Correlation of Microenvironmental Drug Concentration With Inhibition of Growth of Microorganisms on Surfaces

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Methodologies have been developed to study the concept of microenvironmental drug concentration (C^*) near or around microorganisms. C^* may be calculated from data on drug release from a depot site by using appropriate diffusion relationships. By following C^* and correlating this with the minimum inhibitory concentration (C_{MIC}), one could attempt to predict the effectiveness of an antiplaque agent. When C^* is less than C_{MIC} , growth would be expected to occur; when C^* is higher than or equal to C_{MIC} , growth would not be expected. Chlorhexidine diacetate was chosen for this study, which used a system involving microorganisms present on the surface of drug-treated hydroxyapatite pellets. C_{MIC} , defined as the lowest concentration to inhibit bacterial growth, was determined independently and under conditions similar to those used in the C^* experiments. Surface growth of adhering microorganisms (*Streptococcus mutans* SL1) was followed by scanning electron microscopy. The parameters used in the calculation of C^* were determined independently. Diffusion coefficients of the drugs and the diffusion layer thickness were determined under conditions similar to those employed in the release rate studies. Surface growth was generally found to be inhibited whenever C^* was significantly greater than C_{MIC} , and growth occurred whenever C^* was significantly smaller than C_{MIC} . These findings demonstrate how C^* may determine the action of a topically administered antimicrobial agent and how the various physical and chemical factors play roles in influencing this quantity.

Numerous laboratory and clinical investigators have shown that antimicrobial agents are effective in preventing or controlling dental plaque development (2, 13, 18). In antiplaque therapeutics, researchers are aware that (i) the concentration of antimicrobial agent must be higher than a minimum inhibitory concentration (C_{MIC}) to effect a bacteriostatic action and (ii) the maintenance of this concentration is needed to exert a prolonged therapeutic action. This concentration can be maintained in a controlled manner by using various factors in the antiplaque formulations such as pH, temperature, drug concentration, inorganic ions, and polymers (1, 3, 5).

The main objective of this investigation was to develop an in vitro test to screen antiplaque agents and the various formulation factors which may affect their potential activity. The in vitro

test developed here utilizes calculated concentrations of the antimicrobial agent around the adhered microorganisms on the enamel surface (microenvironment). A comparison of microenvironmental drug concentrations (C^* s) for various antiplaque agents with different formulations can serve as a basis to assess their activities and optimal formulations in the screening programs. Application of this calculated C^* for one antiplaque agent, chlorhexidine, was used to demonstrate its validity. Chlorhexidine has been shown previously (2, 4, 16) to exhibit antiplaque activity. Its activity on *Streptococcus mutans* adhered to a compressed hydroxyapatite pellet was used as a model system.

MATERIAL AND METHODS

Antibacterial agent. ^{14}C ring-labeled chlorhexidine diacetate (specific activity, 11.06 mCi/mol) was obtained from the Radiochemical Laboratory of the Imperial Chemical Industries, Ltd., Pharmaceutical Division, Macclesfield, Cheshire, England. In all experiments, the radioactive compound was diluted with cold chlorhexidine diacetate to give optimum scintillation counting.

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Strains. *S. mutans* SL1 (strain D), obtained from W. Loesche, Institute of Dental Research, University of Michigan, Ann Arbor, was cultured in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with excess CaCO_3 (J. T. Baker Chemical Co., Phillipsburg, N.J.) and grown anaerobically at 37°C for 15 to 20 h and then stored at 4°C . Passages were made every 2 weeks into freshly prepared media.

Hydroxyapatite pellets. Hydroxyapatite pellets (0.5-in [1.27-cm] diameter, 1 mm thick) were prepared by compressing 200 mg of hydroxyapatite powder (Stauffer Chemical Co., Victor Division, New York, N.Y.) in a hydraulic press (Fred S. Carver, Inc., Menomonee Falls, Wis.) with a compression force of 10,000 psi. Chlorhexidine diacetate-treated pellets were prepared by soaking in 0.05% chlorhexidine in double-distilled water for 20 min. Untreated pellets were soaked in double-distilled water for 2 to 3 s, dried in an oven at 50°C and sealed on all sides except one with epoxy glue.

Growth of microorganisms on hydroxyapatite pellets. Inocula were prepared as follows. A 0.1-ml volume of stored culture was transferred to 10 ml of freshly prepared Trypticase soy broth with excess CaCO_3 and grown anaerobically at 37°C for 25 h and then subcultured to 250 ml of Trypticase soy broth under 95% N_2 -5% CO_2 and grown to 0.7 optical density at 254 nm. To increase the microbial population deposited onto the hydroxyapatite pellet, the microorganisms were washed twice with phosphate buffer solution (0.1 M KCl, 0.01 M KH_2PO_4 , 0.015 M K_2HPO_4 , 0.003 M D-glucose, pH 6.8). The cells were finally suspended in the buffer to $\frac{1}{10}$ the original volume by vortexing (Scientific Industries, Bohemia, N.Y.). Each treated or untreated hydroxyapatite pellet was exposed to 1 ml of the bacterial suspension at 37°C for 30 min. Approximately 4×10^3 *S. mutans* cells adhered to each pellet. The pellet with adhered bacteria was finally rinsed with the buffer solution for 2 s, and the microorganisms were allowed to grow in 50 ml of Trypticase soy broth with 3% sucrose under 95% N_2 -5% CO_2 . Each pellet was repeatedly transferred to fresh medium to minimize drug concentration buildup in the medium. At every 3rd hour, a pellet was sampled and prepared for scanning electron microscopy. For scanning electron microscopy, the samples were fixed with 10% neutralized formaldehyde (14). The pellet surfaces were coated with a 20-nm-thick layer of gold and scanned (model JSM-43; Japan Electron Optics, Tokyo, Japan) for microbial growth.

Determination of C_{MIC} for microbial growth on pellet surfaces. For determination of minimum inhibitory concentrations (C_{MIC}), nontreated pellets were placed in bacterial suspensions prepared as described above at 37°C for 30 min. The pellets with adhered bacteria (ca. 4×10^3) were rinsed with the phosphate buffer solution for 2 to 3 s. Each pellet was placed in 50 ml of Trypticase soy broth with 3% sucrose with various concentrations of chlorhexidine preequilibrated at 37°C and grown for 9 h at 37°C .

Theory. A physical model was utilized to estimate the C^* of a drug at the surface of a treated hydroxyapatite pellet. The drug may be absorbed or partitioned near the surface region. When a treated hydroxyapatite pellet or tooth enamel is immersed in an agitated liquid, several steps are expected to occur: (i) contact of the drug with the aqueous solution, (ii) solvation of

the drug, (iii) partition of the drug between the hydroxyapatite and the media, and finally (iv) the transport of the drug molecules away from the hydroxyapatite surface (9, 11). It can be assumed that steps i, ii, and iii are fast and step iv is the rate-limiting step. There is a stagnant liquid layer of thickness h next to the enamel (or pellet) surface. C^* is defined as the concentration in this stagnant layer adjacent to the surface where bacteria are also adsorbed and anchored. The following Nernst equation (15) can therefore be utilized to describe release of a drug adsorbed onto the tooth:

$$J = SD(C^* - C_B)/h \quad (1)$$

where J is the flux (amount of drug [grams] desorbing per unit time [seconds]), S is the apparent surface area (centimeters squared) exposed to the medium, C^* is in grams per cubic centimeters, C_B is the bulk concentration (grams per cubic centimeters) of the drug, h is the Nernst diffusion layer thickness (centimeters), and D is the effective diffusion coefficient of the drug (centimeters squared per second). Assuming $C_B \ll C^*$, equation 1 may be simplified to

$$J = SDC^*/h \quad (2)$$

During desorption, the drug molecules diffuse to the surrounding medium; thus, the C^* decreases with time. If the drug diffuses into a dilute protein solution, a drug-protein interaction may occur. Protein-bound drug and free drug molecules may diffuse through the medium at different rates. Assuming that an equilibrium exists between the concentrations of bound and unbound drug along the diffusion path and that the diffusion coefficients are independent of concentration, equation 2 can be extended to accommodate this protein-binding factor:

$$J_{\text{Total}} = J_{\text{FD}} + J_{\text{BD}} = \frac{SD_{\text{FD}}C^*_{\text{FD}}}{SD_{\text{BD}}C^*_{\text{BD}}/h} \quad (3)$$

where J_{FD} and J_{BD} are fluxes of the free drug and the protein-bound drug, respectively.

The free and the bound drug species are related by the equilibrium constant K , defined as:

$$K = C^*_{\text{BD}}/C^*_{\text{FD}} \quad (4)$$

On the other hand, the total drug concentration, C^*_{T} , is

$$C^*_{\text{T}} = C^*_{\text{FD}} + C^*_{\text{BD}} = C^*_{\text{FD}}(1 + K) \quad (5)$$

The total flux becomes

$$J_{\text{Total}} = (SD_e/h) C^*_{\text{T}} \quad (6)$$

where D_e is the effective diffusion coefficient:

$$D_e = D_{\text{FD}} + D_{\text{BD}} K/(1 + K) \quad (7)$$

If the diffusion coefficients are equal, then

$$D_e = D_{\text{FD}} = D_{\text{BD}} \quad (8)$$

When K is very large, $D_e \approx D_{BD}$ and $C^*_T \approx C^*_{BD} \approx C^*$. When K is 0, i.e., there is no binding, $D_e = D_{FD}$ and $C^*_T = C^*_{FD} = C^*$.

In both cases, the general form of the working equation is

$$J_{\text{Total}} = SD_e C^*/h \quad (9)$$

All terms on the right side of the equation can be determined independently. The effective diffusion lay-

er thickness, h , is obtained by the benzoic acid experiment (11, 12). The effective diffusivity, D_e , is obtained in the same media used in the desorption profile experiments, employing a small-volume diaphragm cell (8, 10).

As the adsorbed drug molecules diffuse into the bulk solution, increase of the bulk concentration is expected with time. To maintain the bulk concentration below C_{MIC} , it is necessary to use either a large volume of the medium or to change the desorption medium frequently.

FIG. 1. Effects of various concentrations of chlorhexidine diacetate on *S. mutans* SL1 cells adhering to a hydroxyapatite pellet. $\times 1,000$ magnification.

FIG. 2. Desorption and microenvironmental profiles of chlorhexidine diacetate.

FIG. 3. Growth of *S. mutans* SL1 on a chlorhexidine-treated hydroxyapatite pellet. Scanning electron micrographs, $\times 1,000$ magnification.

RESULTS AND DISCUSSION

C_{MIC} . Several experiments were conducted to determine the C_{MIC} of chlorhexidine diacetate for surface-bound *S. mutans* SL1. In all of these experiments, concentrations above 0.2 $\mu\text{g/ml}$ were found to inhibit surface growth. A typical example of the experimental results is shown in Fig. 1. Up to 9 h there was very little growth when the chlorhexidine diacetate concentration was maintained at 0.4 $\mu\text{g/ml}$ or greater, and substantial growth occurred when the drug concentration was 0.2 $\mu\text{g/ml}$ or lower. For this study, 0.2 to 0.4 $\mu\text{g/ml}$ could be taken as the C_{MIC} of chlorhexidine when ca. 4×10^3 microorganisms were allowed to grow for 9 h on the hydroxyapatite pellet.

Using an agar dilution technique (6), the C_{MIC} was found to be 0.19 $\mu\text{g/ml}$ (for 10^3 cells of *S. mutans*); therefore, 0.2 to 0.4 $\mu\text{g/ml}$ was reasonable as the C_{MIC} in subsequent studies.

C^* . Utilizing equation 9, the C^* was calculated at different times. The effective diffusion coefficient, D_e , was calculated to be $4.58 \times 10^{-6} \text{ cm}^2/\text{s}$ (8, 10). The effective diffusion layer could be calculated by benzoic acid experiments (11, 12). The viscosity was found to have minimal effect on the calculated h value (8, 12). S was obtained simply from measuring the pellet surface and was found to be 1.27 cm^2 per pellet. The desorption rates, J_T , were determined from the slopes of a smooth curve drawn along the desorption profile (Fig. 2).

Figure 2 shows the calculated C^* for the hydroxyapatite treated with 0.05% chlorhexidine diacetate and how C^* decreases with time. Since C_{MIC} was shown to be 0.2 to 0.4 $\mu\text{g/ml}$, microbial growth on the treated pellet was therefore expected to commence after 6 h.

The concentration of chlorhexidine diacetate in the culture media during the microbial growth studies was also determined. The bulk drug concentration was always below the C_{MIC} . Inhibition could only occur in the microenvironmental region where the drug concentration was substantially higher.

S. mutans SL1 cells were examined and found to be generally distributed as a uniform monolayer throughout the hydroxyapatite pellet surfaces. Bacterial growth could be determined by electron microscope examination. The results illustrated in Fig. 3 show that growth was indeed inhibited up to 6 h on the treated pellet, whereas bacterial growth was observed as early as 3 h for the untreated pellet. This agreed well with the prediction from the C^* -time data (Fig. 2). This supports the utility of the concept of C^* as a means to determine the effect of chlorhexidine on the surface growth of bacteria.

Antimicrobial agents have been used to prevent plaque formation. Chlorhexidine had been

shown to exhibit antiplaque activity primarily because of its adsorption to various parts inside the oral cavity (3, 4, 17). There is a need to determine the exact drug concentration near the tooth surface that would inhibit bacterial growth. The in vitro test developed here and the concept of C^* of an antiplaque agent near the tooth surface can be used to assess the potency of individual therapeutic agents.

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