Method for Preclinical Evaluation of Antiplaque Agents

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A preclinical method for the evaluation of antibacterial agents for use against dental plaques associated with caries and periodontal disease is proposed. The method is applicable to screening agents and to defining, in vitro, the minimal conditions required for maximal antiplaque effect. As a model of antiplaque agents, chlorhexidine was assessed in vitro against preformed plaques of microorganisms conducive to dental caries and periodontal disease. The agent was bactericidal to plaques of nine strains of Streptococcus mutans and one strain of Actinomyces viscosus when used in a single treatment for 20 min at $2 \times 10^{-1} \text{%}$, in two 2-min treatments on the same day, or in daily 2-min treatments at this same concentration. Using the last of these experimental conditions, we then tested chlorhexidine in vivo by topical application to the maxillary teeth of infected hamsters and found it to be effective in controlling plaques of S. mutans and A. viscosus.

Microorganisms forming adhesive colonial masses on teeth (plaque) have been strongly implicated as infectious agents in dental caries (4, 5, 8, 9, 19, 21, 22, 28) and periodontal disease (15, 18) of experimental animals and man. Notable among these microorganisms are Streptococcus mutans (2, 10) and Actinomyces viscosus (6, 12). Great potential exists for the utilization of antibacterial agents to control such plaque infections in man. Progress in the development of antiplaque agents, however, has been slow, owing in part to the empirical means commonly employed to establish clinical dosage conditions.

Because the infectious microorganisms causing caries and periodontal disease reside in dense masses on the surfaces of the teeth, topical rather than systemically administered agents seem indicated, unless the latter are secreted in high concentration in the saliva or are peculiarly permeant through the oral mucosa. Hence, there are two special considerations in the evaluation of antibacterial agents in the control of plaque-dependent oral disease: (i) antiplaque effects must be rapid since oral topical administration routinely permits only brief duration of treatment, and (ii) stringent evaluation of an agent's efficacy requires its use with intact plaques since the dense adhesive aggregates constitute a significant barrier to the diffusion of substances to its depth (31; J. M. Tanzer and J. Savel, unpublished data). The commonly employed in vitro technique of "diluting out" an agent and determining the lowest concentration that inhibits the growth of a freshly inoculated culture seems inappropriate. Similarly, growth of plaques in the continuous presence of an antibacterial agent (29) assumes a condition not readily achievable in the mouth. Thus, a relevant model for testing antiplaque agents should utilize pregrown intact bacterial plaques and transient exposure to a topically acceptable agent.

The biguanide chlorhexidine, which has recently been shown effective as an anticaries agent in rats (25) and in controlling plaque associated with gingivitis in man (1, 7, 23, 26), presents a convenient model drug for establishing a procedure for screening potential antiplaque agents and identifying the minimal conditions of use required for maximal antiplaque effect. Accordingly, this paper describes a preclinical evaluation technique in which this agent and intact plaque were used in vitro. The minimal experimental conditions determined to be maximally efficacious in vitro were then used to test the agent for the control of plaque infections associated with both dental caries and periodontal disease in vivo.

MATERIALS AND METHODS

In vitro studies. The following strains of S. mutans, isolated from either humans or rodents, were studied: 6715-11 (Tanzer et al., Int. Ass. Dent. Res. Abstr., 1972, p. 376-380)
In vitro plaques were grown on stainless-steel wires, as previously detailed (30), by use of complex medium (14) supplemented with 5% (w/v) sucrose and 5 mg of Na₂CO₃ per 100 ml. After plaques on wires had grown to a rating of 4, as judged by the method of McCabe et al. (24), the wires and adherent plaques were transferred for 2 hr to fresh broth, rinsed in water, and then immersed in various concentrations of the aqueous di-glucanate salt of 1,6-di-(4-chlorophenyl-diguanido)-hexane (chlorhexidine gluconate, Hibitane; Imperial Chemical Industries, Macclesfield, Cheshire, England) at pH 7 for various times and frequencies followed by two 10-min rinses in distilled water. Rinsing effected at least a 10²-fold dilution of any carried-over agent. Control plaques were immersed either in water or in 25 mm sodium glucanate, the highest concentration of glucanate possibly resulting from the use of chlorhexidine gluconate in these studies. The effects of the two controls were not different from each other. Plaques were again transferred to fresh nutrient broth containing 1 mg of bromocresol purple pH indicator per 100 ml. This final transfer effected at least another 10²-fold dilution of carried-over chlorhexidine. On prolonged incubation, color change from purple (pH > 6.8) to yellow (pH < 5.2) indicated acid production by the plaques and, consequently, inability of the agent to kill the cells. By contrast, lack of color change indicated an antibacterial effect. To determine whether the antibacterial effect was bacteriostatic or bactericidal, 24 hr after treatment some streptococcal plaques were mechanically disrupted and samples were plated on Mitis salivarius agar (Difco); some diphtheroidal plaques were similarly disrupted and plated on meat extract-blood-starch-agar (11). Absence of growth indicated bactericidal effect. To assure that absence of growth on agar did not merely reflect carry-over of residual chlorhexidine, samples of fluid from broth tubes which had remained purple were spread on agar plates. The agar plates were then inoculated with untreated cultures. Sucinoc inula always grew luxuriantly.

In vivo studies. Thirty-five weanling male golden hamsters, indigenously infected by S. mutans and A. viscosus, were superinfected by oral swabbing with a 24-hr thiglycollate culture of virulent streptocycin-resistant S. mutans 6715-11. All animals consumed caries test diet 2000 (18) and distilled water ad libitum. After the plaque and superinfection were established, as shown by the presence of plaque and the ability to recover 6715-11 by oral swabbing 8 days after infection, 2 × 10⁻¹% chlorhexidine was applied with a soft camel's hair brush to the maxillary molar teeth of 18 animals for 2 min/day, 5 days/week for 3 weeks; water was applied similarly to the teeth of 17 control animals. A mouth prop (13) was employed to allow application of the agent; however, direct application to the mandibular teeth of the hamsters was not possible. Although the experimenter was not told whether he was applying the agent or water, it was not possible to maintain him in the "blind" state because the animals squirmed in response to the apparent taste of the agent. Nonetheless, a strictly timed 2-min application was used, followed by extensive rinsing of the mouth with water.

At the end of the third week of treatment, recovery of wild-type S. mutans, A. viscosus, and S. mutans 6715-11 was tested by oral swabbing and plating on appropriate agar after suitable dilution. Then the animals were sacrificed; the jaws were fixed in Formalin and examined for the presence and distribution of plaque. The skulls were coded by number so that the examiner was unaware of whether the maxillary teeth had been swabbed with chlorhexidine or water.

**RESULTS**

In vitro studies. To determine the minimal concentration of chlorhexidine maximally inhibiting plaques of S. mutans, plaques of strain 6715-11 were immersed for 20 min in concentrations ranging from 2 × 10⁻⁴ to 2 × 10⁻⁵% (w/v), with concentration intervals varying by log units. As shown in Table 1, no inhibition of acid production was observed at 2 × 10⁻⁴%. Although concentrations of 2 × 10⁻³ and 2 × 10⁻² retarded acid production by the plaques, eventually (72 hr after treatment) all plaques treated at these concentrations were able to produce sufficient acid to change the color of the indicator. However, plaques treated with concentrations of 2 × 10⁻¹ and 2 × 10⁻⁰% did not change the color of the indicator even after 120 hr of incubation. Samples of these plaques taken 24 hr after treatment did not grow on agar, and thus, at these concentrations, the agent was judged to be bactericidal; samples of plaques treated at lower concentrations grew on agar.

**TABLE 1. Effect of various chlorhexidine concentrations upon the ability of Streptococcus mutans 6715-11 to depress the pH of culture fluid after a single 20-min treatment**

| Concen (%) | No. of plaques (pH < 5.2/5.2-6.8/ > 6.8) |
|-----------|--------------------------------------|
|           | 12 hr | 24 hr | 48-120 hr |
| 0.0       | 1/3/0 | 4/0/0 | 4/0/0     |
| 2 × 10⁻⁴  | 0/7/0 | 7/0/0 | 7/0/0     |
| 2 × 10⁻³  | 2/4/1 | 7/0/0 | 7/0/0     |
| 2 × 10⁻²  | 0/0/7 | 0/7/0 | 7/0/0     |
| 2 × 10⁻¹  | 0/0/7 | 0/0/7 | 0/0/7     |
| 2 × 10⁰   | 0/0/7 | 0/0/7 | 0/0/7     |

* The number of plaques producing a broth pH of < 5.2, 5.2 to 6.8, or > 6.8 at intervals after treatment is indicated.
By contrast, on adding serial aqueous dilutions of chlorhexidine to freshly inoculated cultures of strain 6715-11, partial inhibition of growth was observed at $2 \times 10^{-1}\%$ and the total bactericidal effect was observed at $2 \times 10^{-4}\%$. These concentrations of the strongly cationic agent were undoubtedly overestimated, since its addition to culture medium leads to partial precipitation as a phosphate salt. Comparison of this result with that obtained with intact plaque (Table 1) thus indicated the inappropriateness of the serial dilution technique in estimating the effective dose of such agents against plaque-forming bacteria.

To determine the minimal duration of a single treatment producing maximal inhibition, plaques of 6715-11 were immersed in $2 \times 10^{-1}\%$ chlorhexidine for 1, 2, 5, 10, 20, or 30 min. Single exposures for 1 to 10 min retarded indicator color change transiently (Table 2). Permanent color change inhibition (for 120 hr) was not always observed until 20 or 30 min of exposure to the agent was employed. Plating on agar confirmed the bactericidal effect.

To determine whether more frequent treatment with the agent at 2-min duration and $2 \times 10^{-1}\%$ concentration had a similar effect, two types of experiments were carried out. In the first, plaques were treated for 2 min in $2 \times 10^{-1}\%$ chlorhexidine, rinsed, and incubated for 6 hr in growth medium; they were then retreated for 2 min in $2 \times 10^{-1}\%$ agent, rinsed, and reincubated in broth with indicator. Control and once-treated plaques were studied for comparison. Table 3 shows that the two treatments on the same day were effective in inhibiting the plaques, whereas a single treatment was only transiently inhibitory. Samples of the twice-treated plaques did not grow on agar, though once-treated samples and watertreated controls did.

**Table 2. Effect of various durations of a single 2 × 10⁻¹% chlorhexidine treatment upon the ability of Streptococcus mutans 6715-11 to depress the pH of culture fluid***

| Duration of treatment (min) | No. of plaques (pH < 5.2/5.2-6.8/ > 6.8) |
|----------------------------|---------------------------------------------|
|                            | 24 hr | 48 hr | 72-95 hr | 120 hr |
| 0                          | 4/0/0 | 4/0/0 | 4/0/0    | 4/0/0  |
| 1                          | 4/0/0 | 6/0/0 | 6/0/0    | 6/0/0  |
| 2                          | 0/5/1 | 6/0/0 | 6/0/0    | 6/0/0  |
| 5                          | 0/3/3 | 0/4/2 | 0/5/1    | 0/6/0  |
| 10                         | 0/1/5 | 1/1/4 | 1/1/4    | 1/1/4  |
| 20                         | 0/0/6 | 0/0/6 | 0/0/6    | 0/0/6  |
| 30                         | 0/0/6 | 0/0/6 | 0/0/6    | 0/0/6  |

* The number of plaques producing a broth pH of <5.2, 5.2 to 6.8, or >6.8 at intervals after treatment is indicated.

**Table 3. Effect of various frequencies of a 2-min treatment with 2 × 10⁻¹% chlorhexidine upon the ability of Streptococcus mutans 6715-11 to depress the pH of culture fluid***

| Treatment                | No. of plaques (pH > 5.2/5.2-6.8/ > 6.8) |
|--------------------------|---------------------------------------------|
|                          | 24 hr | 48-120 hr |
| Water, once              | 7/0/0 | 7/0/0     |
| Chlorhexidine, once      | 0/7/0 | 7/0/0     |
| Chlorhexidine, twice     | 0/0/7 | 0/0/7     |
|                          | 378   | 378       |

* The number of plaques producing a broth pH of <5.2, 5.2 to 6.8, or >6.8 at intervals after treatment is indicated.

**Table 4. Effect of daily 2-min treatment with 2 × 10⁻¹% chlorhexidine upon the ability of S. mutans 6715-11 to depress the pH of culture fluid***

| Treatment                | No. of plaques (pH < 5.2/5.2-6.8/ > 6.8) |
|--------------------------|---------------------------------------------|
|                          | 24 hr | 48 hr | 72-120 hr  |
| Water, once              | 12/0/0| 12/0/0| 12/0/0     |
| Chlorhexidine, once      | 12/0/0| 12/0/0| 12/0/0     |
| Chlorhexidine, once daily| 24/0/0| 0/24/0| 0/0/24     |

* The number of plaques producing a broth pH of <5.2, 5.2 to 6.8, or >6.8 at intervals after treatment is indicated.

In the second type of experiment, plaques were treated either once or once daily in $2 \times 10^{-1}\%$ chlorhexidine for 2 min. Table 4 shows that after 72 hr (prior to the fourth treatment) inhibition of plaque appeared complete. Plating on agar revealed no growth after the third treatment.

All eight other strains of S. mutans were bactericidally affected by chlorhexidine under the same conditions of concentration, duration, and frequency of treatment as described for 6715-11. A single 20-min exposure to $10^{-1}\%$, while inhibitory, was not totally bactericidal. In vitro plaques of A. viscosus T-6-1600 were similarly affected, with the exception that $10^{-1}\%$ chlorhexidine was bactericidal. In all cases, chlorhexidine tended to cause partial disruption of the plaques.

**In vivo studies.** For in vivo evaluation of chlorhexidine, we used the hamster dental caries- periodontal disease model (17), employing the concentration, duration, and frequency determined to be effective in vivo. After 3 weeks of
treatment, wild-type *S. mutans*, *A. viscosus*, and *S. mutans* 6715-11 were all recovered from oral swabbings of both chlorhexidine-treated and placebo-treated animals. Subgingival plaque and gingival distortion were often present in both groups of hamsters. However, animals treated with chlorhexidine showed great reduction of plaque on the crowns of teeth compared with placebo-treated controls (Fig. 1). This suggested that chlorhexidine, in the treatment regimen employed, was potent in controlling the plaque infection so long as it had been delivered to the colonized tooth surfaces. However, when the agent had not been effectively delivered, as for example to the gingival crevice or to the mandibular dentition, the plaque at these sites was not always controlled. This probably contributes to the continuing positive recovery of microorganisms of interest from oral swabbings.

**DISCUSSION**

In the preclinical method for evaluating antiplaque agents presented here, intact pregrown plaques of *S. mutans* and *A. viscosus* were briefly exposed to chlorhexidine which was selected as a model antiplaque agent. The data indicate the potent antiplaque effect of chlorhexidine. In vitro studies established concentration, duration, and frequency of treatment required for maximal antibacterial effect, i.e., killing the intact plaque.

Recent data of Rolla et al. (27) suggest that chlorhexidine may reversibly adsorb to microorganisms and thus may not have been totally removed by the rinsing procedure employed in this study. Such reversible adsorption of chlorhexidine to bacteria does not appear to account for the bactericidal effect shown in this paper, because the growth medium which had been used for incubation of chlorhexidine-treated (and rinsed) plaques did not inhibit the growth of fresh bacteria.

When conditions determined to be effective in vitro against intact plaques were used, potent coronal plaque inhibition was demonstrated in hamsters infected by *S. mutans* and *A. viscosus*. As expected, however, eradication of these microorganisms from the mouth was not achieved, probably due to inability to deliver the agent to all plaque-infected sites.

The data also illustrate the importance of considering the diffusion barrier constituted by intact plaque and the necessarily transient exposure of plaques to topical antibacterial agents. The technique of serially diluting chlorhexidine to observe the minimal concentration inhibiting growth of freshly inoculated dispersed cultures erroneously suggested much higher potency of the agent than was observed against intact plaques for limited times.

This work demonstrates the ability to establish reasonably the minimal concentration, duration,
and frequency of use of an antiplaque agent required for maximal potency in vitro and thereby to indicate the minimal conditions of use required to maximize efficacy in vivo. We hope such an approach will avoid much empiricism in establishing the conditions of use of antiplaque agents in clinical trials.

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