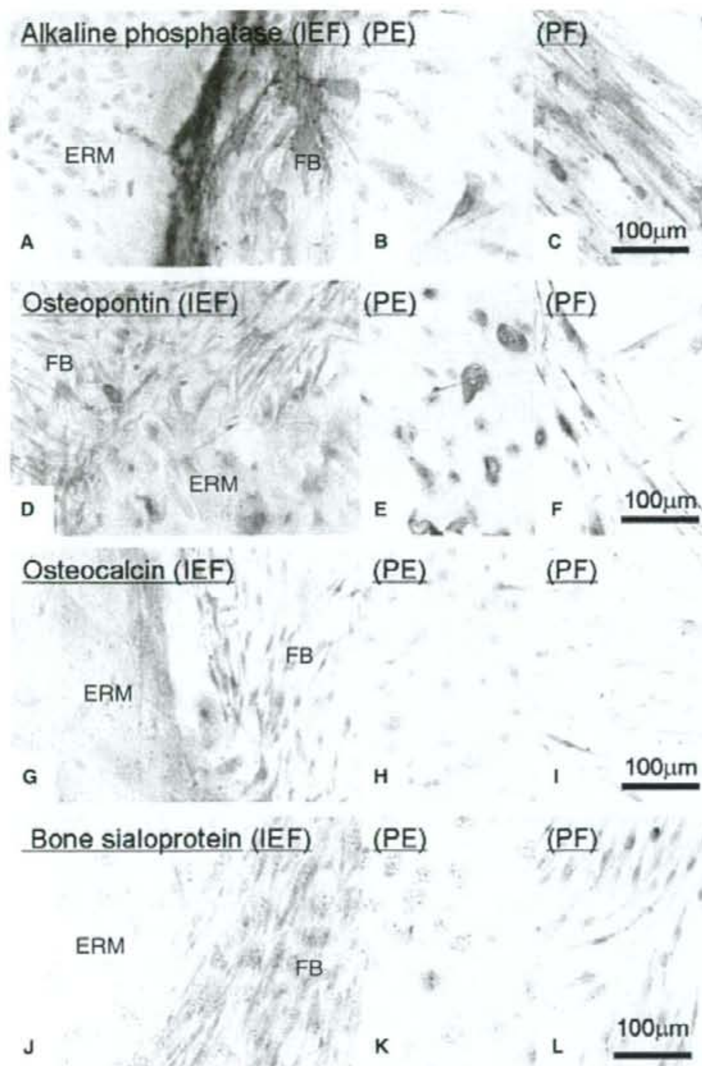


Fig. 3. (A–C) Photomicrographs showing strong alkaline phosphatase activity in interface area cells, in putative epithelial rests of Malassez cells cultured alone and in putative human periodontal ligament fibroblasts cultured alone. (D–F) Photomicrographs showing strong immunoreactivity for osteopontin in interface area cells. Osteopontin was also detected in putative epithelial rests of Malassez cells cultured alone and in putative human periodontal ligament fibroblasts cultured alone. (G–I) Photomicrographs showing strong immunoreactivity for osteocalcin in putative epithelial rests of Malassez cells at the interface area but very weak immunoreactivity for osteocalcin in putative human periodontal ligament fibroblasts cultured alone. Osteocalcin was not detected in putative epithelial rests of Malassez cells cultured alone. (J–L) Photomicrographs showing no immunoreactivity for bone sialoprotein in putative epithelial rests of Malassez of the interface area and in putative epithelial rests of Malassez cells cultured alone, and weak immunoreactivity for bone sialoprotein in human periodontal ligament fibroblasts of the interface area and putative human periodontal ligament fibroblasts cultured alone. ERM, epithelial rests of Malassez cells; FB, human periodontal ligament fibroblasts; IEF, interface area; PE, putative epithelial rests of Malassez cells cultured alone; PF, putative human periodontal ligament fibroblasts cultured alone.

on the root retain a high amount of amelogenin, whereas cementicles contain bone sialoprotein and osteopontin, typically found in bone and cementum (61). Hertwig's epithelial root sheath cells may be involved in both enamel formation and cementum formation, and they may participate in either enamel formation or cementum formation in different situations.

It has been suggested that epithelial rests of Malassez may protect the root surface from resorption, prevent ankylosis and consequently maintain the integrity of the periodontal ligament. Some studies have postulated that proliferating epithelial rests of Malassez cells may be involved in reconstitution of the periodontium (38,39,62). We showed, in the present study, that putative epithelial rests of Malassez cells have a higher proliferation rate, according to the incorporation of 5-bromo-2′-deoxyuridine extensively at the interface (Fig. 2C). Moreover, in our previous study, the synthesis of type IV collagen and laminin including the basement membrane was induced by the interaction at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts (40). The initiation of cementogenesis is at least temporally linked to the disintegration process of Hertwig's epithelial root sheath. It is natural that the basement membrane is broken down in this disintegration. The disruption of a basement membrane may be correlated to the expression of osteopontin and bone sialoprotein. Osteopontin and bone sialoprotein are members of the small integrin-binding ligand, N-linked glycoprotein (SIBLING) family. The SIBLING family can specifically bind pro-matrix metalloproteinases (pro-MMPs) and activate MMPs. MMPs play a crucial role in extracellular matrix degradation during tissue remodeling. Moreover, epithelial growth *in vitro* and during wound healing *in vivo* is intrinsically linked to the expression of MMPs (63,64).

Alkaline phosphatase and noncollagenous bone proteins are considered to have multiple functions related to the formation, turnover and repair of collagen-based mineralized tissues. Previ-

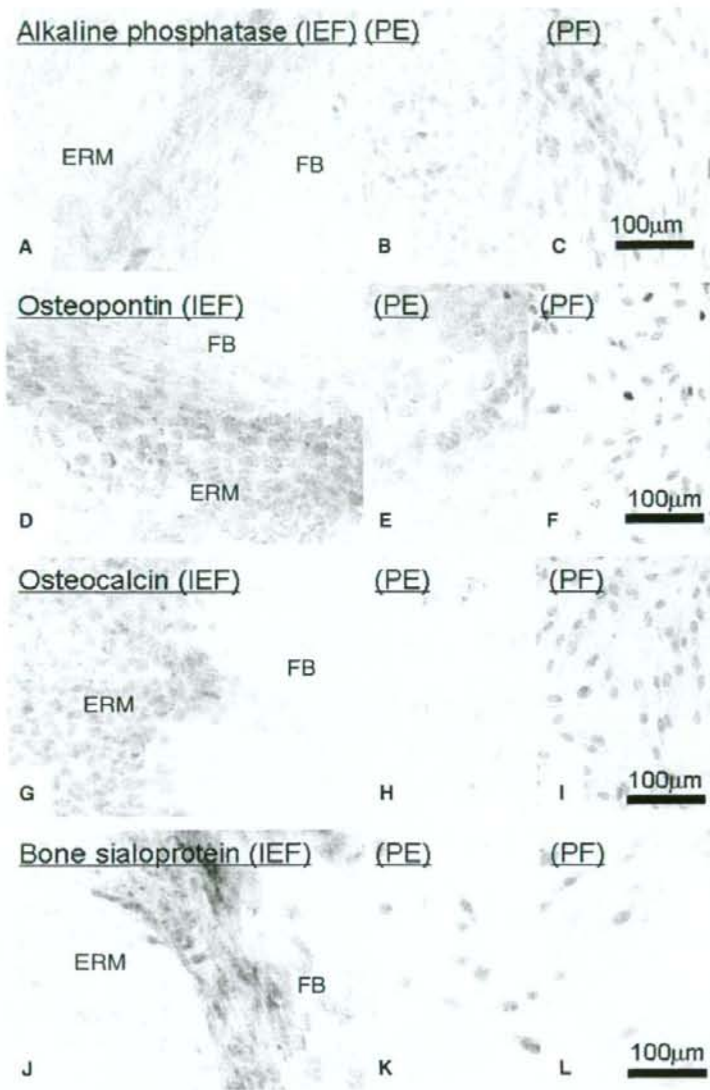


Fig. 4. (A–C) Photomicrographs showing strong immunoreactivity for alkaline phosphatase mRNA in interface area cells, in putative epithelial rests of Malassez cells cultured alone and in putative human periodontal ligament fibroblasts cultured alone. (D–F) Photomicrographs showing strong immunoreactivity for osteopontin mRNA in interface area cells. Osteopontin mRNA was also detected in putative epithelial rests of Malassez cells cultured alone and in putative human periodontal ligament fibroblasts cultured alone. (G–I) Photomicrographs showing strong immunoreactivity for osteocalcin mRNA in putative epithelial rests of Malassez cells of the interface area but very weak immunoreactivity for ostocalcin mRNA in putative human periodontal ligament fibroblasts cultured alone. Osteocalcin mRNA was not detected in putative epithelial rests of Malassez cells cultured alone. (J–L) Photomicrographs showing no immunoreactivity for bone sialoprotein mRNA in putative epithelial rests of Malassez cells of the interface area and in putative epithelial rests of Malassez cells cultured alone, but weak immunoreactivity for bone sialoprotein mRNA in human periodontal ligament fibroblasts of the interface area and in putative human periodontal ligament fibroblasts cultured alone.

ous studies have shown that alkaline phosphatase and noncollagenous bone proteins are present in periodontal ligament, mantle dentin, acellular and cellular cementum, and cementoblasts (10,20,28,52,65). An understanding of the distribution of these proteins within the human periodontium is of fundamental importance. When we set out to determine the level of mRNA expression of alkaline phosphatase and noncollagenous bone proteins in the samples extracted at the interface, only alkaline phosphatase mRNA expression was significantly higher at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts. Although alkaline phosphatase and osteopontin were expressed strongly in putative epithelial rests of Malassez cells cultured alone and in human periodontal ligament fibroblasts cultured alone, the interaction has a strong influence on alkaline phosphatase expression. In fact, it can be easy that alkaline phosphatase expresses under various conditions, e.g., in mineralization medium which includes ascorbic acid, β-glycerophosphate and dexamethasone (66–68). There were no significant differences in osteopontin mRNA expression among the three cell populations. Osteopontin may not be directly associated with the interaction between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts, although it can be associated with mineralization and cell adhesion in each cell (3,10–12,26,31). Tissue nonspecific alkaline phosphatase and osteopontin can be considered as differentiation markers because they are not exclusive to bone and cementum and they can be detected during the differentiation stages on proliferating precursor cells (3,10,52).

On the other hand, osteocalcin and bone sialoprotein have a very restricted tissue distribution and are almost exclusively produced by the hard tissue formation cells. The level of osteocalcin was increased at the interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblast cells, whereas osteocalcin mRNA was not detected in putative epithelial rests of Malassez

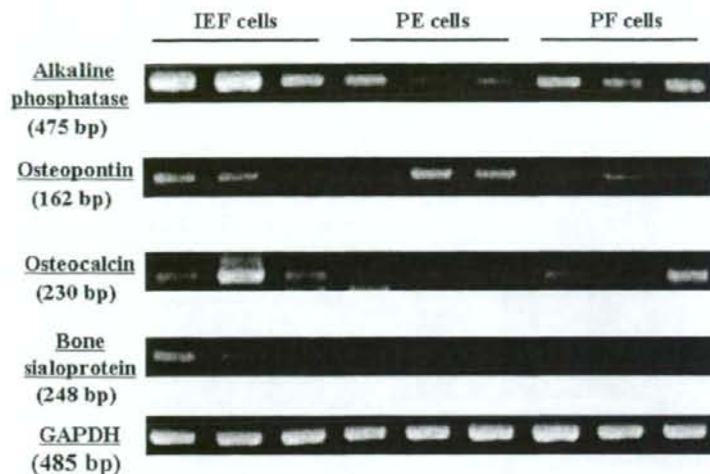


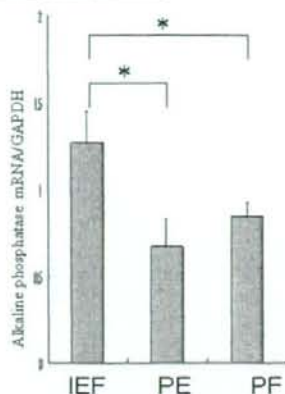
Fig. 5. Reverse transcription-polymerase chain reaction analysis of the mRNA expression of alkaline phosphatase, osteopontin, osteocalcin and bone sialoprotein in cultures of interface area cells, of putative epithelial rests of Malassez cells cultured alone and of putative human periodontal ligament fibroblasts cultured alone. The mRNAs were extracted from the samples including both putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts at the interface (interface area cells), putative epithelial rests of Malassez cells cultured alone, or human periodontal ligament fibroblasts cultured alone. Reverse transcription-polymerase chain reaction products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IEF, the cells including both putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts at the interface; PE, putative epithelial rests of Malassez cells cultured alone, PF, putative human periodontal ligament fibroblasts cultured alone.

cells cultured alone. Moreover, immunohistochemical and *in situ* hybridization data indicated that stronger expression and a wider distribution of osteocalcin were found in putative epithelial rests of Malassez cells at the interface compared with putative epithelial rests of Malassez cells cultured alone, but osteocalcin was not detectable in human periodontal ligament fibroblasts at the interface. The interface between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts showed a stronger expression of bone sialoprotein mRNA compared with human periodontal ligament fibroblasts cultured alone, whereas immunohistochemical and *in situ* hybridization data indicated that bone sialoprotein was not detectable in putative epithelial rests of Malassez cells. When the RT-PCR findings of increased levels of osteocalcin and bone sialoprotein mRNA can reflect the immunohistochemical and *in situ* hybridization data, interaction between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts facilitate osteocalcin expression in putative epithelial rests of Malassez cells and bone sialoprotein expression in human periodontal ligament fibroblasts.

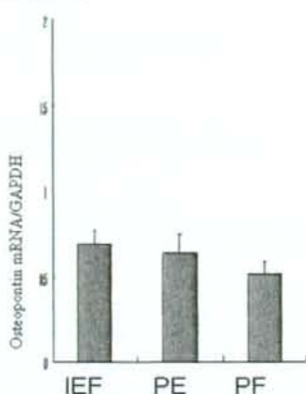
A hypothesis advanced by Slavkin & Boyde proposes that Hertwig's epithelial root sheath cells synthesize proteins that are related to enamel polypeptides, and that these Hertwig's epithelial root sheath-derived proteins are instructive for ectomesenchymal determination, leading to cementoblast differentiation and acellular cementum (23). It has also been suggested that the intermediate layer of the cementum seen in rodent molars is produced by Hertwig's epithelial root sheath cells (23–30). We also indicated that putative epithelial rests of Malassez cells have the ability to produce amelogenin, alkaline phosphatase and the noncollagenous proteins, osteopontin and osteocalcin, supporting their involvement in cementum formation. Bone sialoprotein was detectable in human periodontal ligament fibroblasts, but not in epithelial rests of Malassez cells. Previous reports indicated that bone sialoprotein is detectable in cementum and alveolar bone, but not in periodontal ligament *in vivo*, whereas alkaline phosphatase and osteopontin are detectable in normal periodontal ligament tissue (52,68). The origin of cementogenesis is still controversial. It appears that the extracellular matrix and the constructed cementum are formed by both epithelial rests of Malassez cells and human periodontal ligament fibroblasts, but it is difficult to identify the role of cementoblasts with certainty.

In contrast, immunohistochemical and *in situ* hybridization data indicated that osteocalcin was not detectable in human periodontal ligament fibroblasts in close proximity to epithelial rests of Malassez cells at the interface, while the RT-PCR showed that weak signs of osteocalcin mRNA could be detected in human periodontal ligament fibroblasts cultured alone. These findings suggested that the interaction between epithelial rests of Malassez cells and human periodontal ligament fibroblasts suppressed the production of osteocalcin in human periodontal ligament fibroblasts at the interface. Osteocalcin is one of the extracellular matrix proteins of cementum and bone that has been implicated to have a role in calcification. However, Ducy *et al.* reported that the finding of increased bone density in osteocalcin knockout mutant mice implicates osteocalcin as a potential inhibitor of bone formation (19). Aubin *et al.* showed that over a time course of osteoblast differentiation *in vitro*, the expression of type I collagen was found to be high and then to decrease; the level of alkaline phosphatase increased but decreased when mineralization was well progressed; osteopontin appeared before certain other matrix proteins, including bone sialoprotein and osteocalcin; bone sialoprotein was first detected in differentiated osteoblasts forming

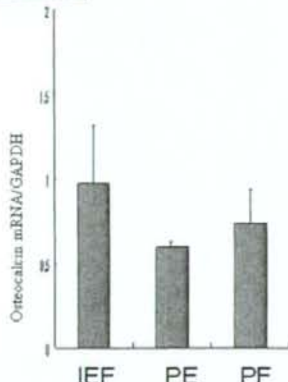
Alkaline phosphatase



Osteopontin



Osteocalcin



Bone sialoprotein

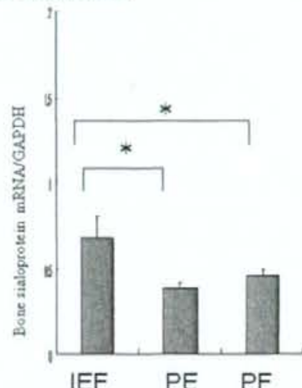


Fig. 6. Graphical representation of relative band intensities standardized by glyceraldehyde-3-phosphate dehydrogenase (mean ± standard deviation, $n = 3$; significant difference: *, $p < 0.05$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IEF, interface area cells, comprising both putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts; PE, putative epithelial rests of Malassez cells cultured alone, PF, putative human periodontal ligament fibroblasts cultured alone.

bone; and osteocalcin appeared with mineralization (5). We showed, in the present study, that bone sialoprotein was detectable, but osteocalcin was not, in human periodontal ligament fibroblasts at the interface. Although human periodontal ligament fibroblasts have a potentially recognizable stage of differentiation, they may not divide and differentiate into the mature cells forming the hard tissues because of the invasion of the proliferating epithelial rests of Malassez cells at the interface.

Cell culture favors growth and selects for the most rapidly proliferating cells. Cell culture data should be cautiously extrapolated to the *in vitro* situation, particularly in providing evidence for cellular origins. However, this should not preclude the fact that valuable information can be obtained from *in vitro* studies, because the results from *in vitro* studies reflect various situations *in vivo*. Moreover, cells derived from periodontal ligament should produce the same proteins and mRNAs as those expressed in periodontal ligament tissues.

In conclusion, the data presented here indicated that the interaction between putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts modulates the expression of alkaline phosphatase, osteocalcin and bone sialoprotein in putative epithelial rests of Malassez cells and human periodontal ligament fibroblasts *in vitro*. Considering the homeostasis of the periodontium, we suggest that epithelial–mesenchymal interactions are highly associated with mineralization in periodontal ligament and play a role in the maintenance of the periodontal ligament.

Acknowledgements

We gratefully acknowledge Shigeki Matsumoto for kindly supplying human extracted third molars. This work was supported by a Grant-in-Aid for Encouragement of Young Scientists (B) (No. 15791088) from the Japan Society for the Promotion of Science, Japan.

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Chairside evaluation of pH-lowering activity and lactic acid production of dental plaque: Correlation with caries experience and incidence in preschool children

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Nobuhiro Takahashi, DDS, PhD[3]

Objectives: Because caries activity may be related to dental plaque acidogenicity, a method was developed for chairside evaluation of pH-lowering activity and lactic acid production by dental plaque. Moreover, this study examined the association of these 2 factors with caries experience on oral examination and with caries activity by following caries incidence for 4 years in a group of preschool children. **Method and Materials:** A dental plaque sample (2.4 μL) was collected from sound buccal surfaces of maxillary primary second molars using a spoon excavator and placed onto the sensor area of a portable pH meter. Sucrose (30 μL, 228 mmol) was mixed with the plaque sample, and pH changes were monitored for 10 minutes. After pH determination, lactic acid concentration in the plaque-sucrose mixture was measured using a portable lactate meter. **Results:** Caries experience of subjects correlated with minimum pH (at 10 minutes after sucrose addition) ($r = -0.53$, $P < .001$) and lactic acid production ($r = 0.38$, $P < .001$). In addition, increments of both primary tooth caries (Δdft) and permanent tooth caries (ΔDFT) for 4 years correlated with minimum pH ($r = -0.47$, $P < .005$ and $r = -0.38$, $P < .05$, respectively). Setting cut-off values of pH and lactic acid concentration at 6.0 and 7.0 mmol/L for Δdft allows screening for caries-susceptible subjects (sensitivity = 0.950 and 0.800, specificity = 0.391 and 0.783, respectively). **Conclusion:** Plaque in caries-susceptible preschool children displays greater pH-lowering activity and lactic acid production. This method can be applied as a chairside screening test for caries activity and susceptibility for preschool children in dental clinics. *(Quintessence Int 2008;39:151–158)*

Key words: caries experience, caries incidence, chairside evaluation, dental plaque, lactic acid, pH

The acid productivity of dental plaque is considered one of the most significant caries risk factors, since dental caries is initiated through demineralization of the tooth surface by organic acid produced by dental plaque bacteria.[1] Previous studies have reported that after rinsing with sugar, dental plaque obtained from caries-active patients shows lower final pH than that from caries-inactive patients.[2,3] Lingström et al[4] reported that dental plaque covering enamel caries lesions displays higher pH-lowering activity than that from healthy tooth surfaces. All these findings suggest that assessment of pH-lowering activity of dental plaque is one of the first steps in evaluating dental caries risk.

Microorganisms in dental plaque produce various organic acids by sugar fermentation, including lactic, acetic, and formic acids.[5] Of these, lactic acid is the main product

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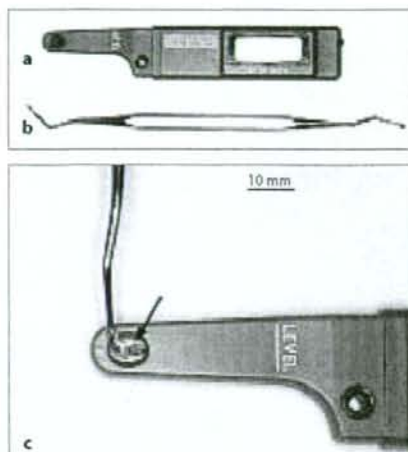

Fig 1 Portable pH meter (*a*, model pH BOY-P1, Sindengen Kogyo) and dental spoon excavator (*b* model No. 1, Yamaura Seisakusyo). A spoon-excavator-cup of dental plaque (2.4 µL) was immediately placed on the sensor of the pH meter (*c*, *arrow*) using a sterilized toothpick.

contributing to reductions in dental plaque pH at the initial phase of the Stephan curve.[6,7] In starved plaque from adults, concentration of lactate was higher in caries-positive subjects than in caries-free subjects, although the proportion of lactate among all organic anions was small.[2,3] After sucrose was added to dental plaque, only lactic acid increased among the organic acids,[8,9] and concentrations of lactic acid were higher in caries-positive subjects than in caries-free subjects.[3]

The association of caries activity with pH-lowering activity and lactic acid production of dental plaque as described above strongly suggests that evaluation of these factors in dental plaque is important for caries risk assessment. However, until now, no simple methods have been available for chairside evaluation of these 2 factors as dental plaque acidogenicity, and little information has been accumulated on relationships with dental caries activity in children. In this study, we first attempted to develop a method for chairside evaluation of pH-lowering activity and lactic acid production by dental plaque in preschool children. Second, we examined the association of dental plaque acidogenicity with caries experience by oral examination and with caries activity by following caries incidence for 4 years. Third, we attempted to determine cutoff points for pH-lowering activity and lactic acid production for screening subjects with high caries risk.

METHOD AND MATERIALS

Subjects and oral examination

Subjects comprised 51 preschool children (25 boys, 26 girls; mean age, 4 years 0 months; range, 2 years 10 months to 6 years 4 months) who visited Shimizu Pediatric Dental Clinic in Shizuoka City, Shizuoka Prefecture, Japan. No subjects were using orthodontic appliances, had been diagnosed with fluorosis, or were on medication. Subjects and their parents provided informed consent to participate in this study.

Oral examination was performed by one clinician to determine the number of decayed, filled teeth (dft) and the modified Oral Hygiene Index Simplified (OHI-S)[10] using a dental mirror and an explorer under sufficient light. In addition, dental radiographs were used if necessary. Teeth with caries lesions of C2 (dentin lesion), C3 (perforation to pulp), and C4 (existence of root only) were counted as "dt." However, no incidents of C4 were found in our subjects.

Measurement of dental plaque acidogenicity at first oral examination

After confirming that the subject had not consumed any food for at least 2 hours, dental plaque was sampled from sound buccal surfaces of maxillary primary second molars using a dental spoon excavator (model No. 1, Yamaura Seisakusyo). The volume of one cup of the spoon excavator is equal to 2.4 µL. A spoon-excavator-cup of dental plaque was immediately placed on the sensor area of a portable pH meter (model pH BOY-P1, Sindengen Kogyo) (Fig 1) using a sterilized toothpick and then mixed with 30 µL of 228 mmol/L sucrose solution. Changes in pH were monitored for 10 minutes at room temperature.

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Table 1 Values of dft, minimum pH, lactic acid production, and OHI-S at the first oral examination, and dft, Δdft, DFT, ΔDFT, minimum pH, and OHI-S at the second oral examination

	Range	Mean ± SD
First oral examination (n = 51)		
dft*	0–16	4.1 ± 3.89
Minimum pH	4.2–5.9	4.8 ± 0.44
Lactic acid (mmol)	1.4–18.5	7.5 ± 3.8
OHI-S	0.5–2.0	1.1 ± 0.5
Second oral examination (n = 43)		
dft	0–16	5.8 ± 4.26
Δdft	0–10	2.4 ± 3.04
DFT	0–4	0.5 ± 1.16
ΔDFT	0–4	0.5 ± 1.16
Minimum pH	4.3–5.5	4.7 ± 0.25
OHI-S	0.0–1.0	0.6 ± 0.3

*Filled teeth at the first oral examination = 0.

The pH value at 10 minutes after sucrose addition was designated as minimum pH. As previously reported,[4] pH values reached a minimum within 10 minutes.

Promptly at 10 minutes, 3 μL of 500 mmol/L sodium phosphate buffer (pH 6.8) was added to the reaction mixture to neutralize the acidic mixture; concentrations of lactate were measured using a portable lactate meter (model Lactate Pro, Arcray). The lactate meter adopts an amperometric method using the lactate oxidase reaction with an optimal pH in the neutral area. Lactate concentration was designated as lactic acid production.

Measurement of dental plaque acidogenicity at second oral examination

At 4 years after the first oral examination, 43 of 51 children (19 boys, 24 girls; mean age, 8 years 0 months; range, 6 years 0 months to 10 years 4 months) underwent oral examination and measurement of pH-lowering activity of dental plaque as described for the first oral examination. Increments of primary tooth caries (Δdft) and permanent tooth caries (ΔDFT) were calculated.

Statistical analysis

Correlations among minimum pH, lactic acid production, dft, DT, DFT, Δdft, and ΔDFT were evaluated by regression analysis and analysis of variance (ANOVA). To evaluate the predictability of minimum pH and lactic acid production for future caries incidence, receiver operating characteristic (ROC) curves were plotted from 2 × 2 tables showing distributions of subjects with and without caries increment at each cutoff value of minimum pH or lactic acid production. Sensitivity and specificity were also evaluated by χ^2 analysis.

RESULTS

All clinical data at the first and second oral examinations are summarized in Table 1. At the first oral examination, subjects with higher dft showed lower minimum pH ($r = -0.53$, $P < .001$; Fig 2a) and higher lactic acid production ($r = 0.38$, $P < .001$; Fig 2b). In addition, a significant correlation was identified between minimum pH and lactic acid production ($r = -0.74$, $P < .001$; Fig 3). No significant correlation was apparent between OHI-S and dft, or between OHI-S and minimum pH or lactic acid production. A similar correlation was observed between dft and minimum pH at the second oral examination ($r = -0.54$, $P < .001$).

Caries increment in primary teeth between the first and second oral examinations (Δdft) significantly correlated with minimum pH and lactic acid production at the first oral examination, but not with dft at the first oral

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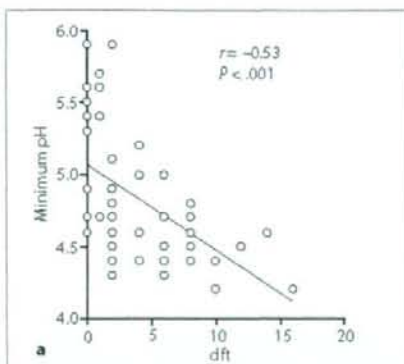

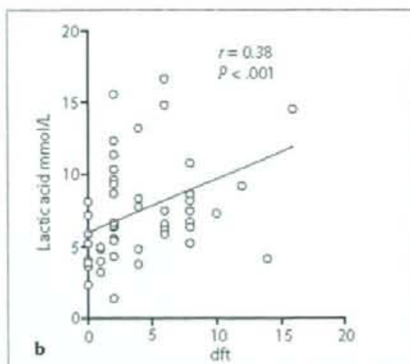

Figs 2a and 2b Relationship of dft with minimum pH *(a)* and lactic acid production *(b)*.

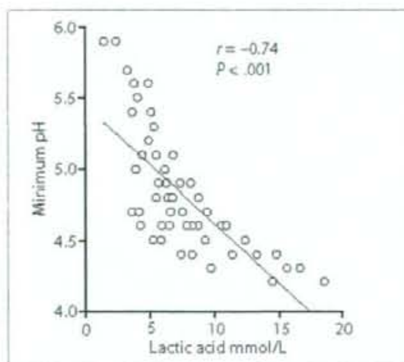

Fig 3 Relationship between lactic acid production and minimum pH.

Table 2 Correlation coefficient and probability of significance

First oral examination	Δdft	ΔDFT
Minimum pH	−0.47**	−0.38*
Lactic acid	0.66***	0.26
dft*	0.27	0.45**

*Filled teeth at the first oral examination = 0.
*$P < .05$
**$P < .005$
***$P < .001$.

examination (Table 2). Caries increment in permanent teeth (ΔDFT) was significantly correlated with minimum pH and dft at the first oral examination, but not with lactic acid production at the first oral examination (see Table 2). No correlation was observed between caries increment and OHI-S (data not shown).

Figure 4a shows ROC curves for Δdft and minimum pH or lactic acid production. In general, the curve for lactic acid production was closer to the x = 0 axis and y = 1 axis, but the sensitivity of minimum pH was higher than that of lactic acid production when specificity was decreased. Setting the cutoff value for minimum pH at pH 5.0 screened subjects with new caries for 4 years with a sensitivity of 0.950 and a specificity of 0.391 ($P < .05$) (Fig 4b). Setting the cutoff value for lactic acid production at 7.0 mmol/L screened subjects with a sensitivity of 0.800 and a specificity of 0.783 ($P < .001$) (Fig 4c). Conversely, ROC curves for ΔDFT were close to the y = x line (Fig 4d), and no significant cutoff value was obtained for minimum pH or lactic acid production (Figs 4e and 4f).

DISCUSSION

Chairside measurement of minimum pH and lactic acid production of dental plaque

Caries-risk testing requires a method that is simple, rapid, and usable from chairside to identify subjects who will become diseased

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Fig 4 Relationship of Δdft and ΔDFT with minimum pH and lactic acid production at the first oral examination. ROC curves of Δdft versus minimum pH and lactic acid production *(a)* and ΔDFT versus minimum pH and lactic acid production *(d)*. Distribution maps showing Δdft versus minimum pH *(b)* and lactic acid production *(c)*, and ΔDFT versus minimum pH *(e)* and lactic acid production *(f)*. Cutoff values are indicated on each panel with corresponding sensitivity, specificity, and *P* value of χ^2 analysis.

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and exclude subjects who will remain healthy.[11] In saliva tests evaluating caries risk, microbial counts of *mutans streptococci* and *lactobacilli* and biochemical properties (volume, pH, buffer capacity, etc) of saliva are usually evaluated. However, since dental caries is a phenomenon occurring at the interface between the tooth surface and dental plaque where salivation is limited, tests using saliva may not always clearly show caries status. In preschool children, the association between dft and salivary rate was not significant, while association between dft and salivary buffer capacity varies from study to study.[12-14] Although significant correlations have been observed between salivary *mutans streptococci* count and dental caries experience in preschool children,[15] this method is not rapid and efficient because of the cultivation procedure, which requires 24 to 48 hours.

Cariostat (Sankin), a caries-risk test evaluating acidogenicity (decreases in pH) of dental plaque by cultivation of dental plaque in sucrose-containing liquid culture medium, has been considered useful for screening high-risk infants[16] and predicting caries occurrence in children.[17,18] In addition, this test uses simple methods for sampling, analyzing, and managing.[19] However, the test requires 48 hours of cultivation to obtain results. Conversely, the method used in the present study was capable of measuring rapid sucrose-dependent falls in pH that occur naturally in the oral cavity. In addition, this method can be completed at chairside using a standardized amount of dental plaque.

Correlation of minimum pH and lactic acid production with caries experience

The results obtained by our method indicate lower minimum pH in preschool children with higher dft ($r = -0.53$, $P < .001$). Minimum pH after adding sucrose reportedly depends on the aging of dental plaque.[20-22] However, each subject in this study performed daily oral care (personal toothbrushing) with relatively low OHI-S (mean, 1.1 ± 0.5; range, 0.5 to 2.0), and no significant difference was apparent between OHI-S and minimum plaque pH. The difference in minimum plaque pH observed in our study may be attributable to individual variation rather than plaque age, eg, differences in acidogenic activity of dental plaque. Lingström et al[4] compared the acidogenicity of dental plaque from sound surfaces of adult subjects having low DMFS (decayed, missing, filled surfaces) with that of adult subjects having high DMFS, and reported that differences in minimum pH are relatively small among adults. This could be due to differences between adults and children, or differences in plaque samples. Subjects in the study by Lingström et al refrained from oral hygiene for 3 days to allow sufficient plaque formation, so that overaccumulation and maturity of dental plaque may have obscured individual variations in dental plaque acidogenicity. The correlation between DFT (= ΔDFT) and minimum pH at the second oral examination in the present study was not significant (see Table 2), also suggesting a difference between caries in primary and permanent teeth.

Minimum pH and lactic acid production as a caries activity indicator

High lactic acid production correlated with minimum plaque pH in this study (see Fig 3), indicating that pH reductions in dental plaque are mainly caused by lactic acid produced from sucrose under the conditions of this study. This is similar to the phenomena occurring in dental plaque after sucrose rinse.[23,24] A correlation between Δdft and both minimum pH and lactic acid production thus seems natural (see Table 2), suggesting the validity of minimum pH and lactic acid production as an indicator of caries activity for preschool children. Both correlation coefficient (see Table 2) and ROC curve (see Fig 4a) indicate that lactic acid production was more strongly related to Δdft, suggesting the superiority of lactic acid production as a caries activity indicator. When a cutoff value of 7.0 mmol/L was set, sensitivity and specificity of lactic acid production as caries predictors were 0.800 and 0.783, respectively, supporting the validity of this method for predicting caries incidence (see Fig 4c).

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Similarly, minimum pH with a cutoff value at 5.0 showed a high sensitivity (0.950) and screened 95% of subjects with caries activity, although specificity was low (0.391 in Fig 4b) and 60.9% of subjects without caries risk may be identified as false positives. Because of the multifactorial etiology of dental caries, accurate prediction of dental caries using a single measure seems unreasonable.[25] Both lactic acid production and minimum pH can be used as primary screening tests for caries risk of preschool children, but may need to be combined with an additional test.

Conversely, the correlation of ΔDFT with minimum pH and lactic acid production was unclear (see Table 2), and predictive power for caries increment was not high (see Figs 4d to 4f). A correlation was identified between ΔDFT and dft at the first oral examination (see Table 2), supporting the notion that dft is a caries predictor for permanent teeth, as reported previously.[26]

Recent studies using schoolchildren as subjects[27,28] have reported that plaque acidogenicity after sugar exposure is unrelated to caries incidence. This may be due to differences in subject age: The acidogenicity of dental plaque may vary among individuals during preschool age and correlate with caries incidence more closely, but during and after school age, the acidogenicity of dental plaque may become similar among individuals. Our observation that minimum pH values obtained at the second oral examination showed a narrower range and smaller standard deviation than those at the first oral examination (see Table 1) may support this speculation.

CONCLUSIONS

It is important for patients and their parents to understand the involvement of dental plaque and its acidogenicity in dental caries etiology to improve lifestyles for dental caries prevention. The method for evaluating dental plaque acidogenicity developed in this study can offer a practical and useful method for this purpose, since the technique is simple to perform chairside in dental clinics and requires only 15 to 20 minutes to show results to patients or their parents, and both minimum pH and lactic acid production show significant correlations with caries experience. In addition, both minimum pH and lactic acid production can be applicable as primary screening tests for preschool children with primary caries risk, although these tests need to be combined with an additional test for the improvement of accuracy.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Science Research (B) no. 14370687 and no. 16390601 from the Ministry of Education, Science, Sports, and Culture, Japan.

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DENTAL MATERIALS XXX (2009) XXX–XXX

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Fluoride released from glass-ionomer cement is responsible to inhibit the acid production of caries-related oral streptococci

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ARTICLE INFO

Article history:
Received 6 March 2008
Received in revised form
12 September 2008
Accepted 30 October 2008

Keywords:
Glass-ionomer cement (GIC)
Eluate
Fluoride
Acid production
Streptococcus

ABSTRACT

Objectives. Glass-ionomer cements (GICs) are known to have inhibitory effects on bacterial growth, but the biochemical mechanism of this property has not been fully understood. This study aimed to evaluate inhibitory effects of GIC on the acid production of caries-related oral streptococci, and to identify the components responsible for the inhibition.

Methods. An eluate was prepared by immersing set GIC in phosphate-buffered saline at 37 °C for 24 h. Fluoride and other elements in the eluate were quantified by fluoride ion electrode and atomic absorption photometry, respectively. *Streptococcus mutans* NCTC 10449 and *Streptococcus sanguinis* NCTC 10556 were used to evaluate the pH fall and the rate of acid production after the addition of glucose in the presence or absence of the eluate. Acidic end products from glucose were also assayed by carboxylic acid analyzer.

Results. The eluate contained silicon (1.24 ± 0.26 mM), fluoride (0.49 ± 0.02 mM) and aluminum (0.06 ± 0.00 mM), and inhibited the pH fall and the acid production rate of both streptococci at acidic pH, with a concomitant decrease in lactic acid production. These effects were comparable to those of a potassium fluoride solution containing the same concentration of fluoride as the eluate.

Significance. These results indicate that the GIC eluate inhibits the acid production of caries-related oral streptococci at acidic pH and that the effect is due to fluoride derived from the GIC. Thus, adjacent to GIC fillings, bacterial acid production and the subsequent bacterial growth may decrease, establishing a cariostatic environment.

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1. Introduction

Fluoride-releasing restorative materials, including conventional glass-ionomer cements (GICs), are frequently used for restorations of permanent or deciduous teeth for the patients with high caries-risk because of their biocompatibility and cariostatic properties.

The eluate of GICs has been found to have no effect on the viability of mouse odontoblast-like cells [1], a weak inhibitory effect on the protein synthesis of human gingival fibroblasts

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doi:10.1016/j.dental.2008.10.014

Please cite this article in press as: Nakajo K, et al. Fluoride released from glass-ionomer cement is responsible to inhibit the acid production of caries-related oral streptococci. Dent Mater (2009), doi:10.1016/j.dental.2008.10.014

[2] and a weak damaging effect on the morphology of human gingival fibroblasts [3]. In addition, GICs are free of cytotoxic components such as 2-hydroxyethyl methacrylate (HEMA), usually contained in resin-modified glass-ionomer [4]. Thus, the available evidence indicates that GICs are less toxic to the cells compared with resin-based materials.

Furthermore, enamel and dentin surfaces adjacent to GIC fillings are protected from acid attack [5,6], indicating that GICs can inhibit acid-induced demineralization of teeth. Furthermore, the demineralized dentin layer adjacent to GIC fillings turns acid resistant with an increase in mineral content such as calcium, and becomes harder [7], suggesting that GICs can promote tooth remineralization [8]. It is considered that these cariostatic properties are due to the fluoride released from GICs; the fluoride can be incorporated in a hydroxyapatite crystal lattice and form fluoroapatite, which has a low acid-solubility [9].

GICs have also been found to have antibacterial effects. It was reported that the population of *Streptococcus mutans* on the surface of GIC fillings was lower than on composite fillings [10,11]. In addition, the pH fall after sucrose fermentation for 4 or 20 h by *S. mutans* cells layered on the surface of a GIC disc was smaller than that on the composite disc [12,13]. These results suggest that GICs inhibit the acid production of *S. mutans* and subsequently decrease the bacterial population. However, the identity and mechanism of this component of GICs is unclear. The observation that the pH of dental plaque decreases within a few minutes after sugar intake [14,15] suggests that the short-term measurement of pH is needed to evaluate the inhibitory effect on microbial acid production.

Therefore, the hypothesis tested was that GIC eluate has a short-term inhibitory effect on the pH fall and the acid production rate of caries-related oral streptococci, *S. mutans* and *Streptococcus sanguinis*, under anaerobic conditions mimicking those in a deep layer of dental plaque, where tooth demineralization occurs. An attempt was also made to identify the components of the GIC eluate that were responsible for these effects.

2. Materials and methods

2.1. *Preparation of GIC eluate and determination of composition*

The powder and liquid of GIC (Fuji IX, GC, Tokyo, Japan) were mixed at the ratio of 1:3.6 for 30 s, and placed into a cylindrical mold (height, 2 mm; diameter, 10 mm). The specimen was allowed to set for 30 min at 25 °C, and immersed in 5 mL of Dulbecco's phosphate-buffered saline containing 154 mM sodium ion, 4 mM potassium ion, 139 mM chloride ion, 10.75 mM hydrogen ion and 9.6 mM phosphate ion (D-PBS; Invitrogen Corp., Rockville, MD, USA) using multi-plates (6 wells). After storage at 37 °C for 24 h, the eluate was collected and the concentration of fluoride was measured using a fluoride ion electrode (F-53, Horiba, Ltd., Kyoto, Japan) after buffering the solution with total ionic strength adjustment buffer (TISAB; Orion Research Inc., Cambridge, MS, USA) at pH 5.3. The concentrations of silicon, aluminum, strontium, magnesium, and calcium in the eluate were determined using atomic absorption spectrometry (AA-6800F, Shimadzu Corp., Kyoto, Japan).

2.2. *Bacterial strains and growth conditions*

S. mutans NCTC 10449 and *S. sanguinis* ATCC 10556 were used. Basal culture medium contained 1.7% tryptone (Difco Laboratories, Detroit, MI, USA), 0.3% yeast extract (Difco Laboratories), and 0.5% NaCl. 0.5% glucose and 50 mM potassium phosphate buffer were added through a sterile membrane filter (pore size 0.22 μm, Millipore Corp., Bedford, Mass, NY, USA) to the autoclaved basal culture medium. This culture medium was kept in an anaerobic chamber (ANB-180L, Hirasawa Works, Tokyo, Japan: 80% N_2, 10% H_2 and 10% CO_2) for at least 3 days before use to remove oxygen. Each strain was pre-cultured in the medium at 37 °C overnight, transferred (5% inoculum size) to medium at pH 7.0 and incubated at 37 °C until the late-log growth phase (O.D. = 0.9–1.0 at 660 nm) in the anaerobic chamber. Bacterial cells were harvested and washed 3 times by centrifuging (21,000 × *g* for 7 min at 4 °C) in D-PBS. Double-sealed centrifuging tubes (Kubota Corp., Tokyo, Japan) were used to protect bacterial cells from air exposure. The cells were suspended in D-PBS in another anaerobic chamber (ANB-180L, Hirasawa Works: 90% N_2 and 10% H_2). The following experiments were performed in this chamber.

2.3. *pH fall by glucose fermentation in the presence of the GIC eluate*

The reaction mixture contained the cell suspension (0.3 mL, O.D. = 10 at 660 nm), D-PBS and the GIC eluate or potassium fluoride (KF) solution. The final concentrations of fluoride in the reaction mixture ranged from 0 to 0.43 mM, which were equivalent to fluoride concentrations in the reaction mixtures for the GIC eluate. The maximum fluoride concentration in the reaction mixture for the GIC eluate containing 0.49 mM fluoride turned to 0.43 mM after mixing with bacterial cell suspension and glucose solution. The reaction mixture was adjusted to pH 7.0, and preincubated at 35 °C for 4 min with agitation by a magnetic stirrer. Then, 10 mM glucose was added to the mixture, and the pH fall was monitored for at least 30 min, using a pH electrode (GS-5015C, Toa Electronics, Tokyo, Japan).

2.4. *Acid production rate from glucose fermentation in the presence of the GIC eluate*

The reaction mixture contained the cell suspension (0.3 mL, O.D. = 3.5 at 660 nm), D-PBS and the GIC eluate or KF solution. The reaction mixture was maintained at pH 5.5 or 7.0 by titration with 60 mM KOH using pH stat (AUT-211S, Toa Electronics) with agitation by a magnetic stirrer, and preincubated at 35 °C for 4 min. The reaction was started by the addition of 10 mM glucose and the acid production was monitored for 10 min.

2.5. *Analysis of acidic end products*

Procedures for analysis of lactic, formic and acetic acids have been detailed previously [16]. At 10 min after the addition of glucose, the reaction mixture was mixed with 0.6N perchloric

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Table 1 – Concentrations of elements in the GIC eluate.

Element	Mean concentration (S.D.)
Fluoride	0.49 (0.02) mM
Silicon	0.82 (0.26) mM
Aluminum	0.01 (0.02) mM
Strontium	<0.001 mM
Magnesium	<0.001 mM
Calcium	<0.001 mM

Data are given in mean (standard deviation) of 3 replicates.

acid. The resultant mixtures were brought out from the anaerobic chamber, filtered through a membrane (pore size 0.20 μm; polypropylene; Toyo Roshi Ltd., Tokyo, Japan) to remove cell debris, diluted with 0.2N HCl and assayed using a carboxylic acid analyzer (Eyela S-3000, Tokyo Rika Co. Ltd., Tokyo, Japan).

2.6. *Statistical analysis*

Statistical significance was assessed by the Dunnett test. A probability of $P<0.05$ was considered to indicate significance.

3. Results

3.1. *Determination of composition in the eluate from GIC*

The eluate contained silicon, fluoride and aluminum with trace amounts of strontium, magnesium and calcium (Table 1).

3.2. *pH fall by glucose fermentation in the presence of the GIC eluate*

Serial pH falls by glucose fermentation of *S. mutans* are shown in Fig. 1. In all experiments using *S. mutans*, pH started to fall immediately after the addition of glucose. However, 10 min after the addition of glucose, the pH of reaction mixtures differed between the various concentrations of fluoride. At 30 min, in reaction mixtures containing eluate with a fluoride concentration of 0.43 mM or KF solution with a fluoride concentration of 0.43 mM, the pH stabilized at 4.93–5.00, which was significantly higher than the pH values of the control reaction mixture, the reaction mixture containing eluate with a fluoride concentration of 0.043 mM, and the reaction mixture containing KF solution with a fluoride concentration of 0.043 mM (Table 2). After 30 min, in the reaction mixture containing eluate with fluoride concentration of 0.043 mM or KF solution with a fluoride concentration of 0.043 mM, the pH continued to fall and approached the control gradually (date not shown). Similar results were obtained for *S. sanguinis* (Table 2).

3.3. *Acid production rate from glucose fermentation in the presence of the GIC eluate*

The rate of acid production of *S. mutans* at pH 5.5 was inhibited significantly by the GIC eluate in a dose-dependent manner, and decreased to about 10% of the control value at pH 5.5 in the presence of the eluate containing 0.43 mM fluoride (Table 3).

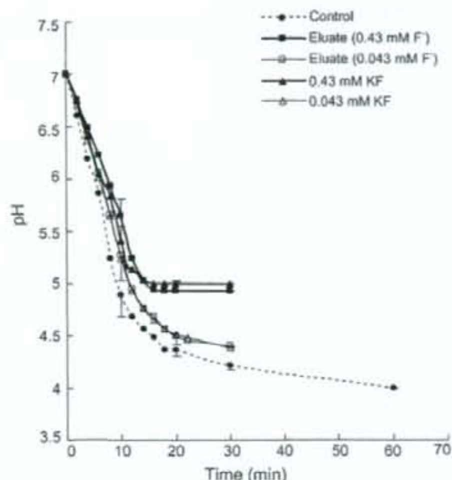

Fig. 1 – Effect of the GIC eluate or KF solution on the pH fall after addition of 10 mM glucose to the cell suspension of *S. mutans* NCTC 10449. The data are the means of 3 independent experiments. Vertical bars indicate standard deviations.

Only a weak inhibition was observed at pH 7.0. All KF solutions inhibited acid production to a degree similar to that of the eluate containing the same concentration of fluoride. There was no significant difference between the Eluate and KF solution containing the same concentration of fluoride. Similar results were obtained for *S. sanguinis* (Table 3).

3.4. *Analysis of acidic end products*

The profiles of acidic end products at pH 5.5 are shown in Fig. 2. As the concentration of fluoride in the eluate or KF solution increased, the proportion of lactic acid decreased, whereas the proportion of formic and acetic acids increased.

Table 2 – pH fall by *S. mutans* and *S. sanguinis* at 30 min after the addition of glucose.

	Mean pH (SD)	
In the presence of:	*S. mutans*	*S. sanguinis*
None (control)	4.21 (0.04)	4.34 (0.1)
Eluate (0.043 mM F^-)	4.35 (0.06)	4.44 (0.12)
Eluate (0.43 mM F^-)	4.93 (0.10)$^{**,\#\#}$	4.79 (0.09)*
0.043 mM KF	4.38 (0.02)	4.46 (0.08)
0.43 mM KF	5.00 (0.03)$^{**,\#}$	4.98 (0.00)$^{**,\#}$

Data are given in mean (standard deviation) of 3 replicates. Significant difference ($^{*}P<0.05$, $^{**}P<0.01$) from the controls. Significant difference ($^{\#}P<0.05$, $^{\#\#}P<0.01$) from the eluate (0.043 mM F^-) and KF solution (0.043 mM F^-).

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Table 3 – Acid production rate by S. mutans and S. sanguinis for 10 min at pH 7.0 and 5.5.

pH	In the presence of	Percentage of acid production rate (S.D.)	
		S. mutans	*S. sanguinis*
7.0	None (control)	100	100
	Eluate		
	0.43 mM F^-	93.4 (4.6)	93.8 (3.9)
	KF		
	0.43 mM	95.4 (4.6)	94.2 (6.2)
5.5	None (control)	61.3 (11.1)*	35.6 (5.4)*
	Eluate		
	0.043 mM F^-	32.5 (5.2)*,**	32.5 (6.1)*
	0.086 mM F^-	28.8 (1.5)*,**	33.4 (1.7)*
	0.215 mM F^-	20.7 (2.6)*,**	15.6 (4.1)*,**
	0.43 mM F^-	6.2 (1.3)*,**	5.4 (3.5)*,**
	KF		
	0.043 mM	31.6 (5.4)*,**	29.0 (8.2)*
	0.086 mM	28.4 (5.5)*,**	28.7 (4.5)*
	0.215 mM	14.4 (2.1)*,**	16.5 (2.2)*,**
	0.43 mM	6.5 (0.6)*,**	4.5(2.0)*,**

Data are given in mean (standard deviation) of 3 replicates. There was no significant difference between the Eluate and KF solution containing same concentration of fluoride.

* Significant difference ($P < 0.01$) from the control at pH 7.0.

** Significant difference ($P < 0.01$) from the controls at pH 5.5.

4. Discussion

In the present study, in addition to fluoride, silicon, aluminum, strontium, magnesium, and calcium were detected in the GIC eluate (Table 1). These elements have previously been reported to leach from GICs [17–19]. The concentrations of these elements are known to differ among GICs [18], probably due to differences in composition. In addition, their concentrations in eluates appear to vary according to eluting conditions. With Fuji IX (a filling cement used in the present study) and Fuji I (a luting cement similar in composition to Fuji IX), the proportion of silicon in the eluate is known to decrease under acidic conditions and increase under neutral pH conditions [17–19]. Consequently, in the present study, D-PBS at pH 7.3 was used for elution, and the proportion of Si in the eluate was relatively high.

The GIC eluate stopped the pH fall completely around pH 4.8–5.0 (Table 2 and Fig. 1) and markedly decreased the rate of acid production at pH 5.5 (Table 3). These findings suggest that streptococcal acid production in areas adjacent to GIC fillings may be inhibited at an acidic pH around the critical pH of tooth demineralization, resulting in a decrease in streptococcal cariogenicity. Furthermore, the present finding that the GIC eluate inhibited acid production to a degree similar to that of the KF solution with the same concentration of fluoride

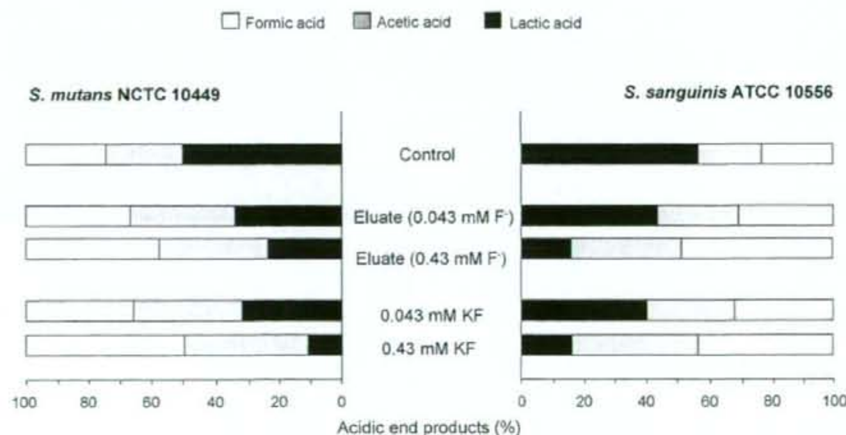

Fig. 2 – Effect of the GIC eluate or KF solution on the profiles of acidic end products for 10 min after addition of 10 mM glucose to the cells of S. mutans NCTC 10449 and S. sanguinis ATCC 10556 at pH 5.5. The data are the means of 3 independent experiments.

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(Tables 2 and 3, Fig. 1) indicates that this effect of the eluate was due to the fluoride derived from the GIC.

The efficient inhibition of fluoride at acidic pH is due to the fact that hydrogen fluoride (HF) behaves according to the equilibrated reaction, $H^+ + F^- \leftrightharpoons HF$ with a pKa value of 3.15. It is possible that fluoride is released from GIC mainly in the form of sodium fluoride (NaF) because the main cation released from Fuji IX is reportedly Na^+ [19], but NaF dissociates to sodium ion (Na^+) and fluoride ion (F^-) immediately at neutral pH. At acidic pH, F^- accepts a proton (H^+) and forms the undissociated form (HF) more easily, according to the equilibrated reaction where the reaction shifts toward the right as the environmental pH is lowered; the concentration of HF is calculated to be about 30 times higher at pH 5.5 than at pH 7.0. Because bacterial cell membranes are more permeable to HF than F^- [20], HF can enter the cells and dissociate into F^- and H^+ again in the relatively alkaline cytoplasm. This intracellular F^- inhibits enolase, a glycolytic enzyme, resulting in a decrease in acid production from glycolysis [21–23]. Thus, an acidic environment can promote entry of HF into bacterial cells and the subsequent inhibition of bacterial acid production via intracellular F^-. Furthermore, H^+ released from HF in the cell can acidify the intracellular pH, leading to a decrease in the entire glycolytic activity. F^- can also inhibit cell membrane-associated H^+-ATPase, a proton pump, resulting in decreased excretion of H^+ from the cells. This can also enhance the acidification of intracellular pH [24–26].

In addition to the inhibitory effect of fluoride, it has been reported that GICs such as Fuji IX are able to neutralize acidic conditions [27], suggesting that there is a buffering potential of released elements from GICs to neutralize acids produced by bacteria.

The present finding, that the proportion of lactic acid was decreased by both the eluate and KF solution (Fig. 2) supports that the eluate and KF solution exert this effect via the same mechanism. Previous researches indicate that this decrease in the proportion of lactic acid coincides with a slowdown in glycolysis through the enolase inhibition by F^- and the subsequent decrease in levels of intracellular glycolytic intermediates [28,29]. A slowdown in glycolysis leads to a decrease in the level of fructose 1,6-bisphosphate (a glycolytic intermediate that activates lactate dehydrogenase), resulting in a decrease in lactic acid production, while the concomitant decrease in levels of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (glycolytic intermediates that inhibit pyruvate formate-lyase), keeps formic and acetic acids production [30].

From the atomic absorption spectrometry, it is not possible to determine the form in which the elements were present in the eluate, i.e. compounds, complexes or ions. Therefore, it is difficult to evaluate the inhibitory effects of elements other than fluoride precisely. However, the most possible ions of the elements in the eluate, SiO_3^- and Al^{3+}, have no inhibitory effects on oral bacterial acid production at the concentrations determined in the present study [31,32]. Although Al^{3+} is known to enhance the inhibitory effects of fluoride on bacterial acid production [33] and H^+-ATPase [25], the concentration of Al^{3+} seemed to be too low to express the synergistic effect. It appears that the concentrations of the other elements were too low to demonstrate the influences on bacterial acid production.

The previous study indicated a possible retention within dental plaque. In the case of resin-modified GIC [8], the plaque fluoride levels increased from the saliva/plaque interface (0.1 mM) towards the plaque/GIC filling interface (1.6 mM). Although fluoride contained in cured GICs is expected to decrease gradually over time [12,34], GIC fillings can undergo a fluoride-recharge by absorbing fluoride from fluoride products such as dentifrices and mouth washes [35,36]. GICs may function as a biomaterial to reserve and release fluoride continuously in the oral cavity.

It is reported that fluoride is bacteriostatic and bactericidal to oral streptococci at concentrations of >15.8 and >160 mM, respectively [37,38]. However, in the current study as well as previous studies [29,39], bacterial acid production from sugar fermentation was inhibited by a lower fluoride concentration, especially at an acidic pH around the critical pH of tooth demineralization. Hallgren et al. [40] observed that dental plaque on the orthodontic brackets retained with GIC had a lower activity of lactic acid production by glucose fermentation as compared to composites. This result supports the antibacterial effect at a low concentration of fluoride as shown in the present study. In addition, they demonstrated that those plaque samples had a lower proportion of mutans streptococci in total viable count [41] and a higher content of fluoride [42].

The inhibition of bacterial acid production not only directly protect against dental caries, but also suppresses the growth of caries-related oral bacteria, which obtain most of the energy for their growth from sugar fermentation. Thus, adjacent to GIC fillings, the acid production and the subsequent growth are expected to decrease. It appears that the inhibition of acid production observed in the present study, together with the inhibition of demineralization and the promotion of remineralization at the tooth surface, would establish a cariostatic environment around GIC fillings *in vivo*. Further study is needed *in vivo* to confirm the expectation.

Acknowledgments

This study was supported in part by Grants-in-Aid for Scientific Research (Nos. 16390601, 17659659, 17791350, 18659562, and 19209060) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

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