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Research Article

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Elaboration of Type b Capsule by *Haemophilus influenzae* as a Determinant of Pathogenicity and Impaired Killing by Trimethoprim-Sulfamethoxazole

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ABSTRACT In vitro, *Haemophilus influenzae* strains have two distinct patterns of susceptibility to trimethoprim-sulfamethoxazole (TMP/SMZ); strains with low minimum inhibitory concentration and high minimum bactericidal concentration (tolerant) and those with both low minimum inhibitory concentration and minimum bactericidal concentration (kill-sensitive). Tolerant *H. influenzae* strains were found to elaborate significantly more type b capsular polysaccharide, a linear polymer of ribosyl ribose phosphate (PRP), than kill-sensitive strains. Tolerant strains became susceptible to killing by TMP/SMZ when type b capsule was physically removed, but reacquired tolerance following growth and reversion to original (mucoid) phenotype. Susceptibility of wild (type a, b, c), isogenic (type b and untypable), and transformed (type b and d) strains indicated that elaboration of type b capsule was associated with TMP/SMZ tolerance.

In a second series of studies, virulence of *H. influenzae* in the infant rat model was correlated with in vitro tolerance. Tolerant strains (13/13) caused systemic disease while none (0/7) of kill-sensitive strains were pathogenic. The efficacy of TMP/SMZ in the treatment of invasive infection was evaluated in rats with established bacteremia and meningitis. TMP/SMZ failed to eradicate *H. influenzae* b from the blood in 85% (17/20) or from the cerebrospinal fluid in 95% (19/20) of infected animals. Thus, in vitro tolerance correlated with therapeutic failure in vivo.

INTRODUCTION

Haemophilus influenzae type b (Hib)[1] is a major pathogen of infancy and childhood resulting in bloodstream infections associated with serious complications such as meningitis. In recent years, attention has been focused on the occurrence of secondary cases of invasive Hib infections in households or day-care centers where susceptible young children are in intimate contact and at high risk of developing invasive disease (1–5). Efforts to interrupt person-to-person spread of Hib in these settings have included the use of antibiotics such as trimethoprim-sulfamethoxazole (TMP/SMZ) (1, 3, 7), rifampin, (6–8), cefaclor (9, 10), and ampicillin (11–13). During an outbreak of Hib carriage and disease in a chronic disease hospital (3), TMP/SMZ failed to eradicate nasopharyngeal carriage in 30% of carriers, although the minimal inhibitory concentration (MIC) of TMP/SMZ for these *H. influenzae* (Hi) strains indicated that they were susceptible. Subsequent investigations by Kirven and Thornsberry (14) showed that although some strains of Hi were killed by low concentrations of TMP/SMZ (≤0.03:0.6 μg/ml), a proportion of strains, hereafter designated tolerant, were inhibited but not killed by concentrations of TMP/SMZ as high as 4:76 μg/ml. The distinction seemed pertinent, since all Hi strains that persisted in the nasopharynx of TMP/SMZ-treated children were tolerant (14).

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[1] *Abbreviations used in this paper*: CSF, cerebrospinal fluid; ELIZA, enzyme-linked immunosorbent assay; Hi, *Haemophilus influenzae*; Hib, Hi type b; MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration; PRP, capsular polysaccharide, a polymer of ribosyl ribose phosphate; sBHI, supplemented brain heart infusion broth; SMZ, sulfamethoxazole; TMP, trimethoprim.

The present studies show that TMP/SMZ tolerance is directly related to elaboration of type b capsule—a linear polymer of ribosyl ribose phosphate (PRP)—which is a major virulence determinant of Hi. Using an experimental model we found that virulence and in vitro tolerance to TMP/SMZ correlated with therapeutic failure.

METHODS

Bacterial strains. 12 Hib isolates were obtained from nasopharynx, blood, or cerebrospinal fluid (CSF) cultures of children in Chicago. Strains 835437, 834502, 838006, and 838091 were donated by Linda Kirven (Center for Disease Control, Atlanta, Ga.). These strains had been isolated from routine cultures using chocolate agar plates. Strain Eag (15) and a spontaneously derived, unencapsulated mutant, strain S (16) were donated by Dr. Porter Anderson (University of Rochester Medical Center, Rochester, N. Y.). Strain Sim (17) produced beta-lactamase and was isolated from the CSF of a child with meningitis in Baltimore. Strains C121 and R274 also produced β-lactamase and were isolated from the blood of children in Boston (donated by Dr. Arnold Smith, Children's Orthopedic and Medical Center, Seattle, Wash.). Strain Morgan (type a) was isolated from the nasopharynx of a child in Vanderbilt (donated by Dr. S. H. Sell, Vanderbilt University, Nashville, Tenn.). A type c strain was obtained through the American Type Culture Collection (N. 9007). Strain KW-20 (untypable) was obtained from Dr. Ken Wilcox (Medical College of Milwaukee, Wis.) who selected a single clone from a multiple-passaged culture of strain Rd (18). Strains of Hi were identified by Gram stain characteristics, nutritional requirements for supplemental X and V factors, inability to grow on blood (rabbit) agar, and the porphyrin production test (21). Serotyping was performed by slide agglutination and counterimmunoelectrophoresis using rabbit type b antisera (Hyland Diagnostics, Div. Travenol Laboratories, Costa Mesa, Calif.). As an additional typing method, Hi strains were inoculated onto supplemented brain heart infusion broth (sBHI) containing 2% agar (Difco Laboratories, Detroit, Mich.) and 10% (vol/vol) hyperimmune burro serum (donated by Dr. J. B. Robbins, Bureau of Biologics, Bethesda, Md.). After 24-h incubation at 38°C, colonies of Hib formed halos (22). Biotyping was performed by biochemical reactions to urease, ornithine decarboxylase, β-galactosidase (ONPG), and indole, a modification of the method described by Kilian (23).

Transformation. To obtain type b and d transformants, we used a single step streptomycin-resistant mutant of strain KW-20 as the recipient. Approximate 10^8 competent (19) KW-20 cells (volume 1 ml) were incubated for 15 min at 38° with 1 μg DNA (20) obtained from strain Eag (type b) or strain ATCC No. 9008 (type d). The DNA exposed cells were incubated in 5 ml sBHI (containing serum) for 120 min at 38° to overcome phenotype lag and to enrich for capsulated transformants (18). We found that $\sim 10^{-5}$–10^{-7} recipient cells were transformed to the capsulated phenotype. As previously reported (18), this frequency is ~1,000-fold less than transformation to an unlinked antibiotic resistance phenotype (e.g., erythromycin resistance). Strain S was transformed to type b in a similar fashion using DNA from strain Eag.

Quantitation of type b antigen. The amount of PRP elaborated by strains of Hi was measured using an enzyme-linked immunosorbent assay (ELISA) (24). This assay is capable of detecting 0.1 ng/ml purified PRP (25) (obtained from Dr. Porter Anderson. Prior incubation with an excess of PRP resulted in loss of reactivity with culture supernatant from type b organisms. Heterologous serotypes of *H. influenzae* were nonreactive. Thus, the assay is both sensitive and specific for PRP. To quantitate amounts of PRP elaborated by different strains of *H. influenzae* a few colonies from a pure culture of Hi were inoculated into sBHI broth (10 ml) and grown at 38°C until late stationary phase (~12 h). After centrifugation at 5,000 *g* for 15 min 0.1 ml of cell-free supernatant was assayed for PRP. A standard curve was obtained using known concentrations of purified PRP.

Susceptibility to TMP/SMZ. A modification of the broth dilution test reported by Kirven and Thornsberry (14) was used for susceptibility tests of Hi to TMP and SMZ alone and in combination. Lysed horse blood was centrifuged at 30,000 rpm for 90 min and filtered (0.22 μm filter-Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). Mueller-Hinton broth (low in thymidine) was supplemented to contain 5% filtered horse blood and 2.5 μg/ml NAD. Stock solutions of TMP and SMZ (obtained from Hoffman-La-Roche, Inc., Nutley, N. J.) were freshly prepared in the appropriate diluents. Further dilutions were made with the supplemented Mueller-Hinton broth. TMP was tested in concentrations ranging from 0.004 to 8 μg/ml, SMZ from 0.15 to 152 μg/ml and TMP/SMZ from 0.004:0.08 to 4:76 μg/ml. From an overnight growth of Hi on a chocolate plate, three colonies were inoculated into 5 ml supplemented Mueller-Hinton broth. The tube was shaken for 4 h at 37°. Appropriate dilutions of the cultured bacterial suspension were made to achieve a final concentration of 10^4 bacteria/ml. 1 ml of this diluted bacterial suspension was inoculated into 1 ml of the antibiotic dilution (final volume 2 ml). The tubes were incubated for 18 h at 35° in 5% CO_2. The MIC was read as the lowest concentration of drug inhibiting visible growth of Hi. To determine the minimal bactericidal concentration (MBC), 0.01 ml from each tube was transferred to a chocolate plate and incubated at 35° in 5% CO_2 for 24 h. The MBC was read as the lowest concentration of the drug which prevented growth. A tolerant strain was defined as a strain which was inhibited but not killed by concentrations of TMP/SMZ as high as 4:76 μg/ml. Strains were also examined for tolerance to other antibiotics including tetracycline, chloramphenicol, rifampin, cefamandole, and ampicillin.

In one series of experiments, the MBC of TMP/SMZ for three tolerant strains (401, Bloch, Eag) and one kill-sensitive strain (S) was measured before and after removal of surface PRP. PRP was removed by suspending ~10^9 bacteria in 10 ml of cold, buffered saline (pH 7.0) and shaking vigorously for 5 min by hand. After shaking, an aliquot of the bacterial suspension was centrifuged at 4,000 *g* at 4° for 10 min. The amount of solubilized PRP in the cell-free supernatant was assayed by ELISA and the MBC of the washed bacteria was measured after every three washes. A total of 12 washes were performed. No significant change in colony counts during washings was observed.

Animal studies. In vivo studies utilized 5-d-old Sprague-Dawley rats (strain COBS/CD obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. The methodology for inducing invasive (bacteremia and meningitis) infection with Hib and the techniques of blood and CSF culture have been described (26). To investigate the efficacy of TMP/SMZ treatment of invasive Hi infection, rats were randomly assigned to a control group (saline) and a treatment group (5:25 mg/kg per dose of TMP/SMZ). All animals received a total of nine doses (volumes of 100 μl) intraperi-

toneally at 6-h intervals. Blood and CSF were obtained before treatment was started and 6 and 54 h after the ninth dose of TMP/SMZ. In one experiment 5-μl duplicate blood and CSF samples were obtained from groups of five rats at 15, 60, and 120 min after the first and ninth doses in order to determine blood and CSF concentrations of TMP. In a separate experiment, 50 μl blood and CSF were obtained from groups of 10 rats at 15 min, 2 h, and 6 h after the first dose, and 15 min, 6, 8, 12, 18, and 24 h after the ninth dose in order to determine blood and CSF concentrations of SMZ. Because 50-μl vol were needed for the SMZ determination, samples of CSF from three to four animals were pooled.

RESULTS

In vitro studies. 20 strains of Hi isolated from nasopharynx, blood, or CSF were tested to determine their in vitro susceptibility to TMP/SMZ using a broth dilution assay (Table I). All strains were inhibited by ≤0.03:0.6 μg/ml. 7 of 20 (35%) strains were killed by ≤0.03:0.6 μg/ml (kill-sensitive), but TMP/SMZ tolerant strains included all blood and CSF isolates and two isolates obtained from nasopharynx. None of the kill-sensitive strains was associated with systemic infection whereas strains exhibiting TMP/SMZ tolerance were associated with systemic Hi infections ($P < 0.01$). None of the 20 strains of Hi showed tolerance when tested against other antibiotics including tetracyline, chloramphenicol, cefamandole, rifampin, and ampicillin.

All 20 strains were serotype b when assayed by slide agglutination and counterimmunoelectrophoresis. However, tolerant strains could easily be distinguished from kill-sensitive strains by growing them on media impregnated with type b antiserum (22). Tolerant strains but not kill-sensitive strains were both iridescent and were surrounded by halos due to interaction of type b antigen and antibody. These observations indicated that tolerant strains elaborated relatively more PRP than sensitive strains. This was confirmed by measuring the amount of PRP elaborated using ELISA (Fig. 1).

To investigate directly the relationship between elaboration of PRP and tolerance to TMP/SMZ, strain 401 was suspended in neutral buffered saline and shaken to remove surface PRP. Successive washes increased the susceptibility of the bacteria to killing by TMP/SMZ (Fig. 2). This alteration in susceptibility was phenotypic because when the washed organisms were allowed to replicate for several hours, they reverted to TMP/SMZ tolerance. Virtually identical results were found with strains Bloch and Eag (data not shown). In contrast, no alternation in susceptibility to TMP/SMZ was found when strain S (kill-sensitive) was washed. Although saline washing removed PRP, it did not restore the phenotype to the kill-sensitive range (0.03:0.6 μg/ml) suggesting either incomplete removal of PRP, a role for other factors in tolerance, or both of these possibilities.

To provide evidence for the association of PRP with TMP/SMZ tolerance, we measured the in vitro TMP/SMZ susceptibility of strain Eag and compared it to that of its isogenic capsule-deficient mutant, strain S. In contrast to strain Eag (tolerant), strain S elaborates substantially reduced amounts of PRP (16), and was inhibited and killed by ≤0.03:0.6 μg/ml TMP/SMZ. DNA extracted from strain Eag transformed strain S to a halo-producing type b transformant (Sb), tolerant to TMP/SMZ (MBC > 4:76 μg/ml).

Strains S and Sb were exposed to 0.25:4.8 μg/ml TMP/SMZ and their relative survival examined at 1, 3, 6, and 18 h (Fig. 3). Growth of Sb was inhibited whereas more than 99% of strain S were killed. At 3 h Gram-stained smears of both S and Sb organisms

TABLE I
Susceptibility to TMP/SMZ of 20 Clinical Isolates of H. influenzae

Tolerant (MBC > 4:76)			Kill-sensitive (MBC ≤ 0.03:0.6)		
Strain	Source	Biotype	Strain	Source	Biotype
Eag	CSF	I	Sanders	nasopharynx	I
Sim	CSF	II	Cuevas	nasopharynx	V
Bloch	Blood	I	Phillips	nasopharynx	I
Fair	Blood	I	Carter	nasopharynx	III
835437	Nasopharynx	I	834502	nasopharynx	V
401	Nasopharynx	I	838006	nasopharynx	III
Foster	CSF	II	838071	nasopharynx	V
McAffee	Blood	II			
McGraw	Blood	I			
Rosa	CSF	I			
Valee	CSF	II			
C121	Blood	I			
R274	Blood	II			

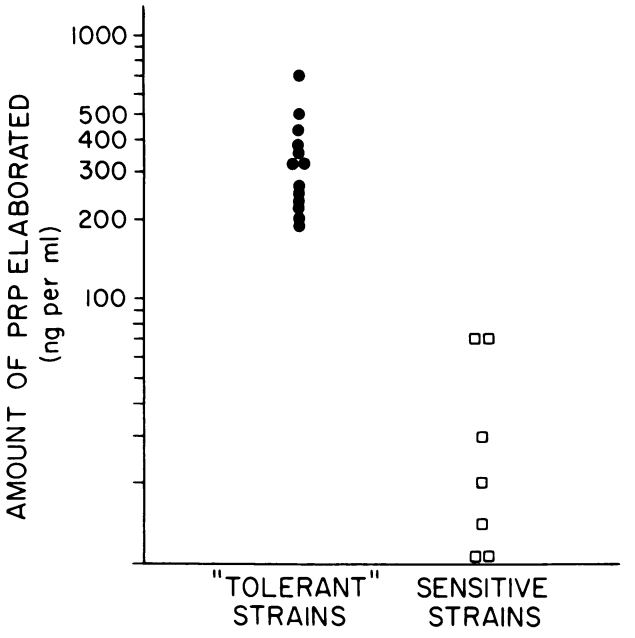

FIGURE 1 Quantity of PRP (nanograms per milliliter) elaborated by *H. influenzae* tolerant (●) and sensitive (□) to killing by TMP/SMZ.

showed a 5 to 10-fold increase in length compared with controls (not exposed to antibiotics). After 6 or more h of TMP/SMZ exposure only Sb—but not S—

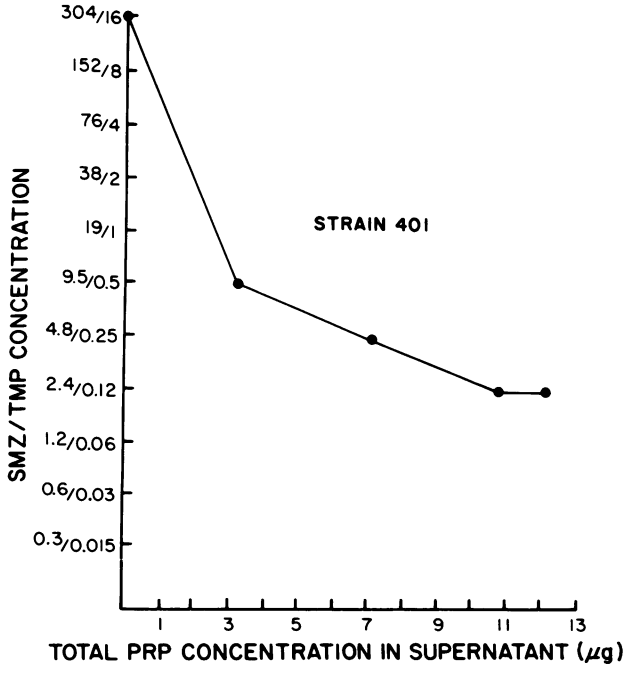

FIGURE 2 Effect of removal of PRP from *H. influenzae* type b (strain 401) by repeated washes with phosphate-buffered saline on the MBC of TMP/SMZ.

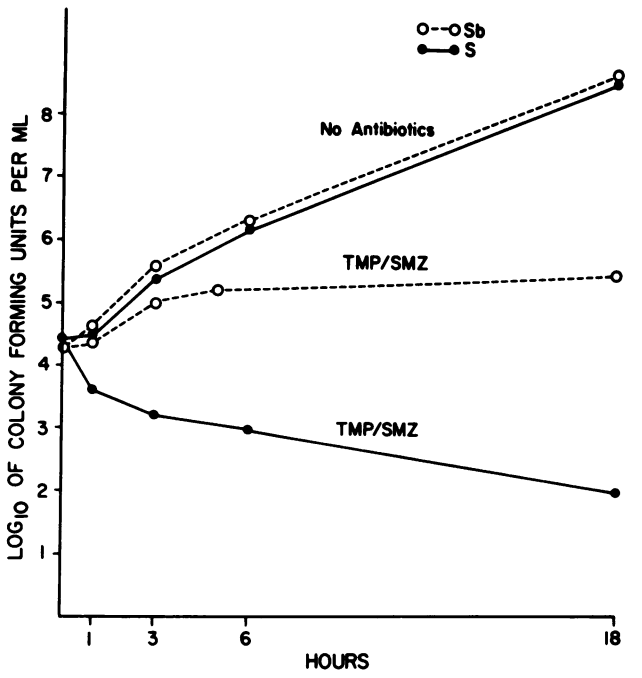

FIGURE 3 Effect of TMP/SMZ on the survival of strain S and its type b transformant. Bacteria were grown at 37°C in the presence or absence of 0.25:4.8 μg/ml TMP/SMZ.

organisms were seen; at 18 h many of the Sb organisms appeared filamentous (Fig. 4).

Because these findings demonstrated a direct relationship between elaboration of PRP and in vitro tolerance to TMP/SMZ, we hypothesized that PRP was limiting the entry of either TMP or SMZ (or both) into the bacterial cell. However, TMP and SMZ, alone or in combination, inhibited both kill-sensitive and tolerant strains at similar concentrations (Table II). Thus PRP did not prevent TMP or SMZ from entering the bacteria to inhibit replication.

We examined the TMP/SMZ susceptibility of different serotypes elaborating copious amounts of capsule as judged by colonial appearance. Wild types a (strain Morg) and c (strain ATCC No. 9007) were inhibited by ≤0.03:0.6 μg/ml and killed by ≤0.12:2.4 μg/ml TMP/SMZ, respectively. Comparison of the genetically related type b and d transformants (derived from strain KW-20) showed that although the MIC of both the type b and d transformants were identical (0.03:0.6 μg/ml), the MBC of the type b transformant was >4:76 whereas the MBC of the type d transformant was 0.25:4.8 μg/ml. Thus, type b but not type d capsule of strain KW-20 was associated with TMP/SMZ tolerance.

In vivo studies. We used a rat model of invasive Hi infection to evaluate the virulence of tolerant- and kill-sensitive strains. All 13 tolerant strains produced invasive infection in rats following intraperitoneal in-

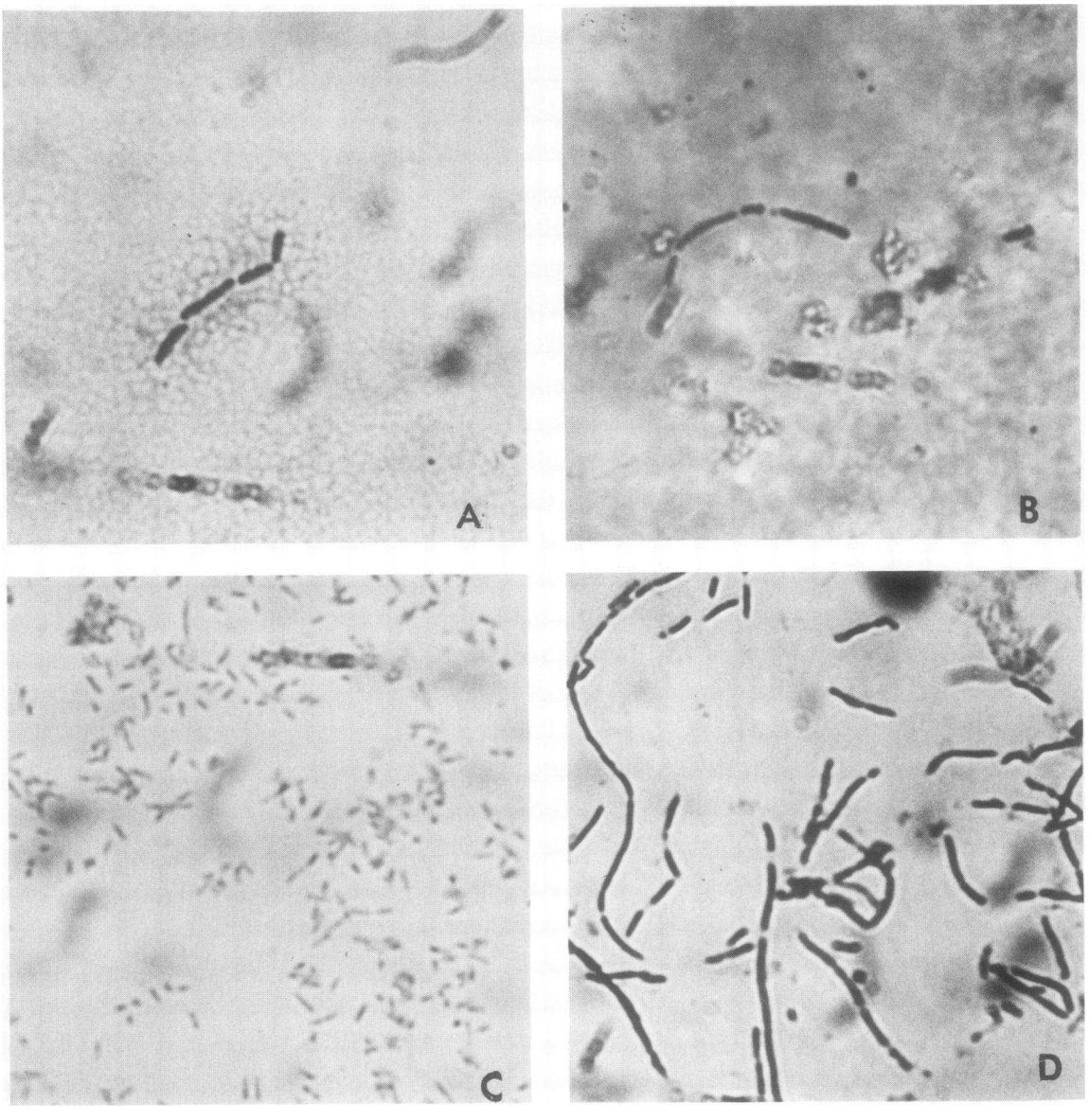

FIGURE 4 Morphology of strain Sb grown in the presence or absence of 0.25:4.8 μg/ml TMP/SMZ. (A) 3 h after exposure to antibiotics; (B) 6 h after exposure to antibiotics; (C) 18 h, no antibiotics; (D) 18 h after exposure to antibiotics. Gram stain ×1,000.

oculation of 10^4 organisms (incidence of bacteremia 90–100%) or intranasal inoculation of 10^7 organisms (incidence of bacteremia 30–60%). In contrast, the seven kill-sensitive strains did not produce detectable bacteremia following either intraperitoneal or intranasal challenge, even with inocula exceeding 10^8 organisms.

We evaluated the relevance of TMP/SMZ tolerance with respect to its therapeutic efficacy for treating Hi bacteremia and meningitis in the rat. In preliminary experiments, we determined the dose of TMP/SMZ resulting in serum and CSF concentrations comparable to those achieved in the treatment of invasive infections in children. Following a single intraperitoneal injection of 5:25 mg/kg TMP/SMZ, the mean peak serum concentration of TMP was 5.7±0.42 μg/ml and that of SMZ was 54.2±1.4 μg/ml. As reported in humans, accumulation of SMZ ($t_{1/2}$ = 12 h) but not TMP ($t_{1/2}$ = 60 min) occurred in the rat when successive doses were given at 6-h intervals. After nine doses (Fig. 5) mean peak serum concentration of TMP/SMZ were 6.3±0.6 and 185.5±16.5 μg/ml, respectively. These serum concentrations approximated those achieved in children treated for invasive infections using 10:50

TABLE II

MIC of TMP/SMZ (Alone or in Combination) against Kill-sensitive and Tolerant Strains of H. influenzae

	TMP	SMZ	TMP/SMZ
		μg/ml	
Kill-sensitive			
Sanders	0.015	0.6	0.004:0.08
Cuevas	0.06	9.5	0.008:0.15
Phillips	0.03	2.4	0.008:0.15
Carter	0.06	4.8	0.008:0.15
Tolerant			
401	0.06	0.6	0.008:0.15
Valle	0.06	0.6	0.004:0.08
Foster	0.06	0.6	0.008:0.15
Fair	0.06	0.6	0.008:0.15

mg/kg TMP/SMZ every 6 h (27). Simultaneous serum/CSF ratios in the rat were ~2/1 for TMP and 2.5/1 for SMZ.

25 suckling rats were infected intraperitoneally with strain 401 (tolerant). Bacteremia and meningitis were documented 36 h later, before treatment (Table III). 20 rats were randomly assigned to the treatment group and 5 served as controls. No significant difference ($P > 0.1$) in the mean numbers of bacteria in blood or CSF was observed between treatment and control groups. 6 h after treatment none of the rats receiving saline (control group) has sterile blood or CSF cultures. In contrast, 13 of 20 treated rats had negative blood cultures ($P < 0.001$), while a 10-fold reduction in the number of bacteria in blood (from $3.9\pm0.6 \times 10^4$/ml to $1.5\pm0.6 \times 10^3$/ml) was seen in the remaining 7 animals. CSF cultures in 7 of 20 treated animals were negative ($P < 0.05$); a 10-fold reduction (from $5.6\pm0.8 \times 10^4$ to $3.6\pm0.7 \times 10^3$/ml) was found in the 13 rats whose CSF cultures remained positive. There was no change in the mean blood or CSF bacterial counts of controls. Cultures were repeated 54 h after cessation of TMP/SMZ treatment. Of the 13 blood and seven CSF cultures negative after 6 h of treatment, 10/13 blood (77%) and 6/7 CSF specimens (86%) had reverted to positive. The mean bacterial counts for blood ($6.3\pm1.6 \times 10^3$/ml) and CSF ($3.9\pm0.6 \times 10^4$/ml) were similar to those of untreated controls ($8.3\pm2.2 \times 10^3$ and $4.8\pm0.9 \times 10^4$/ml, respectively). Thus, despite an initial apparently favorable therapeutic response, TMP/SMZ in the dosages used did not eradicate infection. (Higher dosages of TMP/SMZ caused chemical peritonitis and animal mortality preventing evaluation of higher doses of medication.) These findings indicated that TMP/SMZ tolerance in vitro correlated with therapeutic failure in vivo.

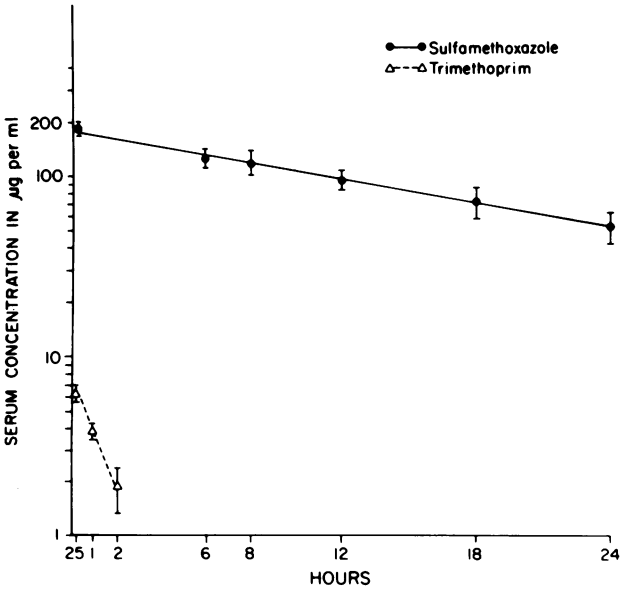

FIGURE 5 Mean serum concentrations of TMP and SMZ after the 10th intraperitoneal injection of 5:25 mg/kg TMP/SMZ given at 6-h intervals.

DISCUSSION

Previous studies showed that 0.03/0.6 μg/ml concentrations of TMP/SMZ inhibited virtually all strains of Hi. However, the majority (~60%) of type b strains are not killed by concentrations of TMP/SMZ exceeding 4/76 μg/ml (14). This in vitro tolerance to TMP/SMZ has an important clinical correlate; tolerant Hi persist in the nasopharynx of children after they have been treated with oral TMP/SMZ. The present studies show that tolerance to TMP/SMZ is associated with elaboration of PRP—the type b capsular polysaccharide and a major virulence determinant of Hi. This is exemplified by the fact that all 13 tolerant strains but none of the kill-sensitive strains produced bacteremia and meningitis in infant rats. In addition, the incidence and magnitude of bacteremia were significantly greater with KW-20 b organisms than with KW-20 d organisms. No detectable bacteremia occurred with the noncapsulated strain KW-20 (18). These observations have implications concerning the use of TMP/SMZ in the prevention and treatment of Hi infections. In addition to the ineffectiveness of TMP/SMZ in eradicating nasopharingeal carriage of Hib (3) our studies in an animal model showed that TMP/SMZ treatment failed to eradicate Hib from either blood or CSF. Concentrations of TMP and SMZ in blood and CSF of the rat were similar to those recommended in treating systemic infections in humans (27). Although restraint should be exercised in extrapolating these results to invasive infections of humans, these data suggest that clinicians should be cautious in selecting TMP/SMZ for the treatment of infections due to Hib,

TABLE III

Blood and CSF Cultures at 6 and 54 h after Treatment with Either TMP/SMZ (5:25 mg/kg/Dose Every 6 h for 54 h) or Saline in Infant Rats with H. influenzae Bacteremia and Meningitis (Strain 401)

	Blood			CSF		
Treatment	Before therapy	6 h after therapy	54 h after therapy	Before therapy	6 h after therapy	54 h after therapy
TMP-SMZ	0/20*	13/20	3/20	0/20	7/20	1/20
Saline	0/5	0/5	0/5	0/5	0/5	0/5

* Number of rats with sterile culture/number of rats tested.

particularly if susceptibility testing shows the infecting strain to be tolerant to TMP/SMZ in vitro.

In describing some strains of Hi as tolerant to TMP/SMZ it should be noted that the term was first coined by Tomasz and his colleagues to describe the unique response of lysis-defective pneumococci to penicillin (28). These insightful investigations showed that β-lactam antibiotics result in inhibition of pneumococci (also streptococci and staphylococci) by mechanisms that are distinct from those resulting in killing and lysis. By analogy, it seems that the mediators and/or target sites for the inhibition of Hi by TMP/SMZ may be distinct from those that determine killing or lysis.

Inhibition of Hi by TMP and SMZ occurs through its action on the enzymes dihydrofolate reductase and dihydropteroate synthetase, respectively. This action limits the availability of folates required as coenzymes in the biosynthesis of nucleic acids; ultimately, DNA synthesis and bacterial cell division ceases. It is also well documented that TMP/SMZ may be bactericidal to Hi in vitro, although the mechanism of killing or lysis is not well understood and the in vitro conditions necessary for demonstrating the lethal action of TMP/SMZ are critical. It has been suggested that death is due to the abnormal growth which occurs when Hi are deprived of thymidine—conditions which permit continued synthesis of protein. Bushby et al. (29) have drawn attention to the morphologic changes occurring in Hi exposed to TMP/SMZ; the bacteria evolve into long, broad, filamentous forms. It was proposed that these abnormalities in structure were consistent with lethal action of the antibiotic caused by thymineless death. In our studies, isogenic, capsulated (strain Sb) and relatively capsule-deficient (strain S) Hi behaved very differently when exposed to identical concentrations of TMP/SMZ (Fig. 3). 6 h after exposure to TMP/SMZ the majority of strain S had undergone lysis (no bacteria visible on stained smears). In contrast, strain Sb did not undergo lysis although TMP/SMZ was bacteriostatic. Importantly, after 18 h of exposure to the antibiotic, inoculation of a washed suspension of the morphologically altered TMP/SMZ-exposed Sb organisms into rats showed that they were viable and virulent. These observations suggest that the morphologic changes may not necessarily be related to thymine deprivation as suggested by Bushby. A further important question is why Hi that elaborate PRP do not lyse when exposed to high concentrations of TMP/SMZ, whereas PRP-deficient strains or strains elaborating heterologous capsules do. The comparison of the type b and type d transformants of KW-20 would seem particularly striking in this regard since these strains would possess near homology of DNA other than in those regions specifying elaboration of capsular polysaccharide (29); outer membrane proteins of these strains were found to be identical.[2] Taken together, our observations using a variety of transformants suggest that elaboration of PRP is related to both TMP/SMZ tolerance and virulence for rats (18). It seems unlikely that tolerance of Hi to TMP/SMZ can be explained by proposing that elaboration of PRP physically impedes the entry of TMP or SMZ into the bacterium. The MIC for either TMP or SMZ alone was not substantially different for tolerant as compared to kill-sensitive strains. Rather, the mechanisms of inhibition and killing or lysis may be determined by distinct mechanisms. A reasonable possibility is that elaboration of PRP influences the structural integrity of the bacterial cell so as to render it less susceptible to killing.

In summary, routine antibiotic susceptibility testing of clinical bacterial isolates has traditionally relied predominantly upon assaying the MIC of bacteria. However, the recognition of tolerance is of substantial importance to the clinician because selection of successful therapy for particular bacteria (e.g., *Staphylococcus aureus*) or of specific infections (e.g., endocarditis) may be critically dependent upon the MBC. The present studies demonstrate a novel example of tolerance in Hi in which a major determinant of its pathogenicity (the type b capsule) promotes enhanced resistance to antibiotic killing in association with ther-

[2] Munson, R. S. Personal communication.

apeutic failure in vivo. Further studies may shed light not only upon the mechanisms by which tolerant strains of Hi resist the lethal action of TMP/SMZ, but may also provide insights into the biochemical basis of virulence in Hi.

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