

Effect of Fluoride on Growth and Acid Production by *Streptococcus mutans* in Dental Plaque

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Received 17 February 1984/Accepted 26 April 1984

The aim of this study was to measure the effect of fluoride on the production of organic acids by *Streptococcus mutans* in dental plaque. The effect was studied in a simplified model of dental plaque with gnotobiotic rats monoinfected with *S. mutans* Ny341. Adaptation of *S. mutans* to fluoride was induced by feeding one group of the rats on fluoride-containing diet and drinking water. No difference was found in the accumulation of *S. mutans* on the teeth between the fluoride-adapted and the control groups. However, there was a significant difference in the amount of lactic acid in metabolically resting plaque between the groups, lactic acid being lower in the fluoride-adapted plaque. At 5 min after a rinse containing 10% sucrose, a high level of lactic acid was found in plaque from animals not exposed to fluoride. Rinses containing 4 or 20 mM fluoride before the sucrose rinse significantly inhibited the lactic acid production in the control group. In the plaque from rats on fluoridated diet and drinking water the sucrose-induced production of lactic acid was not inhibited by a 4 mM fluoride rinse. Moreover, the production of lactic acid in the fluoride-adapted plaque was prolonged. The results indicate that due to fluoride adaptation the inhibition of acid production is unlikely to be important for the caries-preventive action of fluoride.

Several mechanisms have been proposed to explain the cariostatic effect of fluoride. Fluoride can be incorporated into hydroxyapatite, yielding a fluorapatite, which is more acid resistant. Fluoride can also promote the remineralization of early carious lesions (3, 14). In addition, fluoride may reduce the production of acids by bacteria in dental plaque as it is known to inhibit the degradation of glucose by suspensions of dental plaque and salivary sediments at an acidic pH (12, 15). There is little evidence for an effect of fluoride on plaque development. Few differences have been shown between the microbial composition of the plaque from subjects exposed to fluoride and from those in non-fluoridated areas (5, 13, 17). This suggested that the majority of the bacteria in dental plaque are either insensitive to fluoride or able to adapt or mutate in its presence. The oral *Actinomyces* spp. are insensitive to fluoride, whereas *Streptococcus* spp. can adapt to relatively high concentrations of fluoride (1, 9). Hamilton and Bowden (10) found that a freshly isolated strain of *Streptococcus mutans* developed phenotypical resistance against fluoride during growth in a chemostat.

The present experiment was undertaken to measure the effect of fluoride on the acid production of *S. mutans* in dental plaque. This effect was studied in a simplified model of dental plaque with gnotobiotic rats monoinfected with *S. mutans*. Adaptation of *S. mutans* to fluoride was induced by feeding the rats on fluoridated diet and drinking water.

MATERIALS AND METHODS

Microorganisms. *S. mutans* Ny341 (serotype c) was isolated in our laboratory from human dental plaque. The plaque was collected in an area with no fluoride added to the drinking water. Before the animal experiment the strain had only been transferred four times in the laboratory since its isolation. For inoculation of the rats the organism was grown in TPY broth supplemented with 0.1% glucose. The TPY broth is composed of Trypticase peptone (BBL Microbiology

Systems; 20 g/liter) and yeast extract (Difco Laboratories; 10 g/liter). The culture was incubated in a screw-cap vial for 18 h at 37°C. The rats were individually inoculated by application of 100 µl of the *S. mutans* culture into the mouth with a pipette with a disposable plastic tip.

Experimental design. The experiment was designed to monitor the effect of fluoride on the production of acids by *S. mutans* in the plaque of gnotobiotic rats. Germ-free Osborne-Mendel rats 25 to 27 days old and kept in plastic isolators were used. The rats originated from 15 litters that were each reduced to six rats. The litters were distributed at random among six experimental groups consisting of 15 rats each. Groups 1 through 3 served to compare the effects of a fluoride rinse on the acid production by *S. mutans* in dental plaque. The effect of fluoride rinses superimposed on the continuous supply of fluoride in the diet and drinking water was tested in groups 4 through 6. No fluoride rinse was given in groups 1 and 4; groups 2 and 5 received 4 mM fluoride, and groups 3 and 6 received 20 mM fluoride in the rinse. The groups of rats (1 through 6) were subdivided into three subgroups aimed to measure acid production in the plaque before and at 5 and 15 min after a sucrose rinse. Each subgroup contained five rats.

The experimental diet consisted of sucrose (16%), skim-milk powder (32%), wheat flour (44%), lyophilized yeast (7%), and vegetable oil (1%). Sodium fluoride (20 mg/kg) was added to the diet for groups 4 through 6. The drinking water for these groups was also supplied with sodium fluoride (20 mg/liter).

Collection of plaque. At the end of a 3-week period the effect of fluoride on the acid production of the plaque was measured as follows. The rats were starved for 4 h to exhaust the external substrates in the plaque. Then, a fluoride solution containing 4 or 20 mM NaF was administered to the rats by using a peristaltic pump provided with a silicon tube. The fluoride solution was pumped into the rat's mouth for 1 min at a delivery rate of 1 ml/min, during which the rats were restrained. To stimulate acid production a 10% sucrose solution was subsequently given to the rats. The

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sucrose solution was administered in the same way, i.e., for 1 min at a delivery rate of 1 ml/min. The interval between the administration of fluoride and sucrose solutions was 5 min. After the administration of sucrose, rats were periodically sacrificed for the collection of fissure plaque. Plaque samples were taken at 5 and 15 min after the sucrose rinse. These times refer to the time elapsed between the administration of sucrose and the transfer of the plaque into buffer. The time required to kill the rat, to dissect the lower jaw, and to collect the plaque was 2 min. Thus, for the 5-min samples the rats were decapitated 3 min after the stop of sucrose administration. At each time five rats per group were sampled. Plaque was also collected from five rats per group before the application of the sucrose solution (0-min samples). To allow (semi)quantitative removal of plaque, the fissures were split with a scalpel. The plaque was then removed from the fissures of the three molars on the left side of the lower jaw by using a dental probe, to give one pooled sample per rat. The pooled plaque from each rat was treated as a separate sample. The plaque samples were suspended in 100 μ l of 0.1 M histidine at 0°C and ultrasonically dispersed for 30 s under cooling using a Kontes cell disruptor K-881440 (output, 4.3 W).

Bacteriological assays. The total accumulation of *S. mutans* on the dentition of the rats was determined as follows. The three molars on the right side of the lower jaw were extracted and ground in a mortar in 1 ml of 0.85% saline. The suspension was ultrasonically treated for 30 s at 0°C with a Kontes cell disruptor K881440 to disperse the adherent plaque. Microscopic evaluation showed that the vast majority of bacteria occurred as single cells or in pairs after sonication. Sonication for 30 s gave maximal recovery of viable counts from plaque samples. The samples were subsequently diluted in 0.85% saline, and several 10-fold dilutions were plated onto sheep blood agar. The agar plates were incubated for 48 h in an atmosphere of 90% N₂-5% H₂-5% CO₂ at 37°C. Colonies of *S. mutans* Ny341 were counted.

Chemical analysis. Organic acids in the suspended plaques were analyzed by isotachopheresis as described before (18, 19). The isotachopheretic analysis has the advantage that no pretreatment of the samples was required, causing no loss of material from the small samples. To account for the size of the samples, the amounts of acid were expressed in nanomoles per microgram of DNA. DNA was fluorimetrically assayed with ethidium bromide (6). DNA from calf thymus (Boehringer Mannheim Biochemicals; 104175) was used as a standard.

The data were subjected to analysis of variance, and the differences between the means of groups were tested by using a *t*-test.

RESULTS

The total viable counts of *S. mutans* in the control dietary group were 9.4 ± 0.3 (mean log CFU \pm standard error), whereas the log₁₀ CFU of *S. mutans* in the fluoride group amounted up to 9.5 ± 0.2 . Thus, no significant difference occurred in the accumulation of *S. mutans* between the two dietary groups.

In this study we have monitored the effect on acid production of pulse addition of fluoride as well as continuous supply of fluoride in diet and drinking water. Plaque samples obtained from the latter group will be referred to as fluoride-adapted plaque.

The amounts of formic, acetic, and lactic acids in plaque samples from the control dietary group are given in Table 1. After a rinse with sucrose solution, a significant increase of lactic acid was observed at 5 min after the rinse ($P < 0.0001$). Lactic acid had returned to its baseline level at 15 min after the rinse. Sucrose caused no statistically significant changes in the amounts of formic or acetic acids.

Rinsing with 4 mM fluoride solution before sucrose administration depressed the lactic acid peak at 5 min after sucrose from 34.8 to 18.2 nmol of lactic acid per μ g of DNA ($P < 0.0003$). No significant increases of lactic acid were observed after 20 mM fluoride rinses. Fluoride rinses containing 4 or 20 mM fluoride had no statistically significant effects on the amounts of formic or acetic acids.

The amounts of formic, acetic, and lactic acids in samples from the fluoride dietary group are presented in Table 2. The data demonstrated several differences between these samples and those from the control groups 1 through 3. In samples of resting plaque (0 min) the amounts of acids were lower than in the control group. The difference was statistically significant for formic, acetic, and lactic acids ($P < 0.01$).

Further, the amount of lactic acid at 5 min after sucrose was significantly lower in the fluoride-adapted than in the control group (20.4 versus 34.8 nmol/ μ g of DNA; $F(1, 78) = 10.64$; $P < 0.0016$). However, in the fluoride-adapted group the levels of lactic acid and also of formic and acetic acids were significantly increased at 15 min after the sucrose pulse, whereas in the corresponding control group (group 1) lactic acid had dropped to the baseline level.

The adaptation of *S. mutans* to fluoride was revealed by monitoring the effects of fluoride rinses on the amounts of acids in plaque. Sucrose-induced production of lactic acid even occurred after a 20 mM fluoride rinse as indicated by the level of lactic acid at 5 min after sucrose (15.6 versus the baseline level of 5.8 nmol/ μ g of DNA; $F(1, 78) = 5.84$; $P < 0.018$). A major difference with the control group was that in

TABLE 1. Formic, acetic, and lactic acids in *S. mutans* plaque in rats on control diet^a

Group	Fluoride in rinse (mM)	Time after sucrose (min)	Formic acid	Acetic acid	Lactic acid
1		0	11.6 \pm 2.8	10.9 \pm 3.4	12.8 \pm 3.1
		5	9.7 \pm 2.5	12.1 \pm 1.9	34.8 \pm 3.4
		15	4.2 \pm 0.3	5.2 \pm 1.4	12.2 \pm 3.2
2	4	0	10.7 \pm 5.4	6.9 \pm 1.6	14.0 \pm 4.5
		5	4.1 \pm 0.7	8.4 \pm 1.6	18.2 \pm 1.1
		15	4.0 \pm 0.5	8.2 \pm 0.9	16.6 \pm 2.9
3	20	0	6.1 \pm 3.3	6.0 \pm 3.1	13.5 \pm 4.9
		5	7.1 \pm 1.2	7.5 \pm 0.9	15.6 \pm 2.9
		15	5.0 \pm 0.8	3.6 \pm 2.1	16.6 \pm 3.1

^a Nanomoles of acid per microgram of DNA; mean value \pm standard error.

TABLE 2. Formic, acetic, and lactic acids in *S. mutans* plaque in rats on fluoride-containing diet^a

Group	Fluoride in rinse (mM)	Time after sucrose (min)	Formic acid	Acetic acid	Lactic acid
4		0	2.9 ± 0.4	3.5 ± 1.2	5.8 ± 1.1
		5	3.0 ± 0.4	4.6 ± 1.1	20.4 ± 3.1
		15	13.8 ± 6.1	12.8 ± 6.0	27.5 ± 1.1
5	4	0	7.1 ± 1.7	9.0 ± 1.0	5.6 ± 0.5
	4	5	7.2 ± 1.8	10.0 ± 1.1	26.0 ± 3.7
	4	15	6.7 ± 0.9	8.0 ± 1.4	21.0 ± 6.1
6	20	0	3.0 ± 1.0	5.3 ± 1.1	5.5 ± 0.7
	20	5	6.5 ± 2.0	7.8 ± 2.8	15.4 ± 1.0
	20	15	8.9 ± 2.7	8.9 ± 1.4	20.2 ± 4.8

^a Nanomoles of acid per microgram of DNA; mean value ± standard error.

the 15-min samples the lactic acid levels were always significantly higher than the 0-min levels irrespective of the fluoride concentration in the rinse (groups 4 through 6).

DISCUSSION

Cross-sectional microbiological studies of plaque from subjects exposed to fluoride from natural sources or by fluoride supplements have not revealed significant differences between the plaque from these subjects and that from those without fluoride (13, 17, 20). The reason for this was suggested to be that a variety of bacteria in the dental plaque are either resistant or can adapt to fluoride (2). The present observation that *S. mutans* accumulated as well in the presence of fluoride as in its absence was in accordance with the above findings.

The main acids in plaque formed by *S. mutans* included formic, acetic, and lactic acids. The ratio of formic to acetic acid in the plaque is approximately 1:1 and deviates from the expected value of 2 that is based on the conversion of pyruvate by the pyruvate-formate lyase system (21). Recently, we found that in aerobic chemostat cultures of *S. mutans* the main fermentation product was acetic acid. The excess of acetate relative to formate in dental plaque might reflect the aerobic conditions in the upper layer of the plaque.

The effectiveness of 1-min fluoride rinses in inhibiting the acid production indicated that fluoride rapidly diffuses into the plaque. Indeed, measurements by Shields and Mühlemann (16) have demonstrated the rapid penetration of fluoride into plaque. Fluoride is thought to interfere with carbohydrate degradation at various levels: directly by inhibition of enolase (9), indirectly by inhibition of sugar uptake via phosphotransferase systems (11), and by its action as a proton carrier. This latter activity reduces the transmembrane ΔpH and causes a lowering of the intracellular pH, thereby inhibiting the activity of various glycolytic enzymes (7, 8). The inhibition of lactic acid production by fluoride rinses indicated that the unadapted test strain was sensitive to 4 mM fluoride in vivo. This sensitivity was in the same range as those of other *S. mutans* strains grown in chemostat cultures under glucose limitation (10).

It was expected that continuous exposure to fluoride would lead to adaptation of *S. mutans* in dental plaque. Hamilton and Bowden (10) reported the development of resistance to fluoride in cells that had been grown in a chemostat in the presence of fluoride. Several observations indicated that the continuous supply of fluoride to the diet and drinking water of the rats significantly affected the carbohydrate metabolism of *S. mutans*. The low levels of acids in resting plaque reflect a lower glycolytic activity of the organism under conditions in which fermentable carbohydrates are limited. However, it seems unlikely that these

lower amounts of acids are meaningful in terms of caries activity, because in resting plaque the pH is near neutral due to the buffering by saliva. The adaptation of *S. mutans* to fluoride became apparent when monitoring the effects of combined fluoride and sucrose rinses. In contrast to the control plaque, *S. mutans* in the fluoride-adapted plaque produced lactic acid even in the presence of high fluoride concentrations in the rinse.

The mechanism underlying resistance to fluoride in streptococci is not known. However, enolase is unlikely to be involved, as enolases from fluoride-sensitive and -resistant strains of *S. mutans* appear to be inhibited by fluoride (4).

The increased level of lactic acid in 15-min samples from the fluoride-adapted group indicated that fermentable carbohydrates were still available. In contrast, the low level of lactic acid in the control plaque at 15 min indicated that external sucrose was exhausted. Thus, it seems that sucrose is taken up by the fluoride-adapted cells as well as by the nonadapted cells, but that the adapted cells degrade sucrose at a slower glycolytic rate.

The present experiment focused attention on the adaptability of *S. mutans* to fluoride under natural conditions in dental plaque. This finding seems important for further studies to the mechanisms of fluoride action. Such studies should be conducted with phenotypically adapted strains in addition to normal strains. The adaptation of *S. mutans* strongly reduced the organism's sensitivity to fluoride. Thus, the inhibition of bacterial acid production is likely not very important in the caries-preventive action of fluoride.

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