In Vitro Effects of Low Concentrations of Penicillin and Sulfadiazine on *Streptococcus mutans*

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Investigations were conducted to determine the in vitro effects of low levels of penicillin and sulfadiazine on the growth, plaque formation, and agglutination of *Streptococcus mutans* and on the synthesis and activity of enzymes synthesizing extracellular polymers. The concentrations tested were equivalent to those expected in the saliva of subjects receiving oral therapy with the agents. Penicillin at 0.5 ng/ml and sulfadiazine at 1 µg/ml substantially inhibited in vitro plaque formation. At these concentrations, sulfadiazine but not penicillin also inhibited growth of the organism. Neither antimicrobial agent affected the agglutination of *S. mutans* with dextran or the synthesis or activity of enzymes synthesizing extracellular polymers. The effect of sulfadiazine on plaque formation was attributed, at least in part, to the inhibitory action of that agent on *S. mutans* growth.

Streptococcus mutans has been strongly implicated as a causative agent in dental caries both because of its ability to cause caries in animals and because of its association with caries in humans (5). A property of *S. mutans* that is believed to contribute to its cariogenicity is its ability to form sticky extracellular polyglucans from sucrose, thereby enabling the organisms to adhere to the teeth (7). Thus, in theory, agents which could interfere with the ability of *S. mutans* to form adherent deposits on teeth might reduce caries experience in humans.

In earlier studies, children receiving 200,000 U of penicillin G orally per day for the prophylaxis of rheumatic fever showed up to 56% fewer new carious lesions than their untreated siblings (8, 9). The present in vitro study sought to determine whether penicillin G, at the concentrations that would be expected in the salivas of such individuals, affects activities of *S. mutans* that might be related to the cariogenicity of that organism. The activities examined were growth, formation of adherent plaques on steel wires, agglutination, and the formation and activity of enzymes capable of producing extracellular polymers from sucrose.

Because sulfadiazine (1 g/day) is also currently used in the prophylaxis of rheumatic fever, parallel in vitro studies were carried out to determine whether that agent might also affect the activities of *S. mutans*. At the pres-

ent time there is no information on whether sulfadiazine therapy has any effect on caries in humans. As with penicillin, the concentrations examined were those expected to occur in the salivas of treated subjects.

MATERIALS AND METHODS

Microorganisms. Type strains of *S. mutans* tested included OMZ-61 (serotype a), BHT (serotype b), JC-2, Ingbritt (serotype c), SL-1, 6715 (serotype d), and LM-7 (serotype e) (2, 13, 14). Fresh isolates were obtained from children who were untreated siblings of outpatients at the Toronto Hospital for Sick Children.

Selection of concentrations of antimicrobial agents. The antimicrobial agents were tested at a range of concentrations chosen to include those expected to occur in saliva. Since previous workers studying the effect of long-term penicillin therapy on caries (8, 9) did not report the salivary penicillin levels in their subjects, it was necessary to estimate the levels from other information in the literature. The oral administration of 200,000 U of penicillin G has been shown to result in a serum level of approximately 0.12 µg/ml (11). Inasmuch as salivary penicillin levels are approximately 1% of serum levels (1), we estimated the maximum salivary concentration from 200,000 U of penicillin G to be between 1 and 2 ng/ml. The concentrations tested ranged from 0.5 to 48 ng/ml.

The sulfadiazine concentrations tested, 1 and 10 µg/ml, were both within the range of reported salivary excretion levels (3, 17).

Effects of penicillin and sulfadiazine on the growth of *S. mutans*. Inocula were prepared by growing *S. mutans* strains for 24 h in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) sup-

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plemented with 0.1% glucose and 0.25% K_2HPO_4 (6). This, as well as all subsequent incubations, was carried out in airtight containers at 37°C in an atmosphere of 95% nitrogen and 5% carbon dioxide.

A 0.20-ml portion of the culture was inoculated into duplicate test tubes containing 5.7 ml of Mueller-Hinton broth supplemented with 1% glucose, 0.25% K_2HPO_4 , and 0.1 ml of the antibiotic solution to be tested. Optical densities of the cultures were read at 550 nm with a spectrophotometer (Turner model 350; G. K. Turner Associates, Palo Alto, Calif.) at regular intervals during incubation.

Effects on plaque formation. Plaque formation in vitro was tested quantitatively using the methods of McCabe et al. (10) and Miller and Kleinman (12). In preparation for each experiment, bacterial strains were grown for 24 h in Mueller-Hinton broth supplemented with 1% glucose and 0.25% K_2HPO_4 . The culture (0.20 ml) was then inoculated into cotton-stoppered test tubes containing 4.9 ml of Mueller-Hinton broth supplemented with 0.25% K_2HPO_4 , 1 ml of a filter-sterilized 30% sucrose solution, and 0.1 ml of the antimicrobial agent. Antimicrobial solutions were prepared daily from sterile frozen stock solutions and freshly autoclaved distilled water.

Each test tube contained a length of stainless steel wire (20 gauge) which projected 32 to 33 mm into the broth. After 24 h of incubation, the wires were aseptically transferred into tubes containing fresh broth and agent. Nine consecutive daily transfers were performed to allow adequate plaque accumulation. The coated portions of the wires were then cut off, oven dried at 80°C for 24 h, and weighed. After weighing, the wires were cleaned and reweighed to determine the net dry weight of the deposit.

Effects on the agglutination of washed cells. The effect of low levels of penicillin and sulfadiazine on the agglutination of washed cells of *S. mutans* by high-molecular-weight dextran was assessed by the method of Gibbons and Fitzgerald (6). Strains were grown overnight in a Trypticase salts medium (6) containing separately autoclaved 0.2% glucose. Organisms were harvested by centrifugation at $10,800 \times g$ and washed twice with 0.9% sodium chloride. They were then resuspended in 0.067 M phosphate buffer (pH 7.0) to make a suspension containing approximately 2×10^8 cells/ml.

To carry out the agglutination testing, 0.3 ml of the cell suspension was combined with 0.1 ml of either distilled water or a dilution of penicillin or sulfadiazine. Then 0.2 ml of a solution containing 100 μg of dextran per ml (molecular weight, 2×10^6 ; from *Leuconostoc mesenteroides*; Sigma Chemical Co., St. Louis, Mo.) was added. The mixtures were incubated in a water bath at 35°C for 2 h, and agglutination was recorded visually on a scale from no agglutination (–) to marked agglutination (4+). Controls containing cells alone and cells plus penicillin or sulfadiazine were also included.

Effects on the activity of cell-bound enzymes synthesizing polymers from sucrose. Cell-bound enzymes capable of synthesizing polymers from sucrose were isolated from *S. mutans* strains JC-2 and 10-1 grown for 18 h in a Trypticase salts medium

containing 0.2% glucose (7) supplemented with 0.01% sucrose to enhance cell-bound dextransucrase activity (16). The cells were harvested by centrifugation at $10,800 \times g$, washed twice with 0.9% sodium chloride, and resuspended at a 50-fold concentration in 0.067 M phosphate buffer (pH 7.0). To release the enzymes from the surfaces of the cells, the cell suspension was treated with a sonic probe (Biosonik IV, Bronwill Scientific Co., Rochester, N.Y.) for 90 s at maximum power and centrifuged, and the supernatant containing the enzyme was collected.

Duplicate reaction mixtures, each containing 0.2 ml of the enzyme preparation, 1.75 ml of phosphate buffer, 0.05 ml of sulfadiazine, and 1.0 ml of a 0.5% sucrose solution, were incubated in a water bath for 2 h at 35°C. Enzyme activity was measured turbidimetrically according to the method of Gibbons and Nygaard (7).

Effects on the synthesis of cell-bound enzymes synthesizing extracellular polymers. Cultures were grown for 18 h in Mueller-Hinton broth supplemented with 0.25% K_2HPO_4 , 0.2% glucose, 0.01% sucrose, and appropriate concentrations of penicillin or sulfadiazine, and growth was assessed spectrophotometrically. Control cultures were also grown without the antibiotics.

Harvesting of the cells, preparation of enzyme suspensions, and determination of enzyme activity were performed as previously described, except that the 0.05-ml additions of the antimicrobial agents were replaced by an equal volume of phosphate buffer.

Statistical analysis. In each experiment, data were obtained from several *S. mutans* strains in the absence of the antimicrobial agent and at several concentrations of the agent. For each strain, the data obtained in the presence and absence of a given concentration of the agent were considered to be paired data. The findings for several strains of *S. mutans* at a given concentration of agent in each experiment provided a paired set of data. The difference between treatment and control data was tested for significance using the paired *t* test (15).

RESULTS

Effects of penicillin and sulfadiazine on growth. Growth of two type strains (JC-2 and Ingbritt) and two fresh isolates (12-1 and 14-1) was studied in the presence of low concentrations of penicillin G. Bacterial growth after 24 h in the presence of the highest penicillin concentration tested (24 ng/ml) did not differ significantly from that in the absence of penicillin ($P > 0.5$).

Growth of 12 strains, including 6 type strains and 6 fresh isolates, was studied in the presence of low levels of sulfadiazine (Table 1). A concentration of 1 $\mu g/ml$, which is well within the reported range of salivary excretion levels, resulted in a mean inhibition of growth of 19.1%, which was significant at $P < 0.005$. The degree of inhibition differed markedly between

strains, with the greatest inhibition demonstrated by strain SL-1 and no inhibition by strain Ingbritt. At a sulfadiazine concentration of 10 $\mu\text{g/ml}$ the mean inhibition was 22.7%, significant at $P < 0.001$.

Effects on plaque formation. Both penicillin and sulfadiazine substantially inhibited in vitro plaque formation (Table 2). At the lowest concentrations tested, 0.5 ng of penicillin per ml and 1 μg of sulfadiazine per ml, which were well within the limits of expected salivary excretions levels, mean inhibitions of 45.0 and 45.3% were observed, significant at $P < 0.005$ and $P < 0.01$, respectively. In the case of sulfadiazine, the inhibition of plaque formation (45.3%) was more than twice the inhibition of growth (19.1%) observed in the experiments de-

scribed in the previous section. Increasing the concentration of penicillin 90-fold (to 18 ng/ml) and that of sulfadiazine 10-fold (to 10 $\mu\text{g/ml}$) produced greater inhibitions of plaque formation than did the lowest concentrations, but the increases in inhibition were not proportional to the increases in concentration of the antimicrobial agents.

Differences were observed in the absolute amount of plaque formed in different experiments. The mean dry weight for plaques in the controls was 19 mg, with a range of 8 to 38 mg in different tests. Despite this variability in the overall amount of plaque formed, the effects of the agents were comparable in each experiment.

Other effects. All *S. mutans* strains tested (type strains 6715 and JC-2 and fresh isolates 10-1 and 12-1) agglutinated strongly (4+) in the presence of dextran but failed to agglutinate in its absence. The dextran-induced agglutination was unaffected by the presence of 0.5 to 48 ng of penicillin G per ml or 1 and 10 μg of sulfadiazine per ml.

Furthermore, the activity of polymer-forming enzymes after their liberation from cells of the two strains of *S. mutans* tested, JC-2 and 10-1, was unaffected by penicillin at 1 or 18 ng/ml or by sulfadiazine at 1 or 10 $\mu\text{g/ml}$. Findings ranged from 6% inhibition to 4% stimulation of activity.

The synthesis of cell-bound, polymer-forming enzymes per wet weight of cells was similarly unaffected by low levels of the agents.

DISCUSSION

The most notable observation in the present study was the marked inhibition of in vitro plaque formation by *S. mutans* in the presence of the very low concentrations of both penicillin

TABLE 1. Effect of sulfadiazine on the growth of *S. mutans*

Strains	Relative growth with sulfadiazine ($\mu\text{g/ml}$) ^a	
	1	10
Type strains		
JC-2	92	80
Ingbritt	104	100
OMZ-61	83	68
BHT	87	75
LM-7	89	80
SL-1	53	47
Fresh isolates		
12-1	90	81
14-1	57	34
7-1	81	78
8-1	93	92
10-1	79	74
13-1	63	58

^a Growth at 24 h, expressed as a percentage of growth in the absence of sulfadiazine.

TABLE 2. Effect of penicillin and sulfadiazine on in vitro plaque formation

Strain	No. of plaques	Relative amounts of plaque formation ^a in the presence of:								
		Penicillin G (ng/ml)						Sulfadiazine (μg/ml)		
		0.5	1	3	6	9	18	36	1	10
Type strain JC-2										
Expt 1	2			55	55	46	36		61	54
Expt 2	4			35	35	43	18		52	47
Expt 3	4	43	52	30		29	24	14		
Expt 4	4	58	58	52		40	34	17		
Fresh isolate 10-1										
Expt 1	2			59	67	52	31		71	58
Expt 2	4			42	48	35	24		35	33
Expt 3	4	69	68	70		82	53	41		
Expt 4	4	50	39	39		26	14	9		

^a Dry weight of plaque expressed as a percentage of that formed in the absence of antimicrobial agents.

and sulfadiazine that might be expected in the saliva of patients on long-term antimicrobial therapy. In the case of penicillin, the diminution of plaque formation was not associated with evidence of a decreased rate of *S. mutans* growth, interference with dextran-mediated agglutination, interference with the synthesis, or activity of cell-bound, polymer-synthesizing enzymes. Therefore, for the present, the effect of penicillin on in vitro plaque formation by *S. mutans* remains unexplained.

The inhibitory effect of sulfadiazine on *S. mutans* growth appeared to be responsible, at least in part, for its inhibitory effect on in vitro plaque formation. However, the finding that plaque formation was inhibited to a greater degree than was growth suggests that other, presently unidentified, factors may have also been involved.

The inhibitory effect of 1 μ g of sulfadiazine per ml on *S. mutans* growth was also unexpected, since in susceptibility tests reported elsewhere (18) we found that *S. mutans* was resistant to a concentration of sulfadiazine 3,000 times higher. Apparently sulfadiazine, even at higher concentrations, causes only a partial inhibition of growth which is undetectable by susceptibility testing procedures that are only able to detect complete abolition of growth. These observations point to the possibility that an antimicrobial agent might reduce dental caries, even though susceptibility tests indicate that *S. mutans* is resistant to the agent.

The findings have additional implications for clinical studies. The observed inhibition of *S. mutans* plaque formation by penicillin could explain the inhibitory effect on caries of long-term daily administration of penicillin observed in clinical studies (8, 9). Additionally, the observed effect of sulfadiazine on plaque formation suggests that the possible effect of that agent on dental caries should be clinically evaluated.

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