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Determination of Bactericidal Activity of Antibacterial Monomer MDPB by a Viability Staining Method

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In this study, the bactericidal activity of antibacterial monomer MDPB (12-methacryloyloxydodecylpyridinium bromide) against *Streptococcus mutans* was tested by a rapid method for monitoring viability. To *S. mutans* culture containing fluorescence staining solution that distinguishes live from dead cells, MDPB was added at a concentration of 250, 100, 50, or 10 μg/ml. Bacterial cells were observed by fluorescence microscopy and the percentage of dead cells was calculated. After 10, 20, or 30 minutes' contact with MDPB, the live/dead ratio was measured by fluorometry and viable counts (CFU) determined by the conventional plating method.

Viability staining revealed that MDPB exhibited significant bactericidal effects at 50 μg/ml or greater (ANOVA, Fisher's PLSD test), and complete killing of the cells at 250 μg/ml of MDPB was demonstrated in conjunction with a plating method. The staining method thus provided a sensitive means to determine loss of viability, and indicated the strong killing effects of MDPB on *S. mutans*.

Keywords: MDPB, Bactericidal activity, Viability staining

INTRODUCTION

Recent developments in adhesive dentistry allow improved preservation of tooth structure with a minimally invasive tissue-saving approach. However, the criteria that reflect the existence of cariogenic bacteria or their virulence in dentin have not yet been established. Further, the lack of a precise diagnosis of the extent of dentinal caries in clinical situations is a problem to obtaining better prognosis. Some studies have indicated that carious dentin was left in many cases which were deemed to be caries-free after caries removal by traditional methods[1,2]. Therefore, the use of restorative materials that have antibacterial effects provides an adjunct treatment contributing to suppression of residual infection and increasing the survival of the restored tooth.

We have developed an antibacterial self-etching primer by incorporating the monomer, 12-methacryloyloxydodecylpyridinium bromide (MDPB)[3,4]. The adhesive system that employs this technology is commercially available as Clearfil Protect Bond[5]. Antibacterial monomer MDPB, a compound synthesized from quaternary ammonium, exhibits strong antibacterial activity when unpolymerized against oral streptococci[6] and obligate anaerobes[7] or organisms clinically isolated from root lesions[8]. It is expected to be useful not only for cavity disinfection, but also for management of caries lesions[9,10].

Recently, the method to differentiate live/dead bacteria by staining with fluorescence dye has been introduced[11]. The staining method has the advantage of giving results rapidly as it is sensitive for the monitoring of bacterial viability. The purpose of this study was to investigate the bactericidal activity of MDPB against *Streptococcus mutans* by a viability staining method, comparing the results with conventional viable count method.

MATERIALS AND METHODS

Viability staining

Streptococcus mutans NCTC10449 was incubated in Todd Hewitt Yeast Extract (Oxoid, Cambridge, UK) broth overnight at 37℃, diluted, and grown to absorbance 1.0 at 550 nm (equivalent to 1×10^9 colony forming units (CFU)/ml). Bacterial culture of 100 μl was added to 900 μl of 20 mM phosphate buffered saline (PBS) and centrifuged for five minutes in a microcentrifuge at 13,000 rpm. The supernatant was removed and the pellet was resuspended in 1 ml of PBS.

For viability staining, LIVE/DEAD BacLight Bacterial Viability Kit (L-7012, Molecular Probes, USA) was used. BacLight stained live bacteria with intact membranes green and dead cells with disrupted membranes red. Components A and B of the kit were mixed thoroughly and 3 μl of this solution was added to the bacterial suspension. After incubation at room temperature in the dark for 15 minutes,

10 mg/ml MDPB solution was added to the bacterial suspension to give a final concentration of 250, 100, 50, or 10 μg/ml. A control without MDPB was included.

Live (green) and dead (red) cells were observed after 20−30 minutes by fluorescence microscopy (ZEISS Universal Photomicroscope, Welwyn Garden City, UK). Three fields of view were photographed for each sample, and live and deal cells were counted. The proportion of live and dead cells was also determined after 10, 20, or 30 minutes' contact with MDPB by measuring fluorescence on a fluorometer (VersaFluor, Bio-Rad, Hemel Hempstead, UK), with excitation filter EX480/20 (470−490 nm) and emission filters EM520/10 (515−525 nm) and EM620/10 (615−625 nm). The ratio of green/red intensities (G/R ratio) was measured for each MDPB concentration in triplicate.

Viable count

For determination of viable count by the plating method, *S. mutans* suspension was made in contact with MDPB as described before. After 10, 20, or 30 minutes, each bacterial suspension was serially diluted and 100 μl aliquots were inoculated onto Brain Heart Infusion agar (Oxoid) plates. Viable count was determined by incubating the plates anaerobically at 37ºC for 48 hours.

Statistical analysis

The results were subjected to ANOVA and Fisher's PLSD test at a significance level of $p<0.05$.

RESULTS

Viability staining

Figure 1 shows the percentage of dead cells calculated from microscopic observation after their contact with MDPB. Control contained 99.6% live (green) cells in the mean value. 100% viability was obtained for two samples (Fig. 2a), and dead cells within chains of live cells were occasionally observed for one sample. Exclusively red cells were observed at a concentration of 250 μg/ml MDPB (Fig. 2b). Decrease in MDPB concentration resulted in significant increase in live cells ($p<0.05$), but killing was evident even at 10 μg/ml with approximately 24% dead cells.

Figure 3 indicates the changes in G/R ratio as a function of time. G/R ratio of bacterial suspension at the starting point was around 1.8−1.9. At a concentration of 250 μg/ml MDPB, G/R ratio decreased to approximately 0 after 10 minutes' contact. MDPB at 100 μg/ml demonstrated significant decrease in G/R ratio ($p<0.05$) for 20 minutes and the mean ratio after 30 minutes was approximately 0.1. Significant decrease in the ratio was also observed for MDPB at 50 μg/ml for 10 minutes, but reduction was less compared with that of 100 μg/ml MDPB. For 10

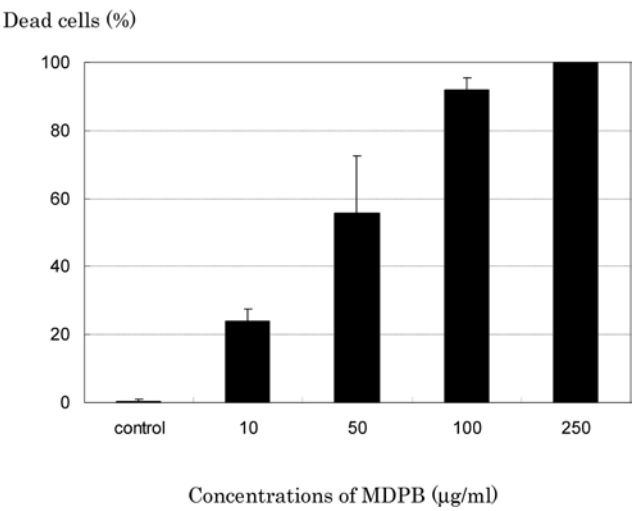

Fig. 1 Percentages of dead cells calculated from microscopic observation after contact with MDPB. The bar indicates standard deviation of the results of triplicate.

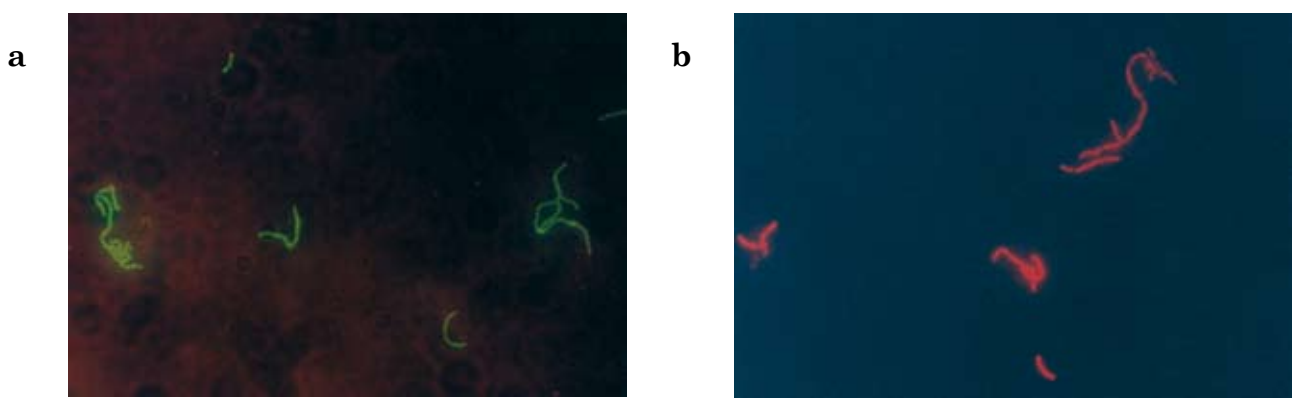

Fig. 2 Microscopic images of the cells after staining with LIVE/DEAD BacLight Bacterial Viability Kit: (a) Control with 100% viability; (b) Exclusively red cells after contact with 250 μg/ml MDPB.

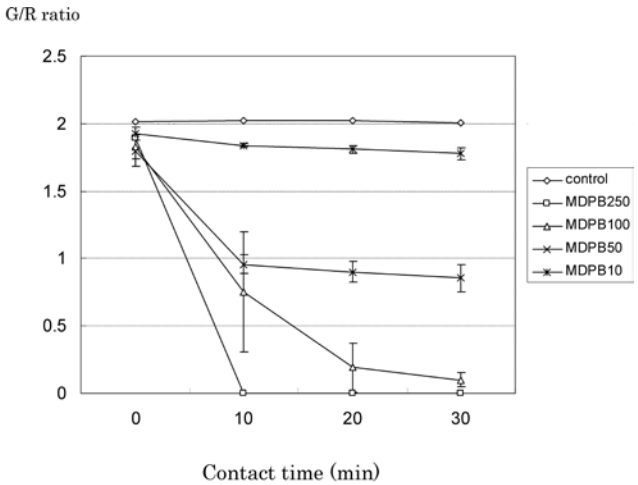

Fig. 3 Changes in G/R ratio as a function of time for each concentration of MDPB. Values are the means of triplicate at each time point. Control indicates culture without MDPB.

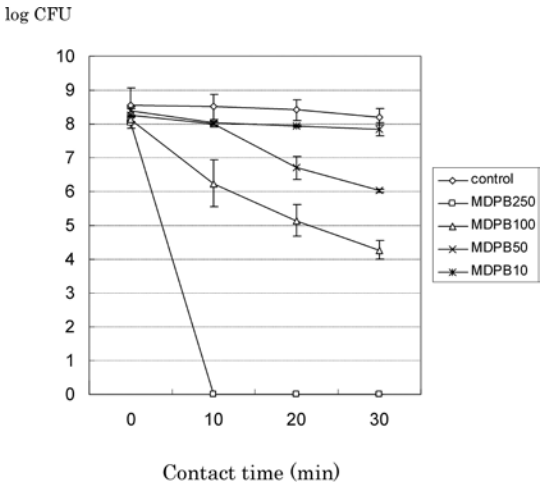

Fig. 4 Bacterial numbers (log CFU) determined by viable count method for each concentration of MDPB. Values are the means of triplicate at each time point. Control indicates culture without MDPB.

μg/ml MDPB, the ratio was slightly decreased after 10 minutes and demonstrated the same level thereafter.

Viable count

Figure 4 shows the number of bacteria (log CFU) determined by the viable count method. Rapid killing of all bacteria was observed after contact with 250 μg/ml MDPB for 10 minutes. Killing effect was dependent upon MDPB concentration, with greater decrease in log CFU for 100 μg/ml than 50 μg/ml MDPB. For these concentrations, a longer contact time resulted in significantly greater reduction in log CFU counts ($p<0.05$). The contact with 10 μg/ml MDPB showed marginal reduction in log CFU at 10−30 minutes.

DISCUSSION

The antibacterial monomer MDPB is a derivative of quaternary ammonium, synthesized by combining dodecylpyridinium bromide with a methacryloyl group. Quaternary ammonium is known to have a wide spectrum of antibacterial activity and is used for various formulations to control bacteria in the dentistry field[12]. Before being polymerized, MDPB acts as the free antimicrobial agent and has been reported to show strong antibacterial activity against oral microorganisms including streptococci, lactobacilli, and a number of anaerobic bacteria[6,7]. MDPB is also inhibitory against bacterial species isolated from root caries, such as *S. mutans, S. oralis, S. salivarius, Lactobacillus* spp., *Actinomyces naeslundii, Actinomyces israelii, Actinomyces gerensceriae, Actinomyces odontolyticus,* and *C. albicans*[8].

To determine the minimum bactericidal concentration (MBC) of antimicrobials, standard broth dilution method according to NCCLS guidelines[13,14] is frequently used. In this method, bacterial viability is determined based on the ability to form colonies on the agar plates after incubation for at least 24 hours. MDPB, a derivative of quaternary ammonium, is considered to show inhibition of bacteria in the same way as other quaternary ammonium; it has high affinity for negatively charged bacterial cells by a nitrogen atom on pyridinium ring and binds to the cell surface, and subsequently disturbs the charge balance, disrupting the cell membrane and leading to the cell death[12].

These actions of MDPB may occur within a short period at high concentrations. Against this background, a rapid assessment of the killing effects of MDPB is useful in elucidating the detailed antibacterial characteristics of this monomer. Therefore, in the present study, we explored the suitability of a viability staining method[11], by which bacterial viability could be observed directly and rapidly. When *S. mutans* stained with *Bac*Light kit was made in contact with MDPB and observed by a fluorescence microscope after 20-30 minutes, it was found that this method was effective in rapidly assessing bacterial viability. MDPB at 250 μg/ml killed all the cells, while killing of almost half of the cells was observed for 50 μg/ml MDPB. The MBC of MDPB for *S. mutans* has been reported to be 50.0−62.5 μg/ml by standard viable count method[6,8]. The staining method employed in this study demonstrated that the bactericidal effect of MDPB was rapidly expressed after contact with *S. mutans* — with 50 μg/ml achieving significant inactivation only after

a short period of 30 minutes. This staining method also has advantage of making it possible to detect the death of individual cells, whereas in the viable count method only one or more cells in a chain need to be alive to form a CFU.

The measurement of G/R ratio by a fluorometer further demonstrated the time-kill relationship. The G/R ratio for the control was calculated to be around 2.0, and it appeared that the values determined by fluorometry did not reflect the number of live/dead cells directly. However, the decreasing pattern of G/R ratio showed good correlation with the results of viable count. The decrease in G/R ratio to almost 0 and no colony formation in the viable count method clearly indicated that killing of all cells by 250 μg/ml MDPB occurred within 10 minutes. With 100 and 50 μg/ml MDPB, the number of live cells was reduced according to the contact time, and the effect was greater for higher concentrations of MDPB. A small reduction in G/R ratio at 10 μg/ml, reflecting a small decrease in CFU count, showed that MDPB at this concentration was still effective at killing some cells.

In conclusion, the fluorescence staining method provided a rapid and sensitive means to determine the loss of viability. Considering the clinical situation that the commercial self-etching primer containing MDPB (Clearfil Protect Bond primer)[5] applied to the cavity is polymerized after 20 seconds, it is further needed to evaluate the killing activity of MDPB within a shorter time period. Especially, evaluation of the effects of MDPB on bacteria in a biofilm using the viability staining method is of importance. Application of this method would be also useful for screening the effects of MDPB on various bacteria, including the viable-but-non-culturable bacteria[15,16] which have been identified in infected dentin.

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