Table 2. Clinical signs and conditions of subjects in this study

	Subjects									
	1	2	3	4	5	6	7	8	9	10
Age	50	50	48	43	66	58	53	62	65	77
Gender	M	M	M	M	F	M	M	F	M	F
Swelling	+	+	+	+	+	+	+	+	+	+
Pain	+	+	–	+	–	+	–	+	–	–
Antibiotic therapy[a]	–	–	–	+	–	+	–	–	+	–
Fluid state	Purulent	Purulent	Purulent	Purulent	Mucous	Mucous	Serous	Serous	Serous	Serous

[a]Application of antibiotic therapy (2 weeks before sampling) is designated as positive.

GCA GCA G-3′; and 907R, 5′-CCG TCA ATT CMT TTR AGT TT-3′. Real-time PCR amplification was performed in an iCycler (Bio-Rad Laboratories) programmed for 3 min at 95°C for initial heat activation, followed by 40 cycles of 15 s at 95°C for denaturation, 30 s at 55°C for annealing and 30 s at 72°C for extension. During the extension step, fluorescence emissions were monitored, and data were analyzed using iCycler iQ Software (Bio-Rad Laboratories). The genomic DNA of *Propionibacterium acnes* ATCC 6919 was used as a standard for quantitative analysis.

Nested PCR

In the first amplification, the 16S rRNA genes were amplified by PCR with universal primers 27F and 1492R (27) and Taq DNA polymerase (HotStarTaq Master mix; Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The primer sequences were: 27F, 5′-AGA GTT TGA TCM TGG CTC AG-3′; and 1492R, 5′-TAC GGY TAC CTT GTT ACG ACT T-3′. PCR amplification was performed in a PCR Thermal Cycler MP (TaKaRa Biomedicals, Ohtsu, Shiga, Japan) programmed for 15 min at 95°C for initial heat activation, followed by 35 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1.5 min at 72°C for extension, and 10 min at 72°C for final extension. The predicted PCR product with the universal primers was 1505 bp in length. In the second amplification (nested PCR), the first PCR products were used as templates and 20 bacteria were identified using species-specific primers based on 16S rRNA gene sequences (Table 1). In order to determine the specificity of the primers *in-silico*, blast searches were performed through the web site of the National Center for Biotechnology Information. PCR amplification was performed in a PCR Thermal Cycler MP (TaKaRa Biomedicals) programmed for 15 min at 95°C for initial heat activation, followed by 35 cycles of 1 min at 94°C for denaturation, 1 min at 58.5°C for annealing, and 1.5 min at 72°C for extension, and 10 min at 72°C for final extension. The predicted sizes of PCR products with species-specific primers are listed in Table 1.

PCR products were separated on 1% agarose gels (Certified Low Range Ultra Agarose, Bio-Rad Laboratories) stained with ethidium bromide and photographed under ultraviolet light. The sizes of PCR products were compared with a molecular size marker (Ready-Load 100-bp DNA Ladder, Invitrogen Corp., Carlsbad, CA), and confirmed to correspond to those listed in Table 1.

Data analysis

Fisher's exact probability tests and Tukey's tests were used to analyze significance. *P*-values of <0.05 were considered statistically significant.

Results

Clinical signs of subjects and the state of cyst fluids are shown in Table 2. All patients had swelling and half had pain in the buccal region and/or mucogingival fold. Three patients received antibiotic therapy less than 2 weeks before sampling. Four of the fluids samples were purulent, two mucous and four serous.

The results of quantitative real-time PCR and nested PCR are shown in Table 3. Bacterial DNA was detected in all samples, and thus bacteria were proven to be present in all samples. The average quantity of bacterial DNA was 5.9 ± 2.1 (purulent), 0.5 ± 0.2 (mucous), and 0.7 ± 0.1 (serous) ng/mg of sample. Purulent fluids contained approximately 8–12 times more bacteria than mucous and serous fluids. Bacterial DNA was detected in the cyst fluids of the patients who received antibiotic therapy less than 2 weeks before sampling (Tables 2 and 3).

P. acnes was detected in nine samples, and its detection frequency was the highest among the bacterial species tested in this study. *Porphyromonas gingivalis*, *Streptococcus constellatus*, and *S. intermedius* were detected in five samples, Anaerococcus sp., *Fusobacterium nucleatum* and *Streptococcus anginosus* in three samples, *Campylobacter rectus*, *Dialister pneumosintes*, *Eikenella corrodens*, *Mogibacterium timidum*, and *Peptostreptococcus micros* in two samples, and *Slackia exigua* in one sample. Anaerobic gram-positive cocci such as *Peptostreptococcus* species, which is frequently detected in oral maxillofacial infections (24), were detected in small numbers in this study. The other seven species tested for were not detected in this study.

In the purulent fluids, 12 bacterial species including anginosus streptococci, which is reported to be associated with abscess formation (25, 26), were detected, while two and five species were detected in the mucous and serous fluids, respectively. *P. gingivalis* was detected with a high frequency (three of four cases) in the serous fluids. In eight cases, two species and/or more than two species were detected. In two cases (one mucous and one serous), *P. acnes* was the only species detected in this study.

In *in-silico* determination of the specificity of the primers used in this study, the primers of *P. gingivalis* were found to cross-react with *Porphyromonas gulae*; those of *P. acnes* to cross-react with Propionibacterium sp. LG and uncultured phylotypes (clones), i.e. lw29, Tc134–108, PE40, PE36, PE34, PE33PE30, PE27, PE22, PE21, PE20, PE15, PE13, PE12, PE06, 47 mm60, ACTINO8A, AT425_EubE10, DZ_D6, 1519, and PH-B24N; those of *C. rectus* to cross-react with *Campylobacter curvus*, *Campylobacter showae*, and *Campylobacter sputorum*; and those of *S. intermedius* to cross-react with *Streptococcus pneumoniae*, *Streptococcus pseudopneumoniae*, and *Streptococcus* sp. MGH.

Discussion

In this study, bacterial DNA was detected by quantitative real-time PCR in all samples (Table 3). A low bacterial detection frequency (< 50%) has been reported in POMC fluids (17, 42), and the discrepancy

Table 3. Total bacterial DNA and detected bacterial species

	Purulent				Mucous		Serous				
	1	2	3	4	5	6	7	8	9	10	
	7.9 [b]	7.5	4.7	3.5	0.4	0.6	0.6	0.7	0.9	0.8	No. of positive samples
Universal[a]	(5.9 ± 2.1[b, c, d])				(0.5 ± 0.2)		(0.7 ± 0.1)				10
Propionibacterium acnes	+	+	+	–	+	+	+	+	+	+	9
Porphyromonas gingivalis	–	–	+	–	+	–	+	+	+	–	5
Streptococcus constellatus	+	+	+	+	–	–	+	–	–	–	5
Streptococcus intermedius	+	+	+	+	–	–	+	–	–	–	5
Anaerococcus sp.	+	+	+	–	–	–	–	–	–	–	3
Fusobacterium nucleatum	+	+	+	–	–	–	–	–	–	–	3
Streptococcus anginosus	+	+	+	–	–	–	–	–	–	–	3
Campylobacter rectus	+	+	–	–	–	–	–	–	–	–	2
Dialister pneumosintes	+	+	–	–	–	–	–	–	–	–	2
Eikenella corrodens	+	+	–	–	–	–	–	–	–	–	2
Peptostreptococcus micros	+	+	–	–	–	–	–	–	–	–	2
Mogibacterium timidum	+	–	–	+	–	–	–	–	–	–	2
Slackia exigua	–	–	–	–	–	–	–	+	–	–	1
Eubacterium saphenum	–	–	–	–	–	–	–	–	–	–	0
Finegordia magna	–	–	–	–	–	–	–	–	–	–	0
Peptostreptococcus anaerobius	–	–	–	–	–	–	–	–	–	–	0
Peptoniphilus asaccharolyticus	–	–	–	–	–	–	–	–	–	–	0
Prevotella intermedia	–	–	–	–	–	–	–	–	–	–	0
Prevotella nigrescens	–	–	–	–	–	–	–	–	–	–	0
Streptococcus mutans	–	–	–	–	–	–	–	–	–	–	0
No. of positive bacterial species	11	10	7	3	2	1	4	3	2	1	

[a] Quantification of total bacterial DNA by real-time PCR with universal primers of 16S ribosomal RNA genes.
[b] ng per mg of sample.
[c] Mean values ± standard deviations.
[d] Significantly different ($P < 0.05$) from mucous and serous sample.

could be because previous reports depended on culturing methods and fastidious bacteria were dominant in POMC fluids. In our study, however, the use of molecular biology made it possible to detect bacteria from mucous and serous cyst fluids despite their small numbers.

P. acnes was detected in nine samples and was the most frequently detected bacterium (Table 3). *P. acnes* is an anaerobic gram-positive rod that is considered to be an indigenous bacteria of skin as well as oral and intestinal mucosa. However, the species has been reported to have pathogenicity based on superantigenicity and mitogen activity of T cells (2, 8, 19). *P. acnes* has been recently detected in various diseases, such as alveolar abscess, sinusitis, osteomyelitis, meningitis, noma, endocarditis, septicemia, hepatitis granuloma, facial acne, and abscess of orbit (5, 28, 29) as well as in opportunistic infection (5).

P. gingivalis, detected in five samples in this study (Table 3), is an obligate anaerobic gram-negative rod, and is reportedly associated with the pathogenicity of periodontitis, alveolar abscesses, and oral infections (7, 23). *P. gingivalis* possesses high proteolytic activity to degrade proteins in periodontal tissues and gingival fluids due to expression of various peptidases, such as gingipain (36, 45). *P. gingivalis* also produces lipopolysaccharide and cytotoxic metabolites such as ammonia, butyrate, and propionate (32, 44). Therefore, *P. gingivalis*, detected in POMC fluids, may be associated with POMC inflammation. *P. gingivalis* was detected with a high frequency in the serous fluids in this study, thus suggesting that the properties of serous fluids resembling those of serum, which is rich in protein and neutral in pH (3), may provide a suitable environment for *P. gingivalis* to survive (43).

S. constellatus and *S. intermedius*, detected in five samples in this study (Table 3), and *S. anginosus,* detected in three samples in this study (Table 3), are designated as the anginosus group of streptococci. The anginosus groups are facultative anaerobic gram-positive cocci and are indigenous bacteria in humans. It has been reported that they possess pathogenicity to form abscesses (4, 24, 48) based on tolerance to polymorphonuclear leukocytes (47), cellular components such as capsules (20), and extracellular enzymes such as hyaluronidase (38). It has also been reported that abscess formation by the anginosus group is enhanced by interaction with other bacteria, such as *F. nucleatum*, because its heat-resistant extracellular materials may enhance the pathogenicity of the anginosus group (25, 26). The growth of *S. constellatus* was enhanced by the presence of *E. corrodens* (4). In this study, particularly in the purulent samples, the anginosus group of streptococci was frequently detected with *F. nucleatum* and *E. corrodens* (Table 3). These results support previous reports, and suggest that bacterial interaction may be a symptom of POMC.

In *in-silico* determination of the specificity of the primers used in this study, the primers of *P. gingivalis, P. acnes, C. rectus,* and *S. intermedius* were found to cross-react with other established species and uncultured phylotypes. Thus, it is possible that some uncultured phylotypes which resemble established species, such as *P. acnes,* were frequently detected by PCR in this study, and that these phylotypes may relate to the pathogenicity of POMC, although further studies on the taxonomy of these phylotypes and their pathogenic roles are required.

Purulent fluids of POMC contained numerous bacteria of various types in this study (Table 3), suggesting that the bacteria in the purulent fluids are associated with POMC symptoms such as pain. Pain was the most common clinical manifestation in purulent cases in this study (Table 2). On the other hand, mucous and serous fluids also contained bacteria, although their numbers were small (Table 3), suggesting that the increase in internal pressure of POMC resulting from increases in the amount of fluid is closely related to POMC symptoms, although bacteria may be related to the progression of POMC symptoms.

It has been reported that various types of bacteria are detected in oral maxillofacial infections and periodontal diseases, and many efforts have been made to determine the pathogenicity of these bacteria. Nevertheless, the relationship between bacteria and POMC symptoms has not been widely investigated. Our results suggest that bacteria, frequently detected in the oral cavity, were present in the fluids of POMC, and that these bacteria were partially responsible for the progression of POMC symptoms, although further studies on the presence of bacterial species other than the 20 species selected in this study and their etiologic roles are required. PCR methods are effective in detecting fastidious bacteria in maxillofacial infections and these methods will assist in conventional bacteriological examination, including culturing methods.

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Synergistic Inhibition by Combination of Fluoride and Xylitol on Glycolysis by Mutans Streptococci and Its Biochemical Mechanism

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Key Words
Fluoride · Glycolysis · Mutans streptococcus · Xylitol

Abstract
The purpose of this study was to evaluate the combined inhibitory effect of fluoride and xylitol on acid production by mutans streptococci, *Streptococcus mutans* NCTC10449 and *Streptococcus sobrinus* 6715, from glucose under strictly anaerobic conditions at fixed pH 5.5 and 7.0. The bacteria were grown in a tryptone-yeast extract broth under strictly anaerobic conditions (N_2: 80%; H_2: 10%; CO_2: 10%). Reaction mixtures for acid production from glucose contained bacterial cells with fluoride (0–6.4 m*M*) and/or xylitol (60 m*M*). Acidic end products of glucose fermentation and intracellular glycolytic intermediates were assayed. The combination of fluoride and xylitol inhibited acid production more effectively than fluoride or xylitol alone. In the presence of fluoride and xylitol, the proportion of lactic acid in the total amount of acidic end products decreased, while the proportion of formic and acetic acids increased. Analyses of intracellular glycolytic intermediates revealed that xylitol inhibited the upper part of the glycolytic pathway, while fluoride inhibited the lower part. This study indicates that fluoride and xylitol together have synergistic inhibitory effects on the acid production of mutans streptococci and suggests that xylitol has the potential to enhance inhibitory effects of low concentrations of fluoride.

Both fluoride and xylitol have been widely used as cariostatic agents. Fluoride inhibits the acid production and growth of oral bacteria such as the cariogenic bacteria mutans streptococci [Kashket et al., 1977; Hamilton and Ellwood, 1978; van Houte, 1980; Okuda and Frostell, 1982; Marsh et al., 1985; Zameck and Tinanoff, 1987], particularly under anaerobic conditions [Hata et al., 1990]. The effects on streptococcal cells of fluoride are partly ascribed to the inhibition of enolase, one of the series of glycolytic enzymes [Jenkins, 1999]. This inhibition decreases the intracellular level of phosphoenolpyruvate (PEP), and thus decreases bacterial sugar uptake via PEP-dependent phosphotransferase system (PEP-PTS) [Jenkins, 1999]. In addition, fluoride can directly inhibit bacterial proton-translocating ATPase (H^+-ATPase) that is considered to partly contribute to the excretion of proton out of the cells, leading to the acidification of intracellular pH. The dissociation of unionized hydrofluoric acid into H^+ and F^- in the cells also promotes intracellular acidification [Jenkins, 1999]. This intracellular acidification can further reduce bacterial metabolic activity.

Xylitol is a valuable sugar alcohol for caries prevention [Scheinin et al., 1976; Trahan, 1995] because the majority of oral bacteria, including mutans streptococci, do not ferment it [Birkhed et al., 1985]. Xylitol is taken into the cells and phosphorylated to xylitol-5-phosphate (X5P) by PEP-PTS [Assev and Rölla, 1984; Trahan et al., 1985; Trahan, 1995]. Since X5P cannot be metabolized further [Hausman et al., 1984; Assev and Rölla, 1986], the intracellularly accumulated X5P inhibits glycolytic enzymes

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such as phosphoglucose isomerase (PGI) and phosphofructokinase (PFK) and subsequently represses sugar metabolism [Assev et al., 1983; Vadeboncoeur et al., 1983; Assev and Rölla, 1986; Bradshaw and Marsh, 1994]. After that, the X5P can be dephosphorylated into xylitol and excreted out of the cells [Trahan et al., 1991]. In this 'futile cycle' [Assev and Rölla, 1986], the bacterial cells are forced to waste energy and their growth is obstructed as a result [Mäkinen and Scheinin, 1976; Assev et al., 1980, 1983; Vadeboncoeur et al., 1983; Loesche, 1984; Assev and Rölla, 1986; Bradshaw and Marsh, 1994].

Since both fluoride and xylitol have inhibitory effects on sugar metabolism of mutans streptococci as stated above, using them together in clinical situations is expected to inhibit acid production of mutans streptococci effectively. Scheie et al. [1988] demonstrated the combined inhibitory effect of fluoride and xylitol on the growth of mutans streptococci and suggested the inhibitory step of glucose metabolism. Moreover, Rogers and Bert [1992] showed the possibility that xylitol enhances the inhibitory effect of fluoride on mutans streptococci growing in continuous culture. However, the biochemical mechanism of the fluoride and xylitol combination on dental plaque bacteria has not been elucidated yet.

Therefore, this study aimed to evaluate the combined inhibitory effect of fluoride and xylitol on the acid production of mutans streptococci from glucose under strictly anaerobic conditions like those of dental plaque with a pH-stat that can exactly keep the constant pH of reaction mixtures. Furthermore, we attempted to elucidate the biochemical mechanism by analyzing the profiles of intracellular glycolytic intermediates.

Materials and Methods

Bacterial Strain

Streptococcus mutans NCTC10449 and *Streptococcus sobrinus* 6715 were used in this study.

Anaerobic Procedure

All the experiments for bacterial cultivation and metabolism were conducted under strictly anaerobic conditions [Yamada and Carlsson, 1975] like those of the deep layers of mature dental plaque [Ritz, 1967; Kenney and Ash, 1969; Katayama et al., 1975], which are closely associated with causation of dental caries. The storage and cultivation of bacteria were performed in an anaerobic chamber (type NHC, gas phase: N_2, 80%; H_2, 10%; CO_2, 10%, Hirasawa Works, Tokyo, Japan). Preparation of bacterial cell suspensions and the bacterial metabolism experiments were carried out in another anaerobic chamber (type NH, gas phase: N_2, 90%; H_2, 10%, Hirasawa Works). During centrifugation outside the anaerobic chamber for harvesting and washing, the bacterial cells were protected from exposure to air by double sealed centrifuge tubes (Kubota Commercial Affairs, Tokyo, Japan) [Miyasawa et al., 2003]. To exclude oxygen, growth media and solutions were placed in the anaerobic chambers for at least 3 days prior to use, while powdered reagents and experimental instruments were placed in the anaerobic chamber for at least 1 day prior to use.

Bacterial Growth Conditions

The bacterial strains, cultured on blood agar plates and stored at 4°C in the anaerobic chamber, were inoculated into 5 ml of tryptone-yeast extract (TYE; pH 7) broth containing 1.7% tryptone (Difco, Detroit, Mich., USA), 0.3% yeast extract (Difco), 85.5 m*M* NaCl, 14.4 m*M* dipotassium phosphate and 11.1 m*M* glucose, and incubated anaerobically at 37°C overnight. The bacterial suspension was subcultured into another 100 ml of TYE broth (inoculum size 5%) for 12 h, and then further cultured into 800 ml of TYE broth (inoculum size 5%). Bacterial growth was monitored by optical density at 660 nm of aliquots from the culture using a spectrophotometer (model UV-160 Shimazu, Ltd., Kyoto, Japan). The cells were harvested at the early exponential phase of growth by centrifugation (10,000 *g* for 10 min at 4°C). The cells were washed twice with a solution containing 150 m*M* KCl and 5 m*M* $MgCl_2$ (4°C) by centrifugation (13,000 *g* for 8 min at 4°C) and the washed cells were finally resuspended in the same solution. The cell suspension was adjusted to 2.7 mg dry weight cells per milliliter and stored at 4°C under anaerobic conditions until the following experiments.

Acid Production from Glucose in the Presence of Fluoride and/or Xylitol

The reaction mixture contained bacterial cells (0.9 mg dry weight cells), 150 m*M* KCl and 5 m*M* MgCl in 2 m*M* potassium phosphate buffer (pH 5.5 or 7.0). Potassium fluoride (0–6.4 m*M*), xylitol (60 m*M*) or both were added into the reaction mixtures. The control reaction mixture contained no fluoride or xylitol. The reaction mixture was maintained at pH 5.5 or 7.0 by titration with 60 m*M* KOH using a pH stat (AUTO pH STAT; model AUT-211S, TOA Electronics, Tokyo, Japan) with agitation by a magnetic stirrer, and then preincubated at 35°C for 4 min. The reaction was started by the addition of 10 m*M* glucose.

Analysis of Carboxylic Acids from Glucose Fermentation

Lactic, formic and acetic acids were assayed following the method of Takahashi et al. [1987]. At 10 min after the addition of glucose, aliquots of the reaction mixture of *S. mutans* NCTC 10449 were sampled and immediately mixed with 0.6 *N* perchloric acid. The resultant mixture was brought out from the anaerobic chamber, filtered to remove cell debris through a membrane filter (pore size 0.20 μm; Advantec, polypropylene, Toyo Roshi Ltd., Tokyo, Japan), diluted with 0.2 *N* HCl and assayed with a carboxylic acid analyzer (model Eyela S-3000, Tokyo Rika Co. Ltd., Tokyo, Japan).

Determination of Glycolytic Intermediates

Glycolytic intermediates were extracted from cells following the method of Iwami et al. [2001]. The cells of *S. mutans* NCTC 10449 were collected by filtering 3.0 ml of reaction mixture through a membrane filter (pore size 0.45 μm; Acrodisc, polyethersulfone, Pall Gelman Laboratory, Ann Arbor, Mich., USA) at 0, 2, 4 and 6 min after the addition of glucose. The glycolytic intermediates

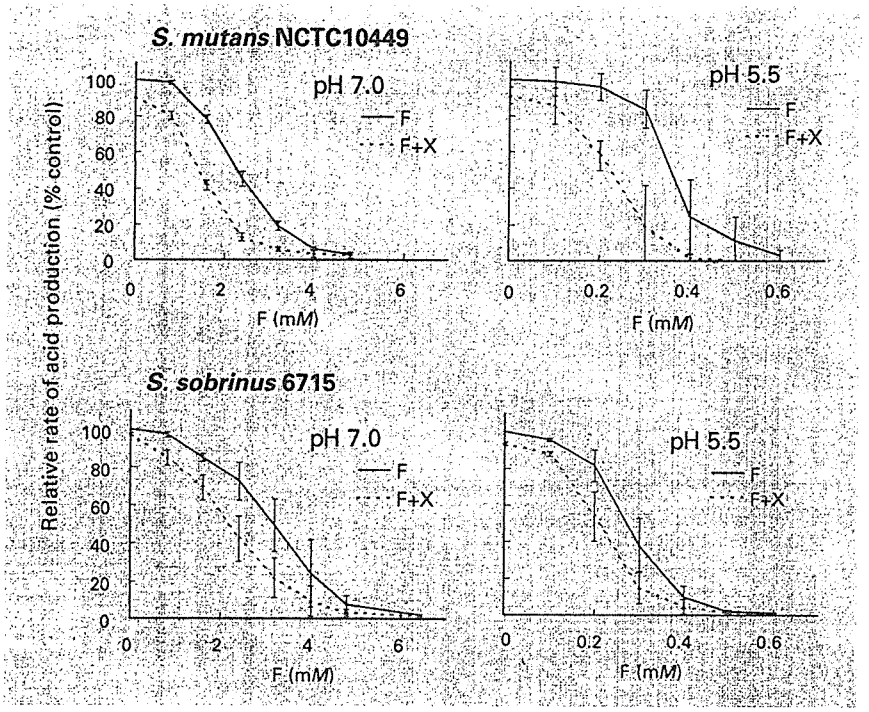

Fig. 1. Effect of fluoride on the acid production for 10 min from 10 m*M* glucose in the presence and absence of 60 m*M* xylitol by the cells of *S. mutans* NCTC 10449 and *S. sobrinus* 6715 at pH 7.0 and 5.5. F = Fluoride alone; F + X = fluoride plus xylitol. The data are the means of three independent experiments. Vertical bars indicate standard deviations from three independent experiments.

were immediately extracted by passing 2.0 ml of 0.6 *N* perchloric acid through the filter. The filter was filtered twice besides with the same perchloric acid solution to extract glycolytic intermediates fully. The extracts were neutralized to pH of 6.0 using 1 *M* triethanolamine-HCl and 5 *M* K_2CO_3 with chilling at 4°C, and filtered through another membrane filter (pore size 0.20 μm; Advantec, polypropylene, Toyo Roshi Ltd., Tokyo, Japan) to remove sediment. The neutralized extracts were stored at 4°C until assayed.

Glycolytic intermediates in the cell extracts were determined at 35°C by the fluorometric methods of Maitra and Estabrook [1964] and Takahashi et al. [1991] with some modifications. A fluorescence spectrophotometer (model CAF-110, Jasco Corp., Tokyo, Japan) was used with a slit width of 10 nm and excitation/emission wavelengths of 340/470 nm. The assay mixture for glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P) contained 20 m*M* triethanolamine hydrochloride-NaOH buffer (TA buffer, pH 7.5), 0.06 m*M* NADP and the cell extract. The amounts of G6P were determined from the increase of NADPH after the addition of 0.6 U/ml glucose 6-phosphate dehydrogenase (EC 1.1.1.49). After NADPH increase reached a plateau, 3.3 U/ml phosphoglucose isomerase (EC 5.3.1.9) was added, and the amount of F6P was then determined from the subsequent increase of NADPH. The assay mixture for dihydroxyacetone phosphate (DHAP), glyceraldehyde 3-phosphate (G3P) and fructose 1,6-bisphosphate (FBP) contained 20 m*M* TA buffer, 0.5 m*M* ethylenediamine tetraacetate, 7.5 m*M* NADH and the cell extract. Glycerol 3-phosphate dehydrogenase (EC 1.1.1.8; 0.85 U/ml), triose phosphate isomerase (EC 5.3.1.1; 25 U/ml) and aldolase (EC 4.1.2.13; 0.23 U/ml) were added sequentially to determine the amounts of DHAP, G3P and FBP from the respective decrease of NADH. The assay mixture for 3-phosphoglycerate (3PG) and 2-phosphoglycerate (2PG) contained 20 m*M* TA buffer, 2 m*M* cysteine, 1.5 m*M* $MgCl_2$, 1.5 m*M* ATP, 0.01 m*M* NADH and the cell extract. Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12; 2.5 U/ml), 3-phosphoglycerate kinase (EC 2.7.2.3; 8.4 U/ml) and phosphoglycerate mutase (EC 5.4.2.1; 4.5 U/ml) were added sequentially to determine the amounts of 3PG and 2PG from the respective decrease of NADH. The assay mixture for PEP and pyruvate (PYR) contained 20 m*M* TA buffer, 10 m*M* KCl, 5 m*M* $MgCl_2$, 0.5 m*M* ADP, 5 μ*M* NADH and the cell extract. Lactate dehydrogenase (EC 2.7.1.40; 3.5 U/ml) and pyruvate kinase (EC 1.1.1.27; 2.4 U/ml) were added sequentially to determine the amounts of PYR and PEP from the respective decrease of NADH.

Statistical Analysis

All numerical data were given as means with standard deviations. Statistical significance was assessed using ANOVA and Dunnette test. Differences were considered significant at the level $p < 0.05$.

Results

Acid Production from Glucose in the Presence of Fluoride and/or Xylitol

In the presence of fluoride, the acid production by *S. mutans* NCTC10449 from glucose for 10 min decreased at both pH 7.0 and 5.5. The decrease of acid production

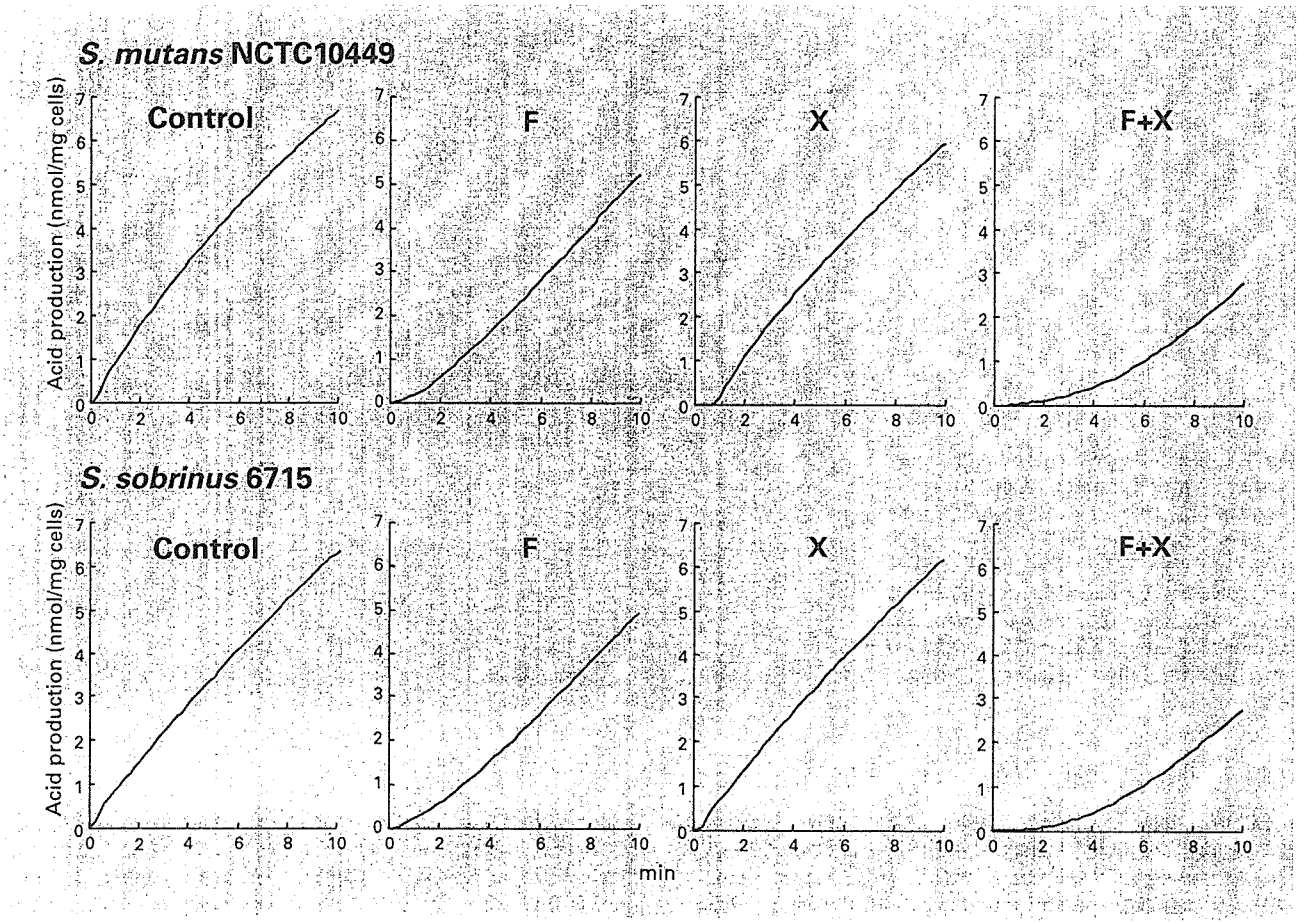

Fig. 2. Effect of fluoride and/or xylitol on the acid production curves of the cells of *S. mutans* NCTC 10449 and *S. sobrinus* 6715 after addition of 10 m*M* glucose at pH 7.0. F = 1.6 and 2.4 m*M* fluoride alone for *S. mutans* and *S. sobrinus*, respectively; X = 60 m*M* xylitol alone; F + X = fluoride plus xylitol. The data represent three independent experiments. The results were similar among three independent experiments.

was dose-dependent (fig. 1). At pH 7.0, 2.4 m*M* fluoride was required to reduce acid production by 50% or more, while only 0.35 m*M* fluoride had a similar effect at pH 5.5. Similar results were obtained in *S. sobrinus* 6715 (fig. 1).

The inhibition of acid production by fluoride was enhanced in the presence of xylitol. At pH 7.0, the acid production by *S. mutans* decreased by 21.7% in the presence of 1.6 m*M* fluoride alone and by 8.9% in the presence of 60 m*M* xylitol alone, while the acid production was decreased by 58.4% in the presence of a combination of 1.6 m*M* fluoride and 60 m*M* xylitol. The enhanced inhibition caused by the addition of xylitol was also observed at pH 5.5, with 16.3% inhibition in the presence of 0.3 m*M* fluoride alone and 80.1% inhibition by the combination of 0.3 m*M* fluoride plus 60 m*M* xylitol. A similar inhibition by the combination of fluoride and xylitol was observed in *S. sobrinus* 6715.

In the absence of fluoride or xylitol, the acid production by *S. mutans* NCTC 10449 started immediately after the addition of glucose and gradually slowed down (fig. 2, control). In the presence of xylitol alone (fig. 2, X), no acid production was detected for about 1 min after the addition of glucose. Once it started, the acid production pattern was similar to the control, though the acid production rate was slower. In the presence of fluoride (fig. 2, F) and the combination of fluoride and xylitol (fig. 2, F + X), the acid production was very low right after the addition

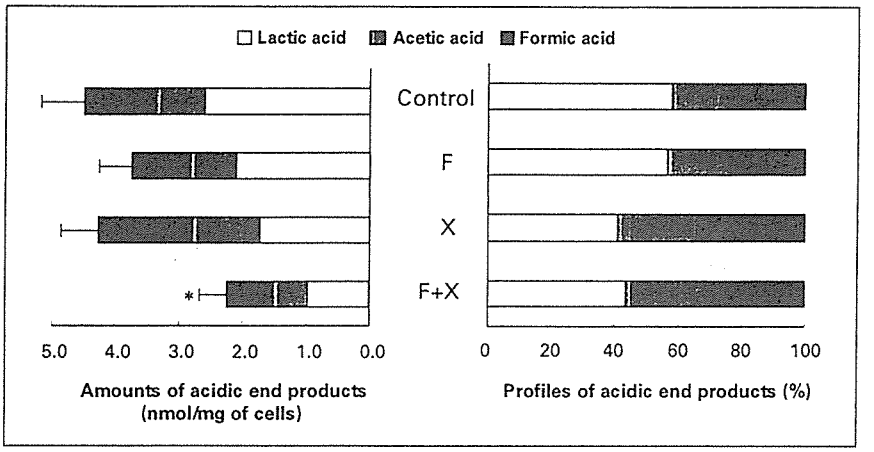

Fig. 3. Effect of fluoride and/or xylitol on the production of acidic end products from 10 m*M* glucose for 10 min by the cells of *S. mutans* NCTC 10449 at pH 7.0. Left = Amounts of acidic end products; right = profiles of acidic end products. F = 1.6 m*M* fluoride alone; X = 60 m*M* xylitol alone; F + X = fluoride plus xylitol. The data are the means of three independent experiments. Error bars indicate standard deviations from three independent experiments. * Significant difference from control ($p < 0.05$).

of glucose and increased gradually and became nearly constant by 10 min. Similar inhibitory effects of fluoride and xylitol were observed in *S. sobrinus* 6715.

Production of Carboxylic Acids from Glucose in the Presence of Fluoride and/or Xylitol

The effects of fluoride and xylitol on the formation of acidic end products from glucose and the profile of intracellular glycolytic intermediates were evaluated at a fluoride concentration of 1.6 m*M* and pH 7.0 using *S. mutans* NCTC 10449, where the combined effect of fluoride and xylitol was evident. The total amount of carboxylic acids (sum of lactic, formic and acetic acids) produced by *S. mutans* NCTC10449 at pH 7.0 for 10 min was 4.49 nmol/mg of cells in the control, while those in the presence of fluoride alone and xylitol alone were 3.75 and 4.27 nmol/mg of cells, respectively. In the presence of fluoride and xylitol combined, the total amount decreased to 2.23 nmol/mg of cells (fig. 3). These results showed that the combination of fluoride and xylitol was more effective in inhibiting the acid production than fluoride or xylitol alone, similar to the results of acid production rate obtained by pH stat (fig. 1). In addition, lactic acid as a proportion of the total amount of carboxylic acids decreased, while the proportion of formic and acetic acids increased in the presence of xylitol alone. A similar result was observed in the presence of a combination of fluoride and xylitol (fig. 3, right).

Profile of Intracellular Glycolytic Intermediates in the Presence of Fluoride and/or Xylitol

In the presence of fluoride alone, the levels of FBP and PYR were lower and the levels of 3PG and 2PG were higher than those in the control during glucose fermentation (fig. 4). In contrast, in the presence of xylitol alone, the levels of glycolytic intermediates except PYR were mostly lower. Moreover, in the presence of the combination of fluoride and xylitol, the levels of G6P through G3P were similar to those in the presence of xylitol alone, while the levels of 3PG through PYR were similar to those in the presence of fluoride alone (fig. 4).

At 0 min (just before glucose addition), the control cells had low levels of glycolytic intermediates, except 3PG, 2PG and PEP (the intermediates which comprise the 'PEP potential' for sugar uptake into cells [Thompson, 1979]). The levels of these 'PEP potential' intermediates (3PG, 2PG and PEP) at 0 min in the presence of fluoride alone were twice as high as those of the control, while the levels of those were negligible in the presence of xylitol alone. The level of 'PEP potential' in the combination of fluoride and xylitol was close to the control at 0 min, though PEP was not detected (fig. 4).

Discussion

Acid production from glucose by mutans streptococci was inhibited in the presence of fluoride or xylitol as previously reported [Kashket et al., 1977; Hamilton and Ellwood, 1978; Assev et al., 1983; Vadeboncoeur et al., 1983; Marsh et al., 1985; Bradshaw and Marsh, 1994]. In addition, this study has demonstrated that the inhibitory effect was enhanced by a combination of fluoride and xylitol. This is consistent with previous results for combined effects of fluoride and xylitol on the growth of mutans streptococci [Scheie et al., 1988; Rogers and Bert, 1992].

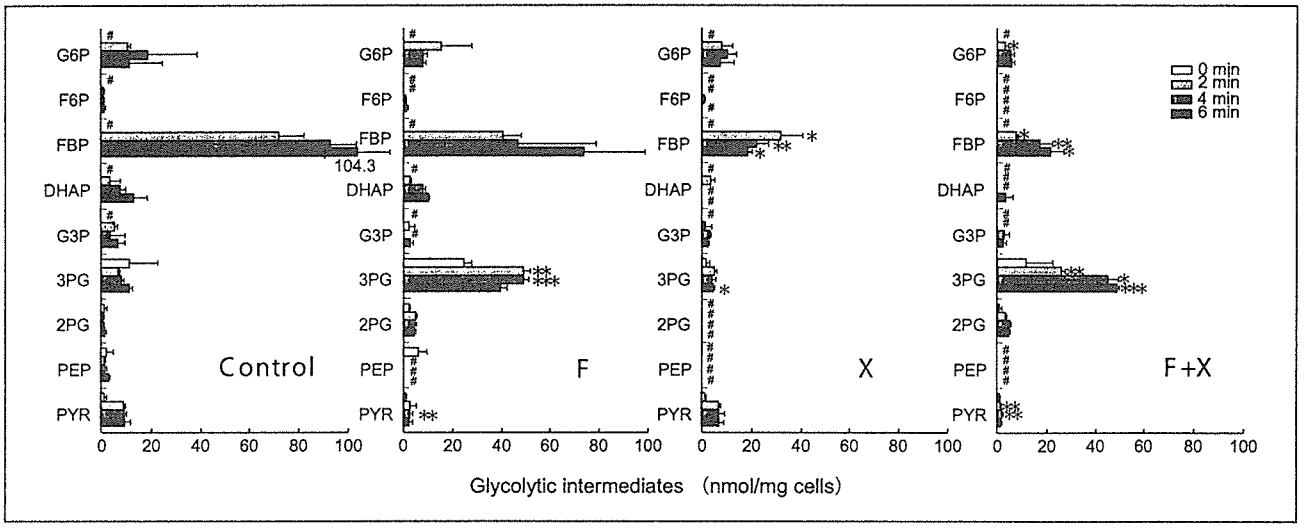

Fig. 4. Effect of fluoride and/or xylitol on the levels of glycolytic intermediates of the cells of *S. mutans* NCTC 10449 during glucose fermentation. The levels of glycolytic intermediates at 0, 2, 4, and 6 min after addition of 10 m*M* glucose to the cells *S. mutans* NCTC 10449 in the presence of 1.6 m*M* fluoride and/or 60 m*M* xylitol. F = 1.6 m*M* fluoride alone; X = 60 m*M* xylitol alone; F + X = fluoride plus xylitol. Error bars indicate standard deviations from three independent experiments. Significant difference from control (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). # = Not detected. The data are the means of three independent experiments.

Moreover, percent inhibition by combination of fluoride and xylitol was larger than the sum of percent inhibition by fluoride alone and percent inhibition by xylitol alone, indicating that the combined effect was synergistic (fig. 1, 2).

The observation that fluoride did not change the profile of end products while xylitol decreased the proportion of lactic acid implies a difference in inhibitory mechanism between xylitol and fluoride (fig. 3). This finding led us to the analysis of glycolytic intermediates to understand the biochemical mechanism for the synergistic effects of xylitol and fluoride.

The presence of fluoride alone increased the level of 2PG and 3PG in the lower part of glycolysis as reported previously [Hata et al., 1990]. This indicates that fluoride inhibited enolase (a glycolytic enzyme catalyzing the conversion of 2PG to PEP) and resulted in the accumulation of 3PG and 2PG and the decrease of PEP (fig. 4). In bacterial cells, PEP is a phosphoryl donor for sugar uptake by PEP-PTS [Schachtele and Mayo, 1973; Dills et al., 1980] and builds 'PEP potential' together with 3PG and 2PG, precursors of PEP. Although 3PG and 2PG levels were high in the presence of fluoride, the slow supply of PEP from 3PG and 2PG caused by the fluoride inhibition could decrease glucose uptake via PEP-PTS and slow down the entire glycolytic process [Kanapka and Hamilton, 1971; Bender et al., 1985] (fig. 2, 4).

In the presence of xylitol alone, in contrast, most glycolytic intermediates decreased as acid production was inhibited, suggesting the repression of sugar uptake by xylitol. In addition, the marked decrease of FBP as previously reported [Miyasawa et al., 2003] may be due to the inhibition by X5P of PGI and PFK catalyzing the conversion of G6P to FBP [Trahan, 1995]. Since FBP is an activator of lactate dehydrogenase, the decrease in FBP caused inactivation of lactate dehydrogenase and consequent reduction of lactic acid production (fig. 3) as previously reported [Miyasawa et al., 2003].

The combination of fluoride and xylitol increased the intracellular levels of 3PG and 2PG as observed in the presence of fluoride alone, while the other glycolytic intermediates decreased similarly to the presence of xylitol alone. These observations indicate that the simultaneous inhibition, e.g. the inhibition of enolase by fluoride and the inhibition of PGI, PFK and glucose uptake by xylitol, repressed the entire glycolytic pathway more efficiently.

These inhibitory effects seemed more efficient in the initial stage of acid production. During preincubation in the presence of fluoride alone, PEP could not be consumed and 3PG and 2PG were accumulated through flu-

oride inhibition of enolase (fig. 4). After addition of glucose, the 'PEP potential' could be used for glucose uptake, however, the enolase inhibition by fluoride continued to interfere with the conversion of 2PG into PEP and consequently slowed down the entire glycolytic flow (fig. 2). In the presence of xylitol alone, the level of 'PEP potential' was low due to the consumption of PEP (fig. 4) through the phosphorylation of xylitol to X5P by PEP-PTS [Assev and Rölla, 1984; Trahan et al., 1985; Trahan, 1995; Miyasawa et al., 2003]. As a result, the lack of 'PEP potential' delayed acid production from glucose for about 1 min after glucose addition (fig. 2). The combination of fluoride and xylitol was more effective. The combination caused both enolase inhibition and low 'PEP potential' (fig. 4), resulting in the prolonged delay of initiation of acid production (fig. 2).

The inhibitory effect of fluoride on dental plaque acidogenicity in vivo is still unclear since there are numerous clinical observations but very few appropriate methods for evaluating antimicrobial activity of fluoride in vivo [van Loveren, 2001]. Reduction of plaque acidogenicity after sucrose challenge has been reported in plaque from subjects who used fluoridated water or rinsed their mouths daily with 48 m*M* (912 ppm) sodium fluoride [Edgar et al., 1970; Geddes and McNee, 1982]. Moreover, Neff [1967] reported that when applied simultaneously with sucrose, 0.52 m*M* (10 ppm) sodium fluoride was sufficient to inhibit acid production of dental plaque. However, Giertsen et al. [1999] argued that mouth rinsing with 12 m*M* (228 ppm) sodium fluoride had no significant effect on the acidogenic potential of dental plaque. In experiments using a continuous culture of mixed oral bacteria [Rogers et al., 1991; Bradshaw and Marsh, 1994] and an artificial biofilm [Bradshaw et al., 2002] that mimic some aspects of the dental plaque micro-ecosystem, fluoride and xylitol as well as a fluoride-xylitol combination were able to reduce the acid production of the micro-ecosystem with a reduction of the number of mutans streptococci. These sophisticated studies support the possibility that the combination of fluoride and xylitol could reduce dental plaque acidogenicity in vivo, though a proper method for clinical application of fluoride and xylitol should be developed as soon as possible.

In conclusion, direct inhibition of glycolytic enzymes, enolase, PGI and PFK and the consequent inhibition on the supply of 'PEP potential' for sugar uptake appears to be the cause of the synergistic inhibition by combination of fluoride and xylitol on glycolysis by mutans streptococci. Attempts have been made to use both fluoride and xylitol together to enhance remineralization of tooth surfaces [Smits and Arends, 1985; Arends et al., 1990; Gaffar et al., 1998; Amaechi et al., 1998]. Besides remineralization, this study suggests that using fluoride and xylitol together can effectively inhibit the sugar metabolism of mutans streptococci and also that xylitol has the potential to enhance the inhibitory effects of low concentrations of fluoride.

Acknowledgments

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ORAL MICROBIOLOGY
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Difference in the xylitol sensitivity of acid production among *Streptococcus mutans* strains and the biochemical mechanism

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Miyasawa-Hori H, Aizawa S, Takahashi N. Difference in the xylitol sensitivity of acid production among Streptococcus mutans *strains and the biochemical mechanism. Oral Microbiol Immunol 2006: 21: 1–6.* © Blackwell Munksgaard, 2006.

Xylitol inhibits the glycolysis and growth of *Streptococcus mutans*, but to different degrees among strains. Thus, we studied the biochemical mechanism through which the inhibition varies, using *S. mutans* strains ATCC 31989, NCTN 10449, and NCIB 11723, which are highly sensitive, moderately sensitive, and resistant to xylitol, respectively, under strictly anaerobic conditions such as those found in deep layers of dental plaque. Xylitol (30 mM) decreased the rate of acid production from glucose (10 mM) in ATCC 31989, NCTC 10449, and NCIB 11723 by 86, 26, and 0%, respectively. The activities of the xylitol : phosphoenolpyruvate phosphotransferase system (PEP-PTS) relative to those of glucose : PEP-PTS were 120, 16, and 3%, respectively. These findings indicated that the higher the xylitol : PEP-PTS activity was and the more effectively xylitol decreased glucose : PEP-PTS activity, the more sensitive the strain was to xylitol. In ATCC 31989 and NCTC 10449, intracellular accumulation of xylitol 5-phosphate and decreases of fructose 1,6-bisphosphate and glucose 6-phosphate were observed. Furthermore, in the presence of xylitol (30 mM), glucose : PEP-PTS activities decreased by 34, 17, and 0%, respectively. These results suggest that the following inhibitory mechanisms are active in the xylitol-sensitive mutans streptococci: direct inhibition of glycolytic enzymes by xylitol 5-phosphate derived from xylitol : PEP-PTS and, possibly, indirect inhibition through competition for the phosphoryl donor, HPr-P, between glucose and xylitol : PEP-PTS.

Key words: acid production; phosphoenolpyruvate-sugar phosphotransferase system; *Streptococcus mutans*; xylitol

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Xylitol is widely used as a noncariogenic sugar substitute because it is not fermented by oral bacteria (6). Xylitol has been reported to inhibit the growth of mutans streptococci in the presence of glucose, galactose, mannose, lactose, maltose, sucrose, sorbitol or mannitol as a carbon source *in vitro* (1, 5, 7, 8, 16, 24, 25), and the acid production from glucose by resting cells of *S. mutans* (7, 13, 25). Xylitol is also known to selectively inhibit the growth of *S. mutans* in mixed culture using a chemostat (3, 15).

The major route of sugar transport by microorganisms is via the phosphoenolpyruvate phosphotransferase system (PEP-PTS). Two sugar-nonspecific proteins, enzyme-I and histidine-containing phosphocarrier protein (HPr), and a sugar-specific protein, enzyme-II are required for PEP-PTSs. PEP phosphorylates enzyme-I to phospho-enzyme-I, which in turn transfers the phosphoryl group to HPr. In many cases, phospho-HPr (HPr-P) generated from phospho-enzyme-I, transfers the phosphoryl group directly to enzyme-II, which in turn phosphorylates incoming sugar (10, 14).

Bacterial cells are thought to incorporate xylitol as xylitol 5-phosphate through xylitol : PEP-PTS and the xylitol 5-phosphate inhibits the enzyme activity of sugar metabolism, resulting in the inhibition of both bacterial growth and acid production (18). In addition, the futile cycle, in which xylitol 5-phosphate is dephosphorylated to xylitol with waste of PEP potential, can also retard the growth of *S. mutans* (18).

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However, some strains are xylitol sensitive whereas others are resistant (19), and the degree of inhibition varies among strains (25). Thus, we studied the biochemical mechanism of the variable inhibition, using three strains of *S. mutans* previously characterized as highly sensitive, moderately sensitive, and resistant to xylitol under strictly anaerobic conditions such as those found in deep layers of dental plaque.

Material and methods

Bacterial strains and growth conditions

We used the following strains of *S. mutans*: NCTC 10449, ATCC 31989, and NCIB 11723. *S. mutans* NCTC 10449 was a gift as a xylitol-sensitive strain from Prof. L. Trahan (Universite Laval, Québec, Canada) (20). Each strain was inoculated into a complex medium containing 1.7% tryptone (Difco Laboratories, Detroit, MI), 0.3% yeast extract (Difco), 85 mM NaCl, and 11 mM glucose as described (25) under strictly anaerobic conditions in an anaerobic chamber (N_2, 80%; H_2, 10%; CO_2, 10%, NHC-type, Hirasawa Works, Tokyo, Japan) and incubated at 35°C overnight. Cell cultures were transferred into the same complex medium and precultured overnight at 35°C. The cell cultures were again transferred into the same complex medium (5% inoculum size) and grown at 35°C. The bacterial cells were harvested by centrifugation (7000 × *g* for 15 min at 4°C) at an early logarithmic phase of growth (optical density at 660 nm [OD_{660}] ≈ 0.3) under anaerobic conditions as described previously (17). Bacterial purity was regularly confirmed by culturing on blood agar plates.

Acid production from glucose in the presence of xylitol

The following experiments were conducted in a different type of anaerobic chamber (N_2, 90%; H_2, 10%, NH-type, Hirasawa Works). Cells were washed twice with cold 2 mM potassium phosphate buffer (pH 7.0) containing 150 mM KCl and 5 mM $MgCl_2$, and suspended in the same buffer. The optical density of the cell suspension at 660 nm was adjusted to 3.5 (1.9 mg of cells [dry weight] per ml).

The cell suspensions were agitated with a magnetic stirrer at 35°C. The reaction was started by adding a mixture of 10 mM glucose and 0 or 30 mM xylitol to the cell suspensions. The rate of acid production by the cells was monitored at pH 7.0 using an automatic pH titrator (model AUT-211S, Toa Electronics Ltd, Kobe, Japan) with 50 mM KOH. The rate of acid production at 2 min after adding glucose or the glucose-xylitol mixture was calculated as μmol of protons per min per mg dry weight of cells.

Before and after the incubation for 10 min, cell suspensions (0.9 ml) were sampled and mixed immediately with 0.1 ml of 6 N perchloric acid. The mixtures were filtered (pore size 0.20 μm, polypropylene; ADVANTEC, Toyo Roshi Ltd, Tokyo, Japan) and cell-free filtrates were diluted with 0.2 N hydrochloric acid and stored at 4°C for the assay of acidic end products.

Analysis of acidic end products

Amounts of acidic end products, lactic, acetic, formic and pyruvic acids were quantified using a carboxylic acid analyzer (model EYELA S-3000X; Tokyo Rikakikai Co., Ltd, Tokyo, Japan) in stored cell-free filtrates, as described previously (17).

PEP-PTS activities for glucose, xylitol and fructose (glucose, xylitol: and fructose: PEP-PTS activities)

The PEP-PTS activities were estimated by a modification of the method of Kornberg & Reeves (9) as described previously (13). Cells were harvested, washed twice as described above and stored at −20°C. After thawing, the cells were suspended in 2 mM potassium phosphate buffer (pH 7.0) containing 150 mM KCl and 5 mM $MgCl_2$ (OD_{660} ≈ 5.0). Toluene was added at a final concentration of 1% to the cell suspension, and mixed vigorously for 1 min. After centrifugation (1200 × *g* for 3 min), the cells were suspended in the same buffer (OD_{660} ≈ 50). The PEP-PTS activities for glucose, xylitol or fructose at pH 7.0 were estimated as a decrease of reduced nicotinamide adenine dinucleotide (NADH) in reaction mixtures containing 0.1 mM NADH, 53 μg of cells (dry weight)/ml, 1 mM phosphoenolpyruvate, 11 U/ml lactate dehydrogenase (EC 1.1.1.27, rabbit muscle; Roche Diagnostics, Indianapolis, IN), 100 mM potassium phosphate buffer (pH 7.0) at 35°C. The reaction was started by adding 5, 10, 30, 60 or 120 mM glucose, xylitol or fructose. The decrease of NADH was monitored using a dual wavelength spectrophotometer (model 557; Hitachi Ltd, Tokyo, Japan) at 340 nm.

Inhibition of glucose : PEP-PTS activity in the presence of xylitol

Glucose : PEP-PTS activity in the presence of xylitol was also determined. Toluene-treated cells as described above were suspended in a reaction mixture containing 1 mM NADP, 53 μg of cells [dry weight]/ml, 1 mM phosphoenolpyruvate, 3.5 U/ml glucose 6-phosphate dehydrogenase (EC 1.1.1.49, yeast; Roche Diagnostics) and 100 mM potassium phosphate buffer (pH 7.0) at 35°C. The reaction was started by adding 10 mM glucose and 0, 10, 30, 60 or 120 mM xylitol to the cell suspensions. The increase of NADPH was monitored spectrophotometrically at 340 nm.

Assays of glycolytic intermediates and xylitol 5-phosphate

Cell suspensions were reacted with 10 mM glucose containing 0 or 30 mM xylitol as described above for the experiment of acid production. After the incubation for 2 min, the cells were collected by passing the reaction mixture through a membrane filter (pore size 0.45 μm, polyethersulfone; Acrodisc, Pall Gelman Laboratory, Ann Arbor, MI). Glycolytic intermediates and xylitol 5-phosphate in the cells were immediately extracted in 0.6 N perchloric acid, and neutralized with 5 M K_2CO_3 in air. The neutralized extracts were stored at 4°C for subsequent assays of the glycolytic intermediates and xylitol 5-phosphate, and at −20°C for 3-phosphoglycerate assays.

The glycolytic intermediates in the cell extracts were enzymatically determined at 35°C by a modification of the enzymatic method of Minakami et al. (12). Xylitol 5-phosphate was estimated as described previously (13). The assay mixture for xylitol 5-phosphate contained 1.1 mM NAD, 5 mM $MgCl_2$, 0.1 mM EDTA, and the extracts in 50 mM Tris-HCl buffer (pH 8.5) at 35°C. The reaction was started by the addition of 4.2 U/ml polyol dehydrogenase (EC 1.1.1.14, sorbitol dehydrogenase, sheep liver; Roche Diagnostics) and 50 U/ml alkaline phosphatase (EC 3.1.3.1, calf intestine; Roche Diagnostics). The increase of NADH was monitored spectrophotometrically at 340 nm.

Statistical methods

Differences in rates of acid production, rates of glucose : PEP-PTS activities with xylitol and in profiles of glycolytic intermediates were analyzed by the Mann–Whitney *U*-test. Differences in amounts of

Table 1. Relative rate of acid production and the formation of acidic end products from 10 mM glucose (G) and 10 mM glucose plus 30 mM xylitol (G +X).

S. mutans strain	Substrate	Relative rate of acid production	Acidic end products		
			Lactate	Acetate	Formate
ATCC 31989	G	100 [a]	1.44 ± 0.28[b]	0.34 ± 0.09	0.34 ± 0.06
	G +X	14 ± 1[#]	0.07 ± 0.01*	0.25 ± 0.04	0.27 ± 0.04
NCTC 10449	G	100	1.07 ± 0.07	0.92 ± 0.04	0.90 ± 0.05
	G +X	74 ± 9[#]	0.42 ± 0.03	1.00 ± 0.07	1.11 ± 0.13
NCIB 11723	G	100	1.72 ± 0.21	0.67 ± 0.08	0.75 ± 0.10
	G +X	102 ± 6	1.62 ± 0.22	0.69 ± 0.09	0.78 ± 0.12

[a] Relative rate of acid production (mean ± standard deviation, %) obtained from six independent experiments. Significant difference between relative rates of acid production in the presence and absence of xylitol: #$P < 0.01$.

[b] Amounts of acidic end products (mean ± standard deviation, μmol/mg cells) obtained from three independent experiments. Significant difference between amounts of acidic end products in the presence and absence of xylitol: *$P < 0.05$.

acidic end products were analyzed by the Dunn test.

Results

Inhibitory effect of xylitol on acid production

Xylitol significantly inhibited the acid production from glucose of *S. mutans* ATCC 31989 and NCTC 10449. In the presence of 30 mM xylitol, the acid production rates of ATCC 31989 and NCTC 10449 were decreased by 86 ± 1% ($P < 0.01$) and 26 ± 9% ($P < 0.01$), respectively, whereas that of NCIB 11723 was not inhibited (Table 1).

The total amounts of acidic end products generated by ATCC 31989 and NCTC 10449 cells decreased in the presence of xylitol. The reduction of lactic acid was remarkable and it was significant in ATCC 31989 ($P < 0.05$), resulting in an end product shift to formate-acetate-dominant (Table 1). Xylitol had no effect on NCIB11723.

Glucose, xylitol and fructose : PEP-PTS activities

All *S. mutans* strains had PEP-PTS activities for glucose, xylitol, and fructose (Fig. 1). In ATCC 31989, fructose : PEP-PTS activity at 5–120 mM fructose and xylitol : PEP-PTS activity at 30–120 mM xylitol were higher than glucose : PEP-PTS activity. In particular, xylitol : PEP-PTS activity at 30 mM xylitol was 120 ± 14% of glucose : PEP-PTS activity at 10 mM glucose. In NCTC 10449, both fructose and xylitol : PEP-PTS activities were lower than glucose : PEP-PTS activity. Xylitol : PEP-PTS activity at 30 mM xylitol was 16 ± 6% of glucose : PEP-PTS activity at 10 mM glucose. Both fructose and xylitol : PEP-PTS activities were low in NCIB 11723, and xylitol : PEP-PTS at 30 mM xylitol activity was only 3 ± 1% of glucose : PEP-PTS activity at 10 mM glucose.

Decrease in glucose : PEP-PTS activity in the presence of xylitol

In the presence of added xylitol, glucose : PEP-PTS activities of ATCC 31989 and NCTC 10449 were decreased (Fig. 2). The presence of 30 mM xylitol decreased glucose : PEP-PTS activity to 67 ± 6 and 83 ± 5%, respectively. As the xylitol concentration increased, the decrease became larger and statistically significant over 60 mM xylitol. However, little inhibition was observed in NCIB 11723.

Effect of xylitol on the profile of glycolytic intermediates during glucose metabolism

When metabolizing glucose only, all of the *S. mutans* strains had large amounts of fructose 1,6-bisphosphate, but the profiles of glycolytic intermediates downstream of

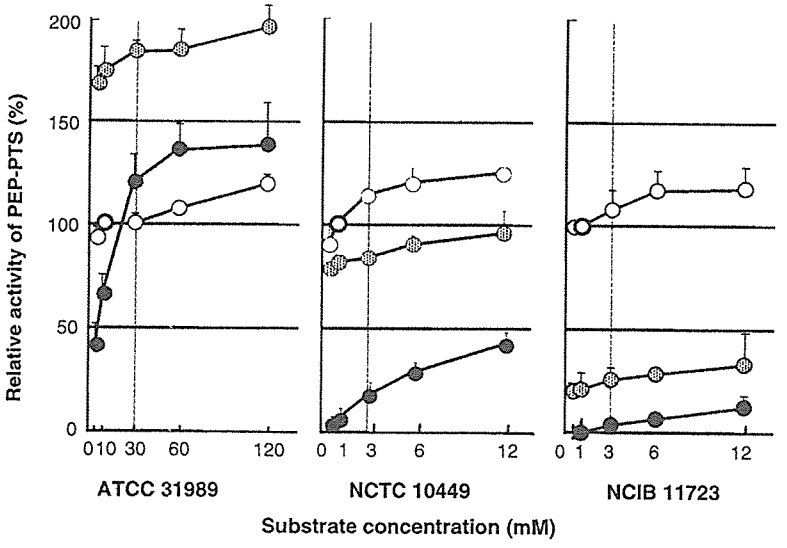

Fig. 1. PEP-PTS activities for glucose (○), xylitol (•) and fructose (shaded circle) of *Streptococcus mutans* ATCC 31989, NCTC 10449, and NCIB 11723. Vertical bars indicate standard deviations from three independent experiments. PEP-PTS activity for 10 mM glucose was regarded as 100%.

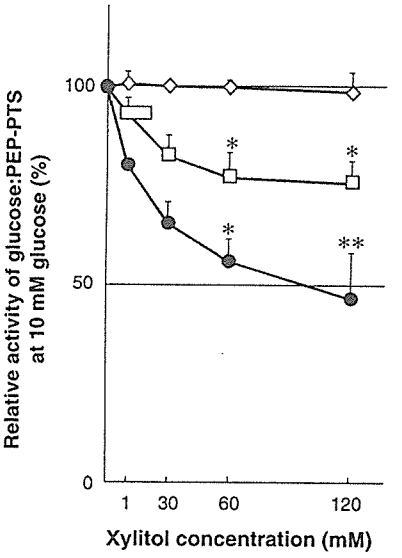

Fig. 2. PEP-PTS activities for 10 mM glucose of *Streptococcus mutans* in the presence of xylitol. ATCC 31989 (•), NCTC 10449 (□) and NCIB 11723 (◇). Significant difference between the PEP-PTS activities in the presence and absence of xylitol: *$P < 0.05$, **$P < 0.01$. Vertical bars indicate standard deviations from three independent experiments.

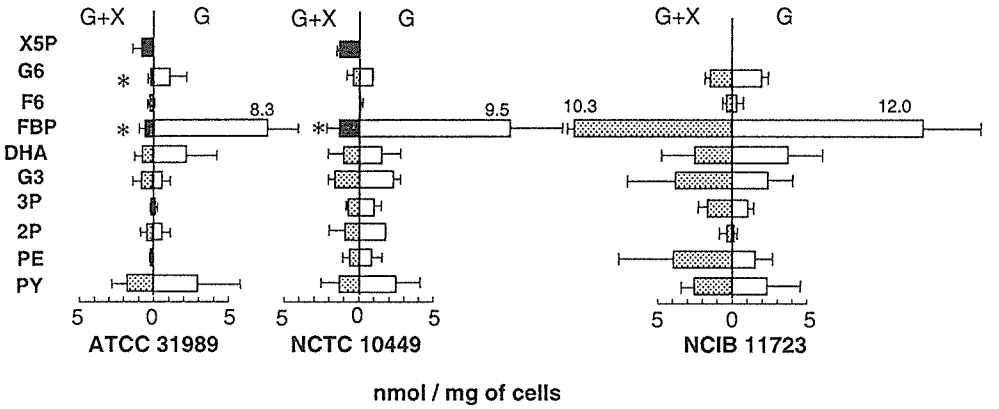

Fig. 3. Glycolytic intermediates and xylitol 5-phosphate of *S. mutans* ATCC 31989, NCTC 10449, and NCIB 11723, 2 min after adding glucose (10 mM) or a mixture of glucose (10 mM) and xylitol (30 mM). Glycolytic intermediates (G, □) in the absence of xylitol. Glycolytic intermediate (G +X) and xylitol 5-phosphate (X) in the presence of xylitol. G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate. Significant difference between intermediate levels in the presence and absence of xylitol: $*P = 0.05$. Horizontal bars indicate standard deviations from four independent experiments.

dihydroxyacetone phosphate were slightly different among strains (Fig. 3).

When glucose was being metabolized by the ATCC 31989 and NCTC 10449 strains in the presence of xylitol, xylitol 5-phosphate accumulated and the amounts of glycolytic intermediates decreased, particularly those of fructose 1,6-bisphosphate ($P = 0.05$) in ATCC 31989 and NCTC 10449 and glucose 6-phosphate (G6P) ($P = 0.05$) in ATCC 31989. However, such inhibition was not evident in NCIB 11723.

Discussion

As previously reported (13, 25), xylitol inhibited the acid production of *S. mutans* strains and decreased the lactate production in strains ATCC 31989 and NCTC 10449 (Table 1), but the degree of these inhibitions varied between strains. On the other hand, in strain NCIB 11723, xylitol did not inhibit acid production or shift the end product profile.

Trahan (18) proposed an inhibitory mechanism of xylitol in which *S. mutans* transports xylitol as xylitol 5-phosphate through the activity of xylitol : PEP-PTS and, consequently, the xylitol 5-phosphate inhibits phosphoglucose isomerase and phosphofructokinase, the glycolytic enzymes for G6P conversion to fructose 1,6-bisphosphate, resulting in a decrease in intracellular levels of fructose 1,6-bisphosphate and the entire glycolytic rate. Miyasawa et al. (13) and Maehara et al. (11) then confirmed this notion, and found the decrease in lactate production was due to the decrease in fructose 1,6-bisphosphate, an absolute activator of streptococcal lactate dehydrogenase. The present study found that higher xylitol : PEP-PTS activity indicated more inhibition of acid production by xylitol (Table 1 and Fig. 1). This observation supports the notion that xylitol 5-phosphate produced by xylitol : PEP-PTS activity is responsible for glycolytic inhibition and suggests that *S. mutans* strains with higher xylitol : PEP-PTS activity are more sensitive to xylitol inhibition. It has been proposed that xylitol is transported via a constitutive fructose : PEP-PES and that xylitol : PEP-PTS activity appears as part of the constitutive fructose : PEP-PTS activity (18, 19, 22). We found here that high fructose : PEP-PTS activities in *S. mutans* were accompanied by high xylitol : PEP-PTS activities (Fig. 1), supporting this notion. Furthermore, it is reported that the *fxpC* gene of the constitutive fructose : PEP-PTS was located in the genomes of xylitol-sensitive streptococci and the *fxpC*-defective mutant was resistant to xylitol (2), although no information is available about *fxpC* gene in the strains used in our study.

Analyses of metabolic intermediates revealed that the xylitol-sensitive strains ATCC 31989 and NCTC 10449 accumulated xylitol 5-phosphate and decreased fructose 1,6-bisphosphate in the presence of xylitol (Fig. 3). These results support the notion that xylitol inhibits the glycolytic enzymes required for G6P conversion to fructose 1,6-bisphosphate. Despite the powerful inhibitory effect of xylitol (Table 1) and high xylitol : PEP-PTS activity (Fig. 1), the accumulation of xylitol 5-phosphate in ATCC 31989 seemed to be smaller than that in NCTC 10449. It is suggested that the glycolytic enzymes of ATCC 31989 are more sensitive to xylitol and a small amount of xylitol 5-phosphate is enough to inhibit the entire glycolytic metabolism.

Not only fructose 1,6-bisphosphate but also G6P significantly decreased in a highly xylitol-sensitive strain ATCC 31989 (Fig. 3), indicating that xylitol itself can inhibit the glucose uptake system (e.g. glucose : PEP-PTS) and result in a decrease in intracellular G6P. This was confirmed by the observation that the presence of xylitol decreased the glucose : PEP-PTS activity in xylitol-sensitive strains, ATCC 31989 and NCTC 10449 (Fig. 2). This could be due to competition for the phosphoryl donor, HPr-P, between the glucose and the xylitol : PEP-PTSs. In the ATCC 31989 strain, with high xylitol : PEP-PTS activity and powerful xylitol inhibition of glucose : PEP-PTS activity, HPr-P could phosphorylate xylitol to xylitol 5-phosphate efficiently and result in a direct inhibition by xylitol 5-phosphate on glycolytic enzymes and an indirect inhibition on glucose phosphorylation by HPr-P.

NCIB 11723 isolated from human dental plaque by Carlsson (4) has natural xylitol resistance like other xylitol-resistant strains isolated from xylitol consumers (21, 23). It has been suggested that the xylitol resistance is due to the absence of a constitutive fructose : PEP-PTS by which xylitol is also incorporated, thus preventing xylitol-resistant strains from incorporating xylitol (19, 21). In the present study, NCIB 11723 had little xylitol : PEP-PTS

activity (Fig. 1) and did not accumulate xylitol 5-phosphate (Fig. 3) in the presence of a low concentration of xylitol, supporting this notion. As the xylitol concentration increased, however, xylitol : PEP-PTS activities of NCIB 11723 appeared (Fig. 1). In the presence of 120 mM xylitol, the xylitol : PEP-PTS activity compared with that of glucose : PEP-PTS reached 12 ± 4%. However, 120 mM xylitol did not inhibit glucose : PEP-PTS activity (Fig. 2) and negligibly inhibited acid production from glucose (data not shown). These findings indicate that xylitol has less affinity for HPr-P than glucose in NCIB 11723. Thus, the strain cannot incorporate xylitol as xylitol 5-phosphate in the presence of both xylitol and glucose.

In conclusion, xylitol sensitivity varies among *S. mutans* strains: the higher the xylitol : PEP-PTS activity, and the more effectively xylitol decreases glucose : PEP-PTS activity, the more sensitive the strain is to xylitol. Xylitol has two inhibitory mechanisms:

- direct inhibition of glycolytic enzymes by xylitol 5-phosphate derived from xylitol : PEP-PTS;
- possibly, indirect inhibition of sugar uptake through competition for the phosphoryl donor, HPr-P, between the glucose and the xylitol : PEP-PTS.

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Food antigen causes T_H2-dependent enteropathy followed by tissue repair in T-cell receptor transgenic mice

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Background: Clarification of the mechanisms underlying the development of food-sensitive intestinal inflammation will provide an important clue to combating food allergies.
Objective: To establish a model of intestinal inflammation caused by oral administration of antigen without additional treatments, we focused on the ovalbumin (OVA) 23-3 T-cell receptor transgenic mouse, which had been reported to have high serum antigen-specific IgE responses to the feeding of an egg white diet.
Methods: Changes in body weight of mice fed an egg white diet were monitored throughout the 28-day experimental period. After the 28-day feeding, intestinal tissues were harvested for histologic examination. Endogenous production of cytokines and histamine in the jejunum, and production of cytokines secreted by OVA-specific $CD4^+$ T cells purified from mesenteric lymph nodes, were analyzed.
Results: Egg white diet–fed OVA23-3 mice developed weight loss and inflammation with villous atrophy and goblet cell hyperplasia, especially in the jejunum. A further characteristic feature was evidence of weight recovery and tissue repair. Jejunal inflammation was also observed in egg white diet–fed recombination activating gene (RAG)-2–deficient OVA23-3 mice. In addition, tissue sections revealed significant infiltration of specific IgE-positive cells and IgE-positive degranulating mast cells. Higher levels of IL-4 and significant levels of histamine were detected in the tissues. In the supernatant of OVA-stimulated T cells, IL-10 levels were also markedly elevated.
Conclusion: We report that high-dose and continuous intake of primitive OVA alone induces enteropathy containing regions under repair in OVA23-3 mice. Antigen-specific T cells and inflammatory cells primed by T_H2 responses play important roles in regulation of development and improvement of the disease.
Clinical implications: Long-term antigen intake causes T_H2-dependent and food-sensitive enteropathy followed by tissue repair. (J Allergy Clin Immunol 2006;117:1125-32.)

Key words: *T-cell receptor transgenic mouse, food-sensitive intestinal inflammation, ovalbumin, food antigen intake, antigen-specific T cells, IgE, mast cell, T_H2, histamine, tissue repair*

Several animal studies have been performed to clarify mechanisms underlying gastrointestinal hypersensitivities or allergies caused by food-derived antigen and to enhance progress toward diagnostic and/or protective measures against them.[1,2] In mouse models of food-sensitive intestinal inflammation, there are numerous examples of IgE-independent[3-7] and IgE-dependent models[8-10] but few of mixed models. Until now, almost all models have been established by using adjuvant-associated antigens, injection of drugs concurrent with dietary antigen, or microbial flora–associated antigen to promote systemic or local sensitization initially and to overcome antigen-induced oral tolerance.[2] However, these procedures do not clarify how the intestinal immune system regulates the induction of tolerance or of food-sensitive intestinal inflammation.

Our previous studies of food allergy prompted us to develop a mouse model of intestinal inflammation.[11,12] We focused on the transgenic mouse with a T-cell receptor (TCR) recognizing ovalbumin (OVA) residues 323-339[13] as a means of amplifying immunologic reactions to orally administered antigens without using adjuvant or other agents, because the mouse has been reported to have specific-IgE responses to the 28-day oral feeding with an egg white (EW) diet.[14] We have noticed that these mice had a trend toward loose feces after commencing the EW diet. Thus, we analyzed the histology of their mucosal and immunologic tissues and succeeded in developing a novel and unique mouse model.

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Abbreviations used
AB: Alcian blue
BALB-mice: BALB/c mice
CN: Casein
CNB: Casein diet–fed BALB/c mice
DFS: Direct fast scarlet
EW: Egg white
EWB: Egg white diet–fed BALB/c mice
EWR: Egg white diet–fed RAG-2–deficient BALB/c mice
FITC: Fluorescein isothiocyanate
MLN: Mesenteric lymph nodes
OVA: Ovalbumin
PAS: Periodic acid-Schiff
TB: Toluidine blue
TCR: T-cell receptor
Tg-mice: Ovalbumin 23-3 mice

METHODS

Animals

Ovalbumin 23-3 mice transgenic for OVA323-339–specific and I-A^d–restricted TCR-αβ (Vα3/Vβ15) on a BALB/c genetic background were kindly provided by S. Habu (Tokai University School of Medicine).[13] These mice were crossed with recombination activating gene (RAG)-2–deficient BALB/c-mice (BALB-mice) to establish RAG-2–deficient OVA23-3 mice (Tg-mice). Animals were maintained in the Hoshino Animal Center (Chiba, Japan). Male mice were used because female mice did not show clear weight changes. Male BALB-mice were purchased from Clea Japan Inc (Tokyo, Japan). They were housed under specific pathogen-free conditions. Guidelines formulated by the University of Tokyo were followed for the care and use of animals.

Ovalbumin administration

Seven-week-old male Tg-mice, RAG-2–deficient BALB-mice, RAG-2–deficient Tg-mice, and BALB-mice were divided into experimental groups as indicated both in figures and figure legends and administered for 28 days with a range of experimental diets: CE2-diet (Clea Japan Inc), EW diet, OVA diet, or casein (CN) diet (Funabashi Farm, Funabashi, Japan). The composition of experimental diets is indicated in the Online Repository at www.jacionline.org. By feeding EW diet and OVA diet, it is calculated that a mouse consumes about 250 mg OVA per day. During the experimental period, the weight of each mouse was measured every 4 or 7 days. After the 28-day feeding, tissues were taken from each mouse for further analysis. Sera were obtained before and after feeding and stored at −80°C. Concentrations of serum total IgE and OVA-specific IgE, IgG_1, and IgG_{2a} were analyzed by ELISA and estimated as described previously.[14]

Histologic analysis

Longitudinal sections of intestinal tissue (3 cm) were taken from the duodenum, the jejunum (11 cm distal to duodenum), the ileum (the superior part of cecum), and the colon (proximal and distal), respectively. Tissues were opened longitudinally, fixed in 10%, formalin and embedded in paraffin. Sections 3 or 5 μm thick were prepared and stained with hematoxylin and eosin for morphologic analysis, direct fast scarlet (DFS) to analyze eosinophils, periodic acid-Schiff (PAS)–Alcian blue (AB) for visualization of goblet cells, and Toluidine blue (TB) to detect mast cells. Parameters and numbers of mice used for quantification of intestinal tissue inflammation are indicated in the Online Repository at www.jacionline.org. For analysis of inflammation by scanning electron microscopy, jejunal sections were fixed in 2.5% glutaraldehyde and coated with gold. Electron micrographs of these specimens were scanned by using an S-3500N scanning electron microscope (Hitachi, Tokyo, Japan) and stored for subsequent observation and analysis.

Immunohistochemical staining

Jejunum sections, 1 cm in length and 4 cm distal to duodenum, were mounted in optimal cutting temperature compound (Tissue-tek; Sakura, Tokyo, Japan). Cryosections (6 μm thick) were fixed with acetone at −20°C. After rehydration with PBS, sections were blocked overnight with antimouse CD16/CD32 Ab (2.4G2; Pharmingen, San Diego, Calif) at 4°C and stained overnight with fluorescein isothiocyanate (FITC)–conjugated rat antimouse IgE (R35-72; Pharmingen) or biotinylated rat antimouse IgE (R35-72; Pharmingen) at 4°C. Biotinylated sections were incubated with streptavidin–horseradish peroxidase (ZYMED, South San Francisco, Calif) followed by DAB (Sigma, St Louis, Mo). IgE-positive mast cells were detected by further counterstaining with TB. To detect ovalbumin-specific IgE, FITC-labeled sections were further stained with biotinylated OVA followed by streptavidin–Texas red (Roche, Tokyo, Japan).

Histamine and cytokine levels in whole gut tissue homogenates

Four-centimeter sections of the jejunum immediately distal to the duodenum were collected and processed according to the method of van Halteren et al.[15] Homogenates were centrifuged and supernatants used for determination of histamine levels by HPLC (Post-Colum Reaction Systems; Waters Co, Milford, Mass).[16] To study the endogenous production of IFN-γ, IL-4, and TNF-α, 6-cm sections of the jejunum located 5 cm distal to the duodenum were collected and processed. Protein lysates of the jejunum were prepared with a cocktail of proteinase inhibitors and centrifuged. Supernatants were collected and concentrations of IL-4 and IFN-γ assessed by ELISA as described previously.[14] The OptEIA ELISA Set was used for TNF-α analysis according to the manufacturer's instructions (Pharmingen). Histamine and cytokine contents were standardized to the weight of jejunum tissue in each sample.

Cell culture and cytokine measurements

Mesenteric lymph node (MLN) lymphocytes isolated from each mouse fed with the CN diet or EW diet were pooled for each experimental group. MLN $CD4^+$ T cells were positively identified by using anti-CD4 mAb conjugated to immunomagnetic beads and selected using a MACS cell separation system (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer's protocol. The proportion of MLN $CD4^+$ T cells was more than 94% in each analysis. The MLN $CD4^+$ T cells (1×10^5 cells/well) were added to 96-well flat-bottom plates and cultured in the absence or presence of OVA (1 mg/mL) with mitomycin-treated splenocytes as antigen-presenting cells (4×10^5 cells/well) in complete RPMI 1640. Supernatants were collected 48 hours later and cytokine concentrations measured by ELISA (IFN-γ, IL-4, IL-2, and IL-5). The OptEIA ELISA Set was used for TNF-α and IL-10 analysis as described.

Statistical analysis

Results are presented as the means ± SDs. Weight change was analyzed by using the Fischer's Protect Least Significant Difference test of multiple comparisons. The Mann-Whitney *U* test was used for nonparametric analysis of levels of serum antibodies, histamine or

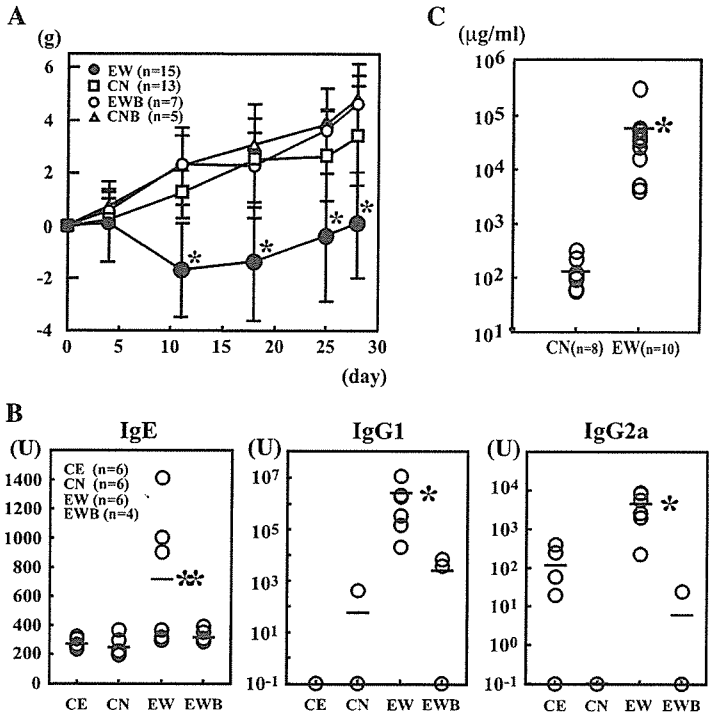

FIG 1. A, Weight loss in EW diet–fed Tg-mice. (*$P < .05$). **B,** Increase in serum OVA-specific antibodies in EW diet–fed Tg-mice. These data represent 4 independent experiments containing 3 to 6 mice in each group. **C,** Increase in total IgE in EW diet–fed Tg-mice. In *B and C,* ○ and bars indicate antibody titers for each mouse and the means, respectively. (*$P < .01$).

cytokine production, and the numbers of inflammatory cells in the intestine. Differences were considered statistically significant for values of $P < .05$.

RESULTS

Feeding of EW diet induces wasting diseases and hyperproduction of serum antibody in Tg-mice

Feeding Tg-mice with the EW diet caused loose feces (see this article's Fig E1 in the Online Repository at www.jacionline.org). Body weight changes from day 0 revealed that EW diet–fed Tg-mice (Fig 1, *A*: EW) failed to thrive from day 4, whereas from day 10 through day 28, weight loss was significant compared with control groups showing normal growth. However, body weights showed a tendency to recover after the initial loss. Control groups were composed of CE2 diet–fed Tg-mice (data not shown), CN diet–fed Tg-mice (Fig 1, *A*: CN), EW diet–fed BALB-mice (Fig 1, *A*: EWB), and CN diet–fed BALB-mice (Fig 1, *A*: CNB), respectively. There were no significant differences in volumes of experimental diet consumed by mice in each group (data not shown).

As shown in Fig 1, *B*, OVA-specific IgE was detected in some (but not all) EW diet–fed Tg-mice (Fig 1, *B*: EW). Extensive induction of OVA-specific IgG_1 and high levels of OVA-specific IgG_{2a} were detected in EW diet–fed Tg-mice compared with control groups (Fig 1, *B*: CN, EWB, and CE, CE2-diet-fed Tg-mice). All of the EW diet–fed Tg-mice (Fig 1, *C*: EW) showed significant total IgE responses compared with control group (Fig 1, *C*: CN).

Villous morphologic changes in the jejunum of EW diet–fed Tg-mice

Analysis of intestinal histology indicated that inflammation associated with morphologic changes was particularly noticeable in sections of the jejunum from EW diet–fed Tg-mice (Fig 2, *A*: EW). Changes to the jejunal tissue consisted of a thickened muscular layer, crypt elongation, and villous atrophy, associated with goblet cell hyperplasia and increased numbers of Paneth cells. However, the severity of inflammation varied from animal to animal in the 28-day feedings: inflammatory regions were identified on all sequential segments in some Tg-mice (Fig 2, *A*: EW-Jejunum; and Fig 2, *B*: EW1), and in discrete areas of sequential segments in others (partial villous atrophy, Fig 2, *B*: EW2). The severity of inflammation showing partial villous atrophy was moderate in general. A characteristic histologic feature in this inflammatory process is villous blunting of the mucosa. Villous blunting without significant inflammatory cell infiltration as indicated in these panels is considered to represent a moderate degree of inflammation under mucosal repair. Thus, these panels provide morphologic evidence of repair (Fig 2, *B*: EW3). PAS-AB staining confirmed excess mucus in EW diet–fed Tg-mice (Fig 2, *B*: EW1-PAS, PAS-AB–stained EW1).