericidal	% of isolates	MBC ( $\mu\text{g/ml}$ )		
			GM <sup>a</sup>	Mode	Range
TMP	Yes <sup>b</sup>	40	0.25	0.25	$\leq 0.008-2.0$
TMP	No	60	>8.0	>8.0	8.0->8.0
SMX	Yes <sup>c</sup>	32	7.15	2.4, 4.8	2.4-19.0
SMX	No	68	>152	>152	32.0->152
SXT	Yes <sup>d</sup>	40	0.6/0.03	0.3/0.015	0.15/0.008-19/1
SXT	No	60	>76/4	>76/4	>76/4

<sup>a</sup> GM, Geometric mean.

<sup>b</sup> MBC  $\leq 2.0 \mu\text{g/ml}$ .

<sup>c</sup> MBC  $\leq 19.0 \mu\text{g/ml}$ .

<sup>d</sup> MBC  $\leq 19/1 \mu\text{g/ml}$ .

TABLE 3. MBCs for 18 California *H. influenzae* isolates

Antimicrobial agent	Bactericidal	MBC ( $\mu\text{g/ml}$ )	% of isolates
TMP	Yes	0.5	6
TMP	No	>16.0	94
SMX	Yes	19.0	6
SMX	No	>76.0	94
SXT	Yes	0.3/0.015	6
SXT	No	>38/2	94

The California *H. influenzae* isolates were all  $\beta$ -lactamase negative, but TMP or SXT was bactericidal for only one isolate.

TMP or SXT was bactericidal for 31% of the 54 Center for Disease Control random *H. influenzae* isolates. For the remaining 69% of the isolates, the MBCs were greater than the highest dilution tested. This group contained both  $\beta$ -lactamase-positive and  $\beta$ -lactamase-negative strains, but there was no apparent correlation between  $\beta$ -lactamase production and SXT bactericidal activity. There was complete correlation between the bactericidal activity of SXT and TMP. If a strain was killed by SXT, it was also killed by TMP alone.

Table 5 shows the MIC and MBC results for the four other antibiotics tested. Most of the 122

isolates had erythromycin MICs of 2 to 4  $\mu\text{g/ml}$  and rifampin, tetracycline, and doxycycline MICs of 0.25 or 0.5  $\mu\text{g/ml}$ . Strains with tetracycline MICs of 16  $\mu\text{g/ml}$  generally had corresponding doxycycline MICs of 4 to 8  $\mu\text{g/ml}$ . The MBCs of these four drugs were either the same as the MIC or one dilution higher.

## DISCUSSION

On the basis of MICs alone (i.e., not considering MBCs), all of the isolates of *H. influenzae* in this study were susceptible to TMP and to SXT when tested either in a broth medium or on an agar medium. Initially, the MICs of TMP and SXT were obtained for the Chicago isolates by the agar dilution method, because at that time we did not have an adequate broth medium for a microdilution test. The end points in the agar dilution method were considered easy to read, because there was a 95 to 100% reduction of growth from that of the control. When the agar plates were held at an angle to a light source, however, a slight haze due to bacteria could be seen through all dilutions with some of the isolates. A haze such as this is not unusual in tests with sulfonamides or TMP and would normally be disregarded. With these organisms, however, the haze occurred only with isolates

TABLE 4. MBCs for 54 Center for Disease Control random *H. influenzae* isolates

Antimicrobial agent	Bactericidal	% of isolates	MBC ( $\mu\text{g/ml}$ )		
			GM <sup>a</sup>	Mode	Range
TMP	Yes <sup>b</sup>	31	0.25	0.25	$\leq 0.008$ -2.0
TMP	No	69	>8.0	>8.0	>8.0
SMX	Yes <sup>c</sup>	13	4.8	2.4	2.4-19.0
SMX	No	87	>152	>152	>152
SXT	Yes <sup>d</sup>	31	0.6/0.03	0.3/0.015	0.15/0.008-19/1
SXT	No	69	>76/4	>76/4	>76/4

<sup>a</sup> GM, Geometric mean.

<sup>b</sup> MBC  $\leq 2 \mu\text{g/ml}$ .

<sup>c</sup> MBC  $\leq 19 \mu\text{g/ml}$ .

<sup>d</sup> MBC  $\leq 19/1 \mu\text{g/ml}$ .

TABLE 5. MICs and MBCs of erythromycin, rifampin, tetracycline, and doxycycline for 122 *H. influenzae* isolates

Antimicrobial agent	MIC ( $\mu\text{g/ml}$ )			MBC ( $\mu\text{g/ml}$ )		
	GM <sup>a</sup>	Mode	Range	GM	Mode	Range
Erythromycin	2	2	0.25-8	2	2	0.5-16
Rifampin	0.5	0.5	0.25-1	0.5	0.5	0.25-1
Tetracycline	0.5	0.25	0.25-16	0.5	0.5	0.25-32
Doxycycline	0.5	0.25	0.25-4	0.5	0.5	0.25-4.0

<sup>a</sup> GM, Geometric mean.

that were  $\beta$ -lactamase positive and that were not eradicated from the carriers with SXT therapy. This unusual finding prompted further study.

Fortuitously, recent studies in our laboratory had shown that susceptibility tests to SXT could be done in a broth medium with organisms other than *H. influenzae* (Swenson and Thornsberry, submitted for publication). We adapted the method for tests on these *H. influenzae* isolates by adding lysed horse blood and nicotinamide adenine dinucleotide, which provided the growth requirements necessary for *H. influenzae*. With this medium and an inoculum of approximately  $10^4$  CFU/ml, microdilution tests could be done that gave clear, easy to read, and reproducible MIC end points. The availability of this method was important because it allowed us not only to reproduce the agar dilution results, but also to determine the MBCs for these isolates.

Two populations of organisms existed in terms of their MBC. TMP or SXT was either bactericidal at low concentrations (geometric means: TMP, 0.25; SXT, 0.6/0.03) or not bactericidal, and growth occurred at the highest concentration tested. It was also observed that if TMP was bactericidal when tested alone against *H. influenzae*, then the combination SXT was also bactericidal. The MBCs could also be correlated with the presence or absence of the haze seen in

the agar dilution tests. The MBCs were low for those strains that did not show the trailing haze; i.e., SXT was bactericidal for these isolates. Conversely, SXT was not bactericidal for those strains that showed trailing end points (haze) on the agar dilution tests.

The MBCs could also be correlated with the results of prophylactic therapy with SXT, as can be shown by examining the history of the Chicago isolates. These isolates could be divided into two groups. One group was  $\beta$ -lactamase negative and had low MBCs for TMP or SXT. When the patients were treated with SXT, all of these isolates were eradicated from the carriers (Yogev et al., in press). The second group of *H. influenzae* isolates produced  $\beta$ -lactamase and had high MBCs; i.e., TMP or SXT was not bactericidal for these organisms in vitro. These strains were not eradicated even with a second treatment with SXT (Yogev et al., in press). There was a similar correlation between MBCs and failure to eradicate the organisms in the California strains (Granoff, personal communication). Our initial studies with the Chicago isolates indicated a relationship between  $\beta$ -lactamase production by *H. influenzae* and the bactericidal effect of SXT on these organisms. The California isolates, however, were  $\beta$ -lactamase negative, and TMP or SXT was not bactericidal in vitro against most of these isolates. Therapy with SXT was ineffective in elim-

inating *H. influenzae* carriage in most of these children (Granoff, personal communication). Furthermore, SXT showed bactericidal activity against slightly less than one-third of the random group of *H. influenzae* isolates from our laboratory, although 61% of these isolates were  $\beta$ -lactamase positive. Thus, there is probably no correlation between  $\beta$ -lactamase production by *H. influenzae* and the bactericidal activity of SXT.

May and Davies (8) reported that 52% of 210 isolates of *H. influenzae* were resistant to TMP by the disk method and that 17 of 18 of these strains were resistant to 10  $\mu$ g or more of TMP per ml. These conclusions were based on the concentrations of TMP necessary to inhibit growth completely, and in both methods the organisms showed either long trailing end points in the agar dilution tests or small colonies within the inhibition zone in the disk tests. Bushby (2) studied 17 of the strains of May and Davies and confirmed their observations on the trailing end points. But, in addition, Bushby reported that the organisms with long trailing end points were morphologically abnormal and mostly dead and that the few viable aberrant forms did not multiply when transferred to medium containing the same concentration of TMP as that in which they initially multiplied, indicating that they represented only a temporary phase of growth.

Bushby also found that in experiments with mice, these allegedly resistant strains were no more resistant in vivo to TMP than those strains judged to be susceptible and that serial passage in the presence of TMP did not alter the end points of strains with either long trailing or clear-cut end points, indicating that the aberrant forms apparently are not a phase in the development of resistance to TMP. He concluded, therefore, that these seemingly resistant strains were susceptible to TMP (2). We have not done the kind of studies that Bushby did, but the correlation of our high MBCs with prophylactic failure in children, and vice versa, indicates that neither TMP nor SXT has bactericidal activity against the strains with high MBCs but both are bactericidal against strains with low MBCs (if TMP is bactericidal, then SXT is bactericidal). The appropriateness of SXT as a prophylactic agent to eliminate *H. influenzae* from children in a closed population would depend upon whether TMP or SXT has bactericidal activity against their isolates. Our knowledge of the three groups of *H. influenzae* we studied indicates that strains that are not killed by TMP or SXT occur randomly. The success of SXT in eradicating *H. influenzae* could be predicted if tests for MBCs were performed on the strains before treatment.

Performance of the MBC test will depend, however, upon the use of broth that is either free of or has only minimal amounts of thymidine. If enough thymidine is present in the medium, it will reduce the activity of the drug and result in higher end points of MICs, and especially in those of MBCs.

The effectiveness of other antimicrobial agents, such as erythromycin, rifampin, or tetracycline, as prophylactic agents against *H. influenzae* is difficult to judge from the MICs we obtained with these isolates. If adequate saliva levels are necessary, as reported for eradicating meningococci from the upper respiratory tract (3), then erythromycin could probably be ruled out, since most MICs are 2  $\mu$ g/ml, that is, more than the levels achievable in the tonsil or saliva (4). The MICs for rifampin are probably borderline in this regard, since most of the MICs for rifampin against *H. influenzae* and the amount of rifampin that can be achieved in the saliva are roughly the same (3). Clinical trials will be necessary to determine the efficacy of rifampin. Preliminary data indicate, however, that rifampin eradicated most of the *H. influenzae* in one institution (Granoff, personal communication) and not in another (A. Todd Davis, personal communication).

Although most of the strains were susceptible to the two tetracyclines tested, tetracyclines are not generally considered drugs of choice for the treatment of young children, because of their undesirable side effects (13). Most of the MICs might be low enough, however, for the organisms to be susceptible, if tetracyclines were used prophylactically.

Thus, eliminating *H. influenzae* carriage has been recommended as a means of controlling outbreaks of systemic infection caused by *H. influenzae* in enclosed populations (5, 6; Melish et al., *Pediatr. Res.* 10:348, 1976). *H. influenzae* type b polysaccharide vaccine was unsuccessful in one study (5), and therapy with ampicillin was not efficacious in other studies (5, 6). SXT was successful in eliminating *H. influenzae* carriage in one study (Melish et al., *Pediatr. Res.* 10:348, 1976), and although we have not examined isolates from this study, the activity of SXT was probably bactericidal. Thus, the efficacy of SXT, as a prophylactic treatment of *H. influenzae*, probably depends upon its bactericidal activity against this organism.

In each of the closed populations (Chicago and California), many of the children may have been colonized by homologous strains that were passed from child to child. This probably accounted for the association of strains that were not killed by SXT and the production of  $\beta$ -lactamase among isolates from Chicago. How-

ever, the occurrence of strains not killed by SXT is not limited, as shown by the data obtained with the random isolates from our laboratory. Since the Chicago and California isolates may represent a limited number of strains, the hypothesis that eradication of the organism correlates with in vitro bactericidal activity should be tested in other clinical situations.

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