APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1976, p. 428-432
Vol. 32, No. 3

Printed in U.S.A.

Use of Hydroxyapatite-Coated Glass Beads for Preclinical Testing of Potential Antiplaque Agents

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Received for publication 9 February 1976

Methods for rapid preclinical testing of antiplaque agents in vitro using hydroxyapatite (HT)-coated glass beads are described. The assays developed could reliably detect (i) prevention of growth in the culture fluid or on the HT surfaces, (ii) the effect of transient exposure of a bactericidal agent on the viability of cells in a preformed bacterial mat, (iii) reversible adsorption of a bactericidal agent on an HT surface, and (iv) the ability of an agent to inhibit adsorption of *Streptococcus sanguis* to an HT surface or to salivary proteins adsorbed to an HT surface.

Antiplaque agents are chemicals that prevent colonization of tooth surfaces by bacteria. Successful colonization requires that a microbe adhere to a surface in the oral cavity (8) and be able to multiply in that environment. Though most antiplaque agents investigated so far are bactericidal, affecting the latter process (4, 9, 13, 18), bacteriostatic agents or chemicals interfering with attachment should be equally effective. Before undertaking time-consuming and expensive testing of potential antiplaque agents in vivo, it is desirable to evaluate the effect of these chemicals in vitro. In this paper, we describe procedures for investigating several properties of potential antiplaque agents. We employed hydroxyapatite (HT)-coated glass beads (16) as the substrate offered for colonization, making it possible to study interactions among the chemicals, selected bacteria, and an HT surface. Chemicals with a known effect on oral microbes, fluoride and chlorohexidine, were used to develop the methods.

MATERIALS AND METHODS

Reagents and media. All chemicals were of reagent grade and were used without further purification. [^{14}C]glucose was obtained from Schwarz/Mann. The antiplaque agents no. 22549 and 22561 were supplied by General Mills, Inc. HT beads were prepared by the method of Sudo et al. (16) using Bio-Glas 1500 (50 to 100 mesh) from Bio-Rad Laboratories. Over 90% of the glass surface was covered with HT (16). The medium for routine culture of microbes consisted of Trypticase soy broth (TSB), from Baltimore Biological Laboratories, supplemented with 0.05% yeast extract (Difco). Sucrose was autoclaved separately and then added to achieve the desired concentration in the medium.

Cultural methods. *Streptococcus mutans* strain 6715 was maintained by daily transfer in TSB-yeast extract medium containing 0.1% glucose (TYG). At 1- to 2-week intervals, the culture was streaked on MS agar (Difco), and a clone was selected for subculture. *S. sanguis* and *Veillonella* species were isolated from dental plaque on MS agar and Rogosa selective medium (14), respectively. Cells were stored on glass beads at −70°C (11). Prior to an experiment, a single bead was transferred into 5 ml of TYG and incubated overnight. All incubations were at 37°C under an atmosphere of 90% N_2-10% CO_2.

Instruments. Several small instruments were manufactured to facilitate the experimental procedures. Stainless-steel scoops of several sizes (Fig. 1a) were constructed so that constant volumes of either dry or colonized HT beads could be measured. Our instruments measured about 2 mg, 11 mg, and 26 mg of dry HT beads. The standard deviation in each case was less than 10% of the mean value. Another instrument, resembling a 3-mm-diameter cork borer (Fig. 1b), was also made of stainless steel. Glassine paper, commonly used for weighing powders, was cut into circles of 90 mm in diameter. Four holes, each 3 mm in diameter, were punched in the paper using the instrument illustrated in Fig. 1b.

Assay for prevention of growth and colonization. Approximately 50 mg of HT beads was autoclaved in 50-ml Erlenmeyer flasks containing 7.5 ml of TSB supplemented with yeast extract. Control flasks received 0.75 ml of autoclaved 50% sucrose. The other flasks received, in addition to sucrose, various amounts of the agent to be tested or of "placebo," the solvent in which the agent was dissolved. An overnight culture of *S. mutans* was diluted 1/60 into each flask, which was then gassed for 60 to 90 s with N_2-CO_2, closed with a sterile rubber stopper, and incubated stationary at 37°C. Care was taken to distribute the beads evenly on the bottom of the flask. After 6.5 to 7 h, the culture fluids were decanted into test tubes, and the HT beads were washed free of nonadhering cells and clumps of bacteria. The number of cells on weighed samples of the HT beads (5 to 20 mg) and in the culture fluid was measured using

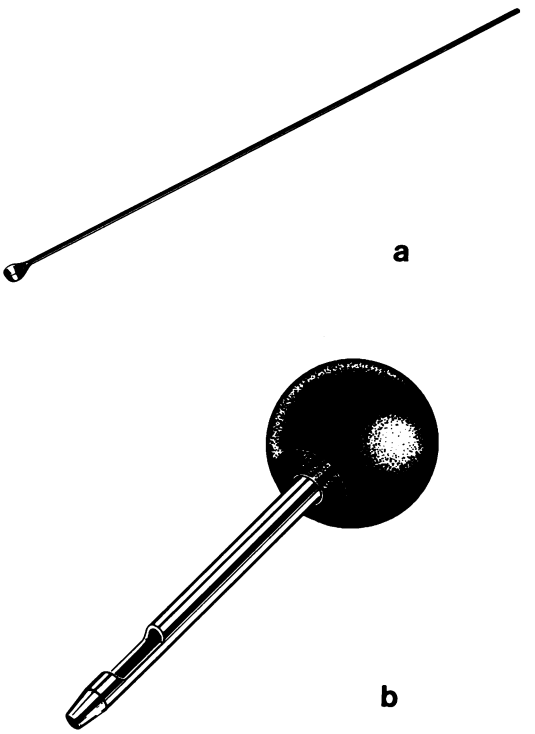

FIG. 1. *Tools manufactured to facilitate testing of antiplaque agents. (a) Stainless-steel scoop for measuring HT beads; (b) tool for making templates from glassine paper.*

the filter assay described by Sudo et al. (16). The average of duplicate samples was used in subsequent calculations. Usually the variation between duplicate samples was small.

Assay for the effect of a lethal agent on the viability of cells in a preformed bacterial mat. HT beads (0.2 g) were colonized with *S. mutans* as described above. When the cells reached stationary phase, the beads were washed free of nonadherent cells, and then weighed samples were placed in small test tubes. Control tubes received 1.0 ml of 0.01 M potassium phosphate buffer, pH 7.2, whereas experimental samples received 1.0 ml of 0.2% chlorohexidine gluconate. After 30 min at 37°C, the beads were washed three times with phosphate buffer, and the bacteria were dislodged from the beads by sonic treatment (Sudo et al. [16]). Colony-forming units were measured on blood agar plates (16).

Assay for reversible adsorption of an antibacterial agent to HT beads. Bacterial lawns of the test organism were prepared as suggested by Bailey and Scott (2). About 26 mg of HT beads was placed into small test tubes, and then 1.0 ml of 0.2% aqueous chlorohexidine gluconate was added. Control tubes received distilled water. After 30 min at 37°C, the beads were washed three times with distilled water. Sterile glassine papers, each containing four holes (3 mm in diameter), were placed on the inoculated plates, and equal volumes (about 2 mg) of the agent-treated HT beads were evenly distributed in each of the circles. The net effect was to spread a constant volume of HT beads in a constant area, creating a uniform disk. The glassine paper was removed, and the plates were incubated for 18 h at 37°C. Zones of inhibition were measured using a Boley gauge.

Assay for the effect of an agent on the adsorption of bacteria to saliva-treated HT beads. An overnight culture of *S. sanguis* was diluted 1/20 into TYG containing 2 μCi of [^{14}C]glucose. After 7 h at 37°C, cells were harvested by centrifugation and washed twice with distilled water. The washed cells were suspended in water at a concentration of 10^9 to 2×10^9 bacteria/ml, determined by microscope count. Fresh saliva was collected from several individuals and pooled. It was clarified by centrifugation for 10 min at 11,000 $\times$ *g*. Samples (26 mg) of HT beads were placed in glass scintillation vials, and 2.5 ml of whole clarified saliva (WCS), along with agent or a placebo solution, was added to each vial. After 30 min at 37°C, the WCS was decanted, and 1.0 ml of fresh WCS and 0.1 ml of ^{14}C-labeled *S. sanguis* were added. Control vials received the appropriate placebo solution, whereas experimental vials received various amounts of agent. After 45 min at 37°C, the beads were washed three times in water, and the residual liquid was carefully wiped from the walls of the vial. A 0.5-ml portion of an NCS tissue solubilizer (Amersham-Searle Corp.) was placed in each vial, and after 45 min at 37°C 5 ml of toluene-based scintillation fluid was added. Samples were counted with a Beckman liquid scintillation system (LS-230).

Scanning electron microscopy. Samples of colonized beads were washed in buffer, fixed in OsO_4, and prepared for scanning electron microscopy (3).

RESULTS

Prevention of growth or of colonization. This assay was developed to determine in a single experiment whether a potential antiplaque agent inhibited bacterial growth and/or colonization of an HT surface. *S. mutans* was cultured as described above for 6.5 to 7 h. Figure 2 is a scanning electron micrograph showing a typical area in one of the control samples. In the medium employed, the control cultures were nearing the end of the logarithmic phase of growth, and the efficiency of the filter assay had not yet declined appreciably (16). Values (Table 1) are expressed relative to the untreated control. The tabulated results agreed well with microscope observations of the samples (data not shown).

Effect of brief exposure to an agent on the viability of cells in a preformed bacterial mat. In the procedure described above, the agent was present throughout the incubation. Many antiplaque agents, however, might be present in the oral cavity only sporadically. Furthermore, they would be required to act on already

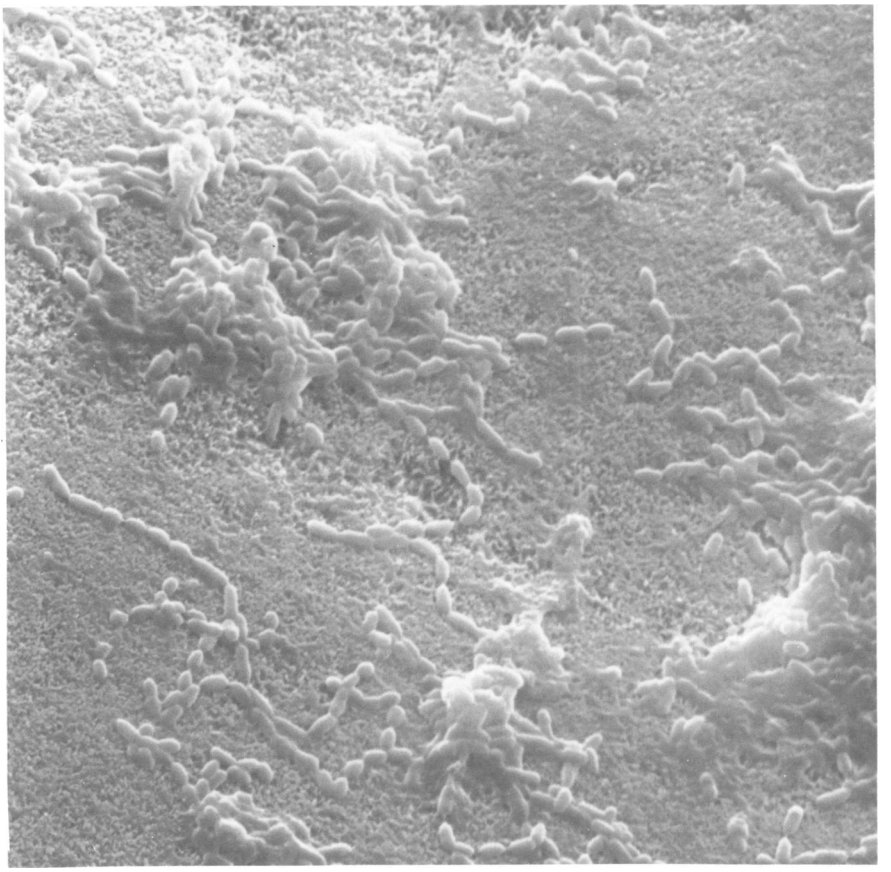

FIG. 2. *Scanning electron micrograph of S. mutans growing on HT beads. ×5,000.*

TABLE 1. *Effect of the continuous presence of agents on bacterial growth and/or colonization of an HT-surface*

Agent	Concn in medium	% of control	
		HT beads	Culture fluid
None	–	100	100
Fluoride[a]	115 μg/ml	<1	<1
Fluoride	50 μg/ml	37	57
Fluoride	10 μg/ml	79	166
22549	20 μl/ml	<1	<1
22549	10 μl/ml	1	6
22549	5 μl/ml	4	25
22549	2 μl/ml	113	167
Placebo[a]	20 μl/ml	84	100

[a] The fluoride was dissolved in a glycerol-water solvent identical with the placebo.

established microbial populations. It has been shown that exposure to chlorohexidine kills cells of *S. mutans* grown on a nichrome wire (17). To test the efficacy of our system, then, we exposed HT beads heavily colonized with *S. mutans* to a solution of 0.2% chlorohexidine gluconate for 30 min. This treatment reduced the number of viable cells from 1.3×10^8/10-mg sample to 8.4×10^6/10-mg sample.

Reversible adsorption of an antibacterial agent to HT beads. Again 0.2% chlorohexidine gluconate was used as the test agent. Using the protocol outlined above, zones on a lawn of *S. mutans* were 7.6 ± 0.4 mm in diameter (mean ± standard deviation, $n = 12$). On a lawn of *Veillonella*, the zone of inhibition was 8.5 ± 0.5 mm ($n = 8$). Pretreatment of the beads with 1 ml of WCS/10 mg of HT beads before exposure to chlorohexidine reduced the average zone size by about 1 mm in both cases. Beads pretreated with buffer only showed no zone of inhibition.

Effect of agent on adsorption of *S. sanguis* to saliva-treated HT beads. The number of cells adhering to the HT beads was increased considerably by precoating the beads with saliva and further increased by using saliva in the second incubation (data not shown). It is unlikely that the entire HT surface was coated with salivary protein, so that adherence to HT as well as adherence to proteins adsorbed to HT

probably occurred. The results in Table 2 indicate that the higher concentrations of the agents tested effectively inhibited both types of adherence. It is unlikely that the radioactivity adhering to the beads resided in small molecules that leaked from the cells during the assay. When ^{14}C-labeled cells were incubated in saliva for 45 min and then removed by centrifugation and the saliva was used in the assay, less than 500 cpm adhered to the saliva-treated HT beads.

DISCUSSION

The methods described are convenient procedures for assessing several important properties of potential antiplaque agents. Quantitative measurements of the effect of an agent on both growth and colonization were obtained after only 7 h of incubation. Previous methods (9, 17) require substantially longer periods or yield only a semiquantitative measure of bactericidal activity. Likewise, the microbial growth suitable for measuring the ability of a bactericidal agent to penetrate a bacterial mat was obtained after 18 h of incubation. It does not seem realistic to employ extremely heavy growth, since that condition occurs in the oral cavity of a minority of the population. Much larger masses can be grown, however, by transferring colonized beads to fresh medium (unpublished data). We have not yet correlated the value for cell number/10 mg of HT beads with average thickness of the microbial mass, although it is possible to derive such a relationship if the need arises.

One desirable characteristic of an antiplaque agent is that it adsorb to an oral surface, thereby creating a reservoir capable of exerting prolonged action. Possible sites for adsorption are epithelial surfaces (4), the salivary pellicle (11), or the HT of teeth (1, 6). The "HT disk" diffusion method allows measurement of reversible adsorption of a bactericidal or bacteriostatic agent to HT. The procedure must be rigidly standardized, the assay being subject to the same variations as those employing cellulose antibiotic disks. The advantage of using HT beads is that it measures interaction among HT, agent, and bacteria rather than cellulose, agent, and bacteria (1).

TABLE 2. *Effect of agents on adherence of S. sanguis to HT or to salivary proteins adsorbed to HT*

Sample	cpm × 10^{3a}	% Placebo
Placebo (20 μl/ml)	14.3	
22549 (20 μl/ml)	0	0
Placebo (5 μl/ml)	21.5	
22549 (5 μl/ml)	6.4	30
Placebo (50 μl/ml)	16.3	
22561 (50 μl/ml)	0.7	5
Placebo (25 μl/ml)	24.8	27
22561 (25 μl/ml)	6.6	

[a] An average of three vials. The background value, derived from vials in which HT beads were omitted from the system, has been substracted from each pair. Typical background values were 10^3 to 4 × 10^3 cpm.

The HT of normal teeth is nearly always coated with adsorbed salivary proteins (15). Ideally, it would be desirable to coat the HT beads with the same or similar proteins (10) and then to assess the influence of these proteins on the adsorption of agents to the "salivary pellicle." We have established that some salivary proteins adsorb rapidly to HT beads (unpublished data). However, there is still considerable variation in the results. Preliminary evidence suggests that differences in the saliva used (10) and the presence of substances in the saliva that inhibit adsorption are two complicating factors. The interaction of HT beads with salivary proteins will require careful study before the system is useful for investigating the influence of antiplaque agents on the adsorption of salivary polymers or for basic studies of the adherence of bacteria to the salivary pellicle. Both the total amount of adsorbed protein and the distribution among different classes of proteins under various circumstances should be determined. It is doubtful that the saliva-treated HT beads used to study the adsorption of ^{14}C-labeled *S. sanguis* were completely coated with salivary proteins. At its present stage of development, then, the assay does not differentiate between adherence to HT and adherence to salivary polymers adsorbed to HT. The agents listed in Table 2 inhibited both types of adherence. Other experiments indicated that these agents moderately reduced the total amount of protein adsorbed to the HT beads (unpublished data). But more detailed studies are necessary before conclusions concerning mechanism of action can be drawn.

The samples of HT beads used in this study present a surface area manyfold larger than the HT surface in the oral cavity. Subsequently, the surface energies are probably quite dissimilar. In some instances, then, one may expect to find quantitative differences between the in vitro results using this system and results with an intact tooth. It would be useful to investigate the magnitude of the difference generated by this factor.

The procedures presented here are an im-

provement over those previously described (1, 9, 17) in that they require only short incubation periods, they yield quantitative results, and the surface offered for colonization is chemically similar to that of the tooth. With further study, HT beads may be useful in elucidating the interactions among HT, salivary proteins, and the bacterial cell surface.

ACKNOWLEDGMENTS

This work was supported in part by a research grant from the Graduate School of the University of Minnesota, by funds supplied by General Mills, Inc., and by Public Health Service general research support grant RR 05322 from the General Research Support Branch, Division of Research Facilities and Resources.

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