Evaluation of three individual glucosyltransferases produced by
Streptococcus mutans using monoclonal antibodies

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Abstract

We previously established murine hybridomas producing a monoclonal antibody monospecific against three glucosyltransferases (I, SI and S) of Streptococcus mutans which contribute to dental caries formation. Here, we developed a new immunochemical technique (cross-dot system) with which individual levels of glucosyltransferases expressed by S. mutans can be evaluated. We also examined glucosyltransferase production and in vitro artificial plaque formation by a reference strain and several clinical isolates of S. mutans. The findings indicate that the levels of glucosyltransferases produced greatly vary with the cells and the culture medium, and that the cells producing high levels of both glucosyltransferase-SI and glucosyltransferase-I enzymes may possess high in vitro artificial plaque forming ability. We suggest that the cross-dot system will be useful for estimating the cariogenic potential of S. mutans isolates.

Keywords: Streptococcus mutans; Monoclonal antibody; Immuno-dot assay

1. Introduction

Among the seven species comprising the mutans streptococci, S. mutans is implicated in the etiology of human dental decay [1]. This organism produces three extracellular glucosyltransferases (EC 2.4.1.5) that synthesize water-insoluble and -soluble glucans from dietary sucrose [2,3]. The ability of this bacterium to firmly adhere to tooth surfaces through de novo water-insoluble glucan synthesis by the three glucosyltransferases is considered to be one of the most important pathogenic factors leading to cariogenic plaque formation and subsequent dental caries [1]. The gtfB, gtfC and gtfD genes encoding glucosyltransferase-I, -SI and -S enzymes, respectively, have been isolated from S. mutans [2,4,5], and the mechanisms of cariogenic plaque and dental caries formation by S. mutans are being clarified by molecular genetic approaches [6–8]. Epidemiological and microbiological studies have revealed different cariogenic potentials and genetic diversity among fresh S. mutans isolates [9,10]. However, there is little information about the glucosyltransferase production by S. mutans, because there are no useful tools available for determining the individual amounts present in
Polyclonal antibodies cannot distinguish between the glucosyltransferase-I and -SI enzymes, which have extensive amino acid homology [11]. We established murine hybridomas producing a monoclonal antibody (mAb) monospecific for each of the three glucosyltransferases, and confirmed their usefulness through Western blotting of glucosyltransferase samples from *S. mutans* isolates [3].

Here we describe a new immunochemical technique (cross-dot system) using monospecific mAbs, which can semi-quantify the relative amount of each glucosyltransferase. We used it to compare the levels of the three glucosyltransferases expressed by the reference strain PS14 and six clinical isolates of *S. mutans* possessing different in vitro artificial plaque forming abilities.

2. Materials and methods

2.1. Bacteria, media and culture conditions

*S. mutans* strain PS14 (serotype c) was mainly used...
throughout this study. Six *S. mutans* isolates were routinely isolated from the saliva of six individuals, identified, and used in the enzyme expression study. Cultured bacteria were inoculated into 5 ml of Todd-Hewitt broth (THB, BBL), THB supplemented with 1% glucose (G-THB), Brain Heart Infusion broth (BHI, Difco), Trypticase Soy broth containing yeast extract (TSY), or M4 semi-synthetic medium (M4) [12], then cultured anaerobically at 37°C for 18 h in candle jars.

2.2. Preparation of glucosyltransferase extracts

In order to recover the total glucosyltransferases (extracellular and cell-associated forms), chilled ethanol (5 ml) was added to each 5 ml culture at the post-stationary phase and left for 30 min. The resultant precipitate was collected by centrifugation at 10,000 × g for 5 min, suspended in 250 μl of 8 M urea in 10 mM potassium phosphate buffer, pH 7.2 (UP buffer), then incubated at 25°C for 1 h under vigorous stirring with a Twin Mixer TM-282 (Iuchi, Osaka). After centrifugation (10,000 × g, 5 min), the precipitate was again extracted with the UP buffer as described above. The supernatants were pooled and used in the cross-dot assay as the sample containing total glucosyltransferases. In some experiments, the urea extraction was repeated three times and each supernatant was separately assayed.

2.3. Preparation of primary antibodies

Culture supernatants of the hybridomas producing mAbs P4, P32 and P72 were prepared as described [3] and used as primary antibodies. The specificity and potency of these mAbs were confirmed by an enzyme-linked immunosorbent assay (ELISA) as described [3], using crude glucosyltransferase-I from *S. milleri* transformant KSB8, crude glucosyltransferase-SI from *S. milleri* transformant KSC43, and pure glucosyltransferase-S from *S. mutans* PS14.

2.4. Cross-dot assay

The assay was performed essentially as described by Alric et al. [13], using a cross-blot/cross-dot apparatus (Boite-a-Blot; Sebia, France). Briefly, a Clear Blot Membrane-P (Atto, Tokyo) was rinsed

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**Fig. 3.** Cross-dot determination of the three glucosyltransferases produced by six clinical isolates (1–6) of *S. mutans*. The ethanol precipitate from each 5 ml culture of the six isolates grown for 18 h in G-THB medium was extracted twice with 250 μl UP buffer and the pooled extract was analyzed by the cross-dot assay as described in Section 2.4.
with methanol, washed with distilled water and assembled into the cross-dot apparatus using a grid D. After the apparatus was filled with UP buffer and individual lanes were aspirated, each lane was filled with 50 μl of the enzyme preparations diluted from 2² to 2³-folds (some to 2¹²-fold) with UP buffer. The membrane was then incubated at 4°C overnight. Following three washes with 200 mM NaCl in 50 mM Tris-HCl buffer, pH 7.4 (TBS), the membrane was removed from the apparatus, incubated for 5 min in 20 ml of blocking buffer (10% skimmed milk in TBS), and re-assembled into the apparatus using grid A (in which the configuration of the wells is perpendicular to that of grid D). The apparatus was then filled with blocking buffer. Individual lanes were aspirated and each was refilled with a dilution of hybridoma culture supernatant containing mAbs P4 (1:50), P32 (1:10) and P72 (1:20) diluted to 500 μl in the blocking buffer, followed by incubation for 1 h at room temperature. The membrane was washed three times with TBS, removed from the apparatus, and incubated for 15 min in 20 ml of the blocking buffer. After blocking, the membrane was incubated for 2 h in 20 ml of a solution of horseradish peroxidase conjugated goat anti-mouse immunoglobulin (Amersham, 1:2000 dilution in 0.1% bovine serum albumin-TBS), washed three times in 20 ml of TBS, followed by incubation in the substrate solution containing 0.05% 4-chloro-1-naphthol. The results were visualized using a Densitograph AE-6920-MLR (Atto, Tokyo).

2.5. In vitro artificial plaque formation

Cultured bacteria were inoculated into 2 ml THB containing 5% sucrose (S-THB) in disposable culture tubes (12×75 mm, Iwaki Glass, Chiba), and cultured anaerobically at 37°C for 18 h at a 30° angle. The tubes with artificial plaque were vortex-mixed for 10 s at maximal speed on a Touch Mixer MT-51 (Yamato, Tokyo), decanted and washed with 2 ml of phosphate buffered saline (PBS). The decanted and wash solutions were pooled as the non- and loose-adhered cell fraction. The cells remaining on the tube surface were suspended by sonication in 4 ml of PBS and are referred to as the adherent fraction. The turbidity at 550 nm of both fractions was determined immediately after sonication (50 W, 10 s). The percentage of firmly adhered cells per total cells (% adherence) was calculated and used as an indication of the in vitro artificial plaque forming ability of S. mutans isolates.

3. Results and discussion

Using the cross-dot assay with three mAbs, the experimental conditions preparing total glucosyltransferases were examined. The solubilization of cell-associated glucosyltransferases was performed primarily by the 8 M urea extraction method described by Hamada et al. [14]. The ethanol precipitate from a G-THB overnight culture of PS14 cells was extracted repeatedly with UP buffer, and each extract was analyzed with the cross-dot assay. As shown in Fig. 1, large portions of the three glucosyltransferases were solubilized by the first extraction, and the remaining portions were recovered by the secondary extraction. This result suggests that the
complete extraction of glucosyltransferases can be achieved by repeating the treatment in which an ethanol precipitate from a 5 ml culture is suspended in 250 µl of UP buffer and incubated at 25°C for 1 h under vigorous stirring.

The total glucosyltransferases produced by PS14 cells grown for 18 h in five different media were extracted with UP buffer, and analyzed with the cross-dot assay. Fig. 2A-E shows the results of a 2-fold dilution series of the five samples prepared from the THB, G-THB, RH1, TSY and M4 cultures that were dotted and immunostained. The mAb P32 (anti-SI) reacted up to 23-, 24-, 22-, 23- and 27-fold dilutions, mAb P72 (anti-I) up to 22-, 23-, 22-, and 23-fold dilutions, and mAb P4 (anti-S) up to 22-, 23-, 25-, 23- and 27-fold dilutions. The results showed that the amounts of the three glucosyltransferases produced by PS14 cells differed with the culture medium. In particular, the differences between the four complex media and the M4 semi-synthetic medium were remarkable. The data indicated that the levels of three glucosyltransferases produced in the M4 medium were 8-60-fold higher than those produced in the complex media. Motoda [15] reported that the extracellular water-insoluble glucan synthesizing activity of S. sobrinus strain B13N is over 5-fold higher in M4 than in THB, TSY or FMC medium. Therefore, it seems of interest to identify the nutritional factor(s) in the M4 medium that stimulates the production of extracellular glucosyltransferases from mutans streptococci.

We examined the glucosyltransferase production and in vitro artificial plaque forming ability of six fresh S. mutans isolates by means of the cross-dot assay and by estimating firmly adherent artificial plaque formation. Figs. 3 and 4 show the results of the glucosyltransferase production and artificial plaque formation by the clinical isolates grown in G-THB and S-THB, respectively.

Although the profiles of glucosyltransferase production and firmly adherent plaque formation greatly differed among the isolates, they were classified approximately into three groups. Isolates 1 and 6, which produced relatively high levels of the three glucosyltransferases, showed the maximal adherence, whereas isolates 2, 4 and 5, which produced relatively high levels of glucosyltransferase-SI and glucosyltransferase-S but relatively low levels of glucosyltransferase-I, showed moderate adherence, in agreement with reference strain PS14. In contrast, isolate 3, which produced a relatively high level of glucosyltransferase-S but little glucosyltransferase-I and no glucosyltransferase-SI, did not form firmly adherent plaque on the glass surface. Similar glucosyltransferase profiles were also obtained when these isolates were cultured in other complex media (data not shown). These findings are comparable to those of an in vitro cariogenicity test with S. milleri transformants indicating that the transformant expressing gtfC (encoding glucosyltransferase-SI) can firmly colonize glass surfaces, whereas the transformants expressing gtfB (encoding glucosyltransferase-I) or gtfD (encoding glucosyltransferase-S) cannot, and that the co-cultures of the transformants expressing gtfC and gtfB result in the maximal sucrose-dependent colonization [7,16]. These results and those of the present study suggest that the glucosyltransferase-SI enzyme plays the most important role in the process of in vitro artificial plaque formation and possibly in cariogenicity, and that the glucosyltransferase-I enzyme also functions as an important accelerating factor in that process. Thus, the cariogenic potential of S. mutans producing high levels of both glucosyltransferase-SI and glucosyltransferase-I enzymes is most likely higher than that of S. mutans producing low levels of those enzymes. In conclusion, the cross-dot assay system employed in this study should be a useful method of semi-quantifying the individual levels of the three glucosyltransferases expressed by S. mutans, and of estimating the cariogenic potential of S. mutans isolates. Currently, in vitro and in vivo studies to clarify the detailed relationship between glucosyltransferase producibility and the exact cariogenicity are in progress in our laboratory using this assay system with many S. mutans isolates.

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References


