In Vitro Susceptibility of Mutans Streptococci to Antimicrobial Substances as Determined by a Membrane Transfer Assay

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Received 19 June 1989/Accepted 2 October 1989

The in vitro susceptibilities of seven representative strains of mutans streptococci to three topicaly applied chemotherapeutic agents were determined by a modified membrane transfer assay. The MBCs of chlorhexidine and I2-KI ranged from 0.5 to 1.0%, while SnF2 was sublethal at the highest concentration tested (8%) in all but one strain (AHT; mode MBC, 8.0%). The proposed in vitro assay may be useful for preclinical screening of potential antimicrobial agents prior to use in the oral cavity.

The phenotypically similar group of bacteria collectively known as the mutans streptococci have been implicated as major etiological agents of dental caries. These bacteria, along with other members of the indigenous biota, are capable of forming dense aggregates (dental plaque) on the surface of the teeth. Accordingly, measures to control den- tomicrobial infections include attempts to reduce the numbers of this organism by mechanical, chemical, or immuno- logical means (4). If the appropriate substance can be identified, chemical elimination of mutans streptococci has the potential of being a cost-effective and relatively simple supplement to other means of caries prevention. The ability of an antimicrobial agent to exert bactericidal effects in a relatively short time (e.g., minutes) is a major factor in the selection of potential agents for topical applications in the oral cavity (4).

Prior to costly clinical testing, in vitro evaluation is an important first step in establishing the potential efficacy of a chemotherapeutic agent. In this regard, it is desirable for in vitro testing to simulate the in vivo situation as closely as possible (2, 4, 10). For example, because plaque microor- ganisms aggregate on teeth in dense masses approaching \(10^{11}\) cells per g (wet weight), in vitro tests should also use dense bacterial masses. Even though many bacteria growing on solid medium can attain a density of growth resembling that of dental plaque, a means of exposing the bacteria to the chemical agent for a short time is also necessary in order to simulate the short periods of exposure possible in the oral cavity. We recently reported a method for exposing dense microbial lawns of bacteria grown on membranes to antimicro- bic agents for a short time (5 min) (2). Here, we report the in vitro bactericidal effects of three chemical compounds proposed as anticaries agents against seven representative strains of the mutans streptococci.

The seven mutans streptococci used for this study are listed in Table 1. Prior to each test, cultures were activated from frozen storage (\(-70^\circ C\)) by overnight anaerobic growth in Trypticase soy broth (TS; Gibco Laboratories, Grand Island, N.Y.). Growth of all cultures occurred at 37°C under an atmosphere of 85% N\(_2\)-10% H\(_2\)-5% CO\(_2\) within an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.).

Aqueous solutions of 2% chlorhexidine gluconate (CH) (from 20% stock; Imperial Chemical Industries, Wilmington, Del.), 2% (wt/vol) iodine-2% (wt/vol) potassium iodide (3) (J. T. Baker Chemical Co., Phillipsburg, N.J.), and 8% (wt/vol) stannous fluoride (in a 1:1 glycerin-water solution [vol/vol]) were prepared and filter sterilized (0.45-\(\mu\)m-pore-size filter unit; Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.). These stock solutions were stored at 4°C in plastic, sealed containers. The antimicrobial test solutions for MBC determinations were obtained by serial twofold dilution of the stock concentration.

A modification of the membrane transfer assay (2) was used to determine the MBCs of the test solutions against mutans streptococci. Briefly, 0.3 ml of inoculum from 24- h-old TS broth was evenly spread onto a sterile 75-mm nitrocellulose-containing membrane (0.3-\(\mu\)m pore size; PHWP 07500; Millipore Corp., Bedford, Mass.) on a TS agar plate. A dense lawn of bacteria was obtained after 24 h of anaerobic incubation. On another freshly made TS agar plate, sterile paper disks were uniformly arranged and 25-\(\mu\)l samples of serially diluted CH, I\(_2\)-KI, or SnF2 were added to the serially arranged disks (see Fig. 1). The bacterium- covered membranes were then transferred to the disk-containing plate (bacteria side up) and left in contact with the agent-saturated disks for exactly 5 min. Membranes were then transferred to fresh TS plates containing 0.04% triphen- yl tetrazolium chloride dye (TTC; Sigma Chemical Co., St. Louis, Mo.). TTC was added to the medium to denote cell viabili- ty (2, 7). After the plates were incubated for 24 h, the MBC was determined by observing the lowest dilution lethal to the bacteria in the area over which the disk was applied (nonreduced TTC is colorless) (2). The membrane transfer assay was intentionally designed to minimize carry-over; however, the plates were incubated for an additional 48 h to confirm that effects were bactericidal and not inhibitory, since any carry-over of antimicrobial agents would diffuse into the agar and allow reactivation of growth if the bacteria were still viable. All determinations were performed on four separate occasions, and results were recorded as the mode MBC for each strain-agent combination tested (2).

A TTC-stained membrane illustrating a MBC determination is shown in Fig. 1. The mode MBCs of the three stock solutions are summarized in Table 2. These results showed that 0.5 to 1.0% CH and I\(_2\)-KI were bactericidal for all strains tested. SnF2, however, exhibited sublethal effects at the 8% concentration for all but one strain (AHT; MBC, 8%).

Conventional in vitro methods commonly proposed for

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testing antibacterial substances (1) may be inappropriate for predicting outcomes against dentomicrobial infections because of the dense masses of bacteria associated with plaque. Conventional in vitro methods are more applicable for serum or tissue infections in which the concentration of bacteria is several orders of magnitude less than those of dentomicrobial infections. The high density of mutants streptococcus cells achieved on the membranes in this investigation more closely resembles the cell densities found in plaque (2).

Another shortcoming of conventional in vitro antimicrobial tests is the duration of exposure to these agents. Conventional disk diffusion assays usually expose antimicrobial agents to bacteria for 16 to 18 h (1). Because plaque-associated diseases tend to remain localized rather than invasive, topically applied rather than systemically administered antimicrobial therapies may be more effective. Topical substances intended for use in the oral cavity are usually applied for brief periods (e.g., minutes), and therefore the in vitro assay should simulate short exposure times. The proposed membrane transfer assay allows for testing of different concentrations of agents for short periods.

Although zones were not scored until 24 h after exposure of the cells to the agents, we observed zones of nonviable cells after 15 min with CH and I$_2$-KI that were comparable to zones after 24 h of incubation. This observation suggests that these agents, when applied at the MBC or above, are immediate in action, and therefore, carry-over of the chemotherapeutic agent is not a confounding factor when results of this assay are interpreted. Moreover, the use of a membrane which restricts carry-over of accumulated agents when the membrane is transferred from contact with the agent-containing disks to a fresh agar plate was done intentionally to minimize carry-over (7).

Other proposed in vitro susceptibility assays, such as that devised by Tanzer et al. (8), have advantages similar to those of the membrane transfer assay. In these susceptibility assays, dense aggregates of mutants streptococci are grown on stainless-steel wires and then exposed to various concentrations of antimicrobial agents for different periods of time (8, 10). In fact, MBCs reported by these investigators for CH and I$_2$-KI are within an order of magnitude of those we report here (9, 10).

Knowing how well a particular in vitro susceptibility test predicts clinical efficacy comes from comparing results of the clinical trials with those of the in vitro test. Data from our assay suggest that concentrations of between 0.5 and 1% for CH and I$_2$-KI and 8.0% and greater for SnF$_2$ represent the MBCs against the mutants streptococci. Several clinical trials indicate that a bactericidal effect occurred when multiple topical applications of chlorhexidine (5), iodine-KI (3), and stannous fluoride (6, 11) were used against *Streptococcus mutans* and other supragingival plaque bacteria. Several studies have also reported the use of fluoride concentrations that may have been bactericidal to *S. mutans* after short-term application. For example, Woods (11) cleaned the buccal surfaces of molar teeth with a prophylaxis paste containing 9% SnF$_2$. One week later, proportions of *S. mutans* were markedly reduced. Studies by Keene et al. (6) indicate that a 10% solution of SnF$_2$ may be bactericidal to *S. mutans*, because treated sites were found to be free of detectable levels of *S. mutans* several days posttreatment.

In conclusion, the membrane transfer assay may be useful for screening many potential antimicrobial agents for bactericidal effects against cariogenic bacterial strains such as mutants streptococci. We see no reason why this method or a modification would not also be useful for testing antimicrobial agents against local infections by pathogens on other
susceptible body surfaces such as skin, genitals, eyes, and nasal tissues.

This investigation was supported by Public Health Service grants DE07026, DE00155, and DE02670 from the National Institute of Dental Research.

LITERATURE CITED


