Microflora and Chemical Composition of Dental Plaque from Subjects with Hereditary Fructose Intolerance

CHARLES I. HOOVER,¹ ERNEST NEWBRUN,^{1*} GERRALD METTRAUX,² AND HANS GRAF²

Department of Oral Medicine and Hospital Dentistry, University of California San Francisco, San Francisco, California 94143,¹ and Department of Periodontology, University of Bern, CH-3010, Bern, Switzerland²

We compared the microbiological and chemical composition of dental plaque from subjects with hereditary fructose intolerance who restrict their dietary sugar intake with that of control subjects who do not. The two groups showed no significant differences in chemical composition of plaque: the mean protein, carbohydrate, calcium, magnesium, and phosphate contents were similar. Dental plaque from both groups contained similar numbers of total colony-forming units per microgram of plaque protein, and *Streptococcus sanguis*, an indigenous nonpathogen, was isolated with equal frequency from plaque samples of both groups. However, potentially odontopathic *Streptococcus mutans* and *Lactobacillus* were isolated three to four times more frequently from plaque samples of control subjects than from plaque samples of subjects with hereditary fructose intolerance. Clearly, diet (sucrose in particular) influences the colonization and multiplication of specific cariogenic organisms in dental plaque.

In 1890, W. D. Miller first postulated that dental caries resulted from the fermentation of carbohydrate by acidogenic microorganisms (39). During the early 1900s, researchers suggested that *Lactobacillus acidophilus* caused dental caries and reported that dietary restriction of carbohydrate concomitantly reduced salivary counts of *L. acidophilus* and the development of new carious lesions (3, 23, 28).

Recently, *Streptococcus mutans* has been recognized as the prime cariogenic microorganism in humans (13, 26, 30, 36). Dietary restriction of carbohydrate also results in the reduction of *S. mutans* and iodophilic polysaccharide-storing microorganisms in dental plaque (14, 50). Children with congenital sucrase deficiency restrict the amount of sucrose in their diet and have fewer caries than normal children of similar age (51). *S. mutans* could not be detected in dental plaque from 40% of these children.

Hereditary fructose intolerance (HFI), first described in 1956 by Chambers and Pratt (11), is characterized by severely reduced activity of fructose 1-phosphate aldolase (41, 43). Ingestion of items containing fructose or sucrose or both results in severe hypoglycemia, nausea, and vomiting. People who have HFI develop a strong aversion to all sweets, sucrose-containing foods, and fruits, and adhere to a lifelong, self-imposed diet that virtually excludes sucrose. We have compared the diet, oral hygiene, caries experience, plaque microflora, and plaque chemical composition of subjects who have HFI with

those of controls. Dietary analyses, oral hygiene status, and caries experience of our HFI and control subjects are reported in detail elsewhere (E. Newbrun, C. Hoover, G. Mettraux, and H. Graf, submitted for publication). This report assesses the effect of dietary sucrose restriction on the bacterial colonization and chemical composition of dental plaque.

MATERIALS AND METHODS

Study population. On four, in some cases five, separate occasions over a 2-year period, we examined 17 HFI subjects (3 from the United States, 14 from Switzerland) and 14 control subjects (2 from the United States, 12 from Switzerland). Oral examinations and plaque collections took place between 8 and 11 a.m. and were usually performed on a pair of subjects, one from each group. The HFI group consisted of 10 male and 7 female subjects whose mean age was 29.1 years (range, 6 to 54 years). The control group consisted of 3 male and 11 female subjects whose mean age was 26.5 years (range, 9 to 55 years). Eight control subjects were blood relatives of persons in the HFI group. Control subjects consumed more than 20 times as much sucrose as HFI subjects and had significantly greater DMFS scores (control, 36.1; HFI, 3.3).

Microbiological analyses of dental plaque. Supragingival dental plaque was collected and assayed from four specific sites in each subject on each occasion. Sterile needles were used to collect plaque from the occlusal surface of the right first mandibular molar and the palatal groove of the left first maxillary molar. Interproximal plaque was obtained from between the left first and second mandibular molars and the right

first and second maxillary molars with sterile unwaxed dental floss (John O. Buttler Co., Chicago, Ill.) held in a sterile plastic support (E-Z Floss, Palm Springs, Calif.). When any of these teeth were carious, restored, or missing, we substituted appropriate alternative sites.

The collected plaque samples were immediately placed in A/S Nunc vials (Almac Cryogenics Inc., Oakland, Calif.) containing 1.0 ml of reduced transport fluid (47). The vials were introduced into an anaerobic glove box (12) containing 85% N₂, 10% H₂, and 5% CO₂, and the samples were dispersed, serially diluted, plated, cultured, and enumerated for total colony-forming units (CFU) of *S. sanguis*, *S. mutans*, and *Lactobacillus* as previously described (27).

We picked three to five representative *S. mutans* colonies at random from *S. mutans*-positive plaque samples and isolated them in pure culture. When more than one colony morphology was apparent, we isolated representative colonies of each morphology for biochemical identification and biotyping. The isolates were identified as *S. mutans* by Facklam's scheme (17) and biochemically typed by the scheme of Shklair and Keene (45).

To compare the numbers of *S. mutans* colonizing the plaque of control subjects with numbers in HFI subjects, we analyzed the protein content of 109 interproximal samples collected for microbiological analysis by using a Coomassie brilliant blue colorimetric assay (37). The volume of each plaque suspension was measured and recorded. The sample was dried in an oven overnight at 85°C and then resuspended in 50 μ l of distilled water. The suspensions were spotted drop by drop with capillary tubes on 25-mm glass fiber filters (Gilman Instrument Co., Ann Arbor, Mich.) and immediately dried with a heat lamp. Protein standards (bovine serum albumin, 1 to 10 μ g) were also spotted and dried on filters. The dried filters were placed in ice-cold 20% trichloroacetic acid for 5 to 10 min and stained for 20 min in a cold aqueous solution containing 0.25% Coomassie brilliant blue R (Sigma Chemical Co., St. Louis, Mo.), 7.5% acetic acid, and 5% methanol. To remove the stain, the filters were immersed for 10 min three times in aqueous 7.5% acetic acid-5% methanol, then transferred to a vacuum filtration manifold and rinsed with the stain removal solution until the blank filter was white. Stained protein spots were cut from the filters with a cork borer and placed in microcentrifuge tubes (Beckman Instruments, Fullerton, Calif.) containing 0.6 ml of 0.12 N NaOH in 20% water-80% methanol (elution buffer). We calculated protein content by linear regression analysis and determined the total CFU per microgram of protein and *S. mutans* CFU per microgram of protein by matching the microbiological analysis of each sample to the protein content.

Plaque samples collected from subjects in the United States were processed and plated on growth media within 1 h. Plaque samples collected from subjects in Switzerland were shipped by air freight to our laboratory. Transit time between collection of samples in Switzerland and processing in our laboratory was usually 3 days. We have previously reported the transport conditions and the survival of selected oral bacteria (27).

Chemical analyses of dental plaque. We com-

pared the amounts of protein, carbohydrate, phosphate, calcium, and magnesium in supragingival dental plaque from HFI and control subjects. Plaque was collected in plastic "scoops" held in sterile hemostats, pooled from as many sites as possible from each subject, and placed in separate, preweighed Nunc vials. The wet weight of each sample was measured, and the specimen was stored at -40°C until use. Samples collected in Switzerland were stored and shipped in liquid nitrogen (-196°C). Before chemical analysis, the plaque was suspended in 1.0 ml of twice-distilled water and dispersed by ultrasonic oscillation at 75 W for 30 s.

We measured protein content by the fluorescamine (Fluram) method (4) and used the method of Dubois et al. (16) to measure the carbohydrate content.

To measure the phosphate content, we used a modified King (31) colorimetric assay. A 0.1-ml portion of plaque suspension was pipetted into platinum crucibles containing 1.0 ml of CaO. The specimens were dried overnight at 85°C and then ashed in a furnace at 575°C for 2 to 3 h (the CaO fixes the phosphate during ashing). The ashed samples were dissolved in 0.6 ml of 30% HClO₄, brought to a final volume of 1.1 ml with twice-distilled water, and transferred to test tubes. While the tube contents were mixing on a Vortex mixer, 0.5 ml of 2.5% ammonium molybdate and 0.25 ml of reducing agent (1-amino-2 naphthol-sulfonic acid) were added to each tube. The absorbance (660 nm) of each sample was immediately measured against a water reagent blank with a Beckman DB-G spectrophotometer. We calculated the phosphate content by linear regression analysis with absorbance readings of KH₂PO₄ standards. The range of the assay is from 0 to 40 μ g.

We analyzed the calcium and magnesium content of dental plaque by atomic absorption spectrophotometry. A 0.2-ml portion of each suspended plaque sample was placed in a preweighed platinum crucible. After the samples dried overnight at 85°C, they were ashed in a furnace for 2 to 3 h at 575°C, allowed to equilibrate to room temperature, and dissolved in 0.1 ml of concentrated HCl followed by 0.9 ml of twice-distilled water. Each suspension was then divided into 0.5-ml portions: one for measurement of Ca²⁺ and the other for measurement of Mg²⁺.

To measure Mg²⁺ content, we aspirated the sample directly into a Perkin-Elmer model 290 B atomic absorption spectrophotometer (Perkin-Elmer Co., Norwalk, Conn.). We calculated the Mg²⁺ content by linear regression analysis with atomic absorption readings of Mg²⁺ standards (0 to 2 μ g of Mg²⁺ per ml).

To measure Ca²⁺ content, we added 0.12 ml of 5% La₂O₃-25% HCl to each 0.5-ml sample before it was aspirated into the atomic absorption spectrophotometer. We calculated the Ca²⁺ content by linear regression analysis with atomic absorption readings of Ca²⁺ standards (0 to 10 μ g of Ca²⁺ per ml).

Both calcium and magnesium standards were prepared from certified atomic absorption standards (Fisher Scientific Co., Fair Lawn, N.J.).

RESULTS

Microbiological analyses of dental plaque. Samples of supragingival dental plaque

(271 from 17 HFI subjects, 220 from 14 control subjects) were collected and analyzed for the presence of *S. sanguis*, *S. mutans*, and *Lactobacillus* (Fig. 1). The isolation frequency of *S. sanguis* was similar for both groups (HFI, 86.0%; control, 84.1%). However, *S. mutans* was isolated from only 26.2% of plaque samples from HFI subjects, compared with 72.3% from controls. *S. mutans* could not be detected in 5 of 17 HFI subjects, but was present in 13 of 14 control subjects. Similarly, *Lactobacillus* was isolated from only 10.3% of plaque samples from HFI subjects, compared with 40% of samples from controls. *Lactobacillus* was not detected in 5 of 17 HFI subjects, but was present in 12 of 14 control subjects. We isolated *Lactobacillus* more often from interproximal plaque samples than from smooth surface samples in both groups (Table 1). No such variation according to site of plaque collection was observed in either group in the isolation frequency of *S. sanguis* or *S. mutans*.

To express the extent of *S. mutans* colonization quantitatively, we compared the *S. mutans* CFU per microgram of plaque protein of HFI subjects with that of control subjects. Dental plaque from both HFI and control subjects contained similar total CFU per microgram of pro-

TABLE 1. Isolation frequency of *S. sanguis*, *S. mutans*, and *Lactobacillus* according to site of plaque collection^a

Bacterium	Population	Isolation frequency (%)	
		Smooth surfaces	Interproximal spaces
<i>S. sanguis</i>	HFI	82.4	89.1
	Control	77.5	88.7
<i>S. mutans</i>	HFI	21.8	27.8
	Control	62.2	78.6
<i>Lactobacillus</i>	HFI	3.7	16.6
	Control	20.5	58.6

^a Represents same data as shown in Fig. 1.

tein (Table 2). But nearly all HFI subjects, on an individual basis, had lower proportions of *S. mutans* in their dental plaque than the mean value for controls (Table 3). Plaque samples from one HFI subject contained proportions of *S. mutans* similar to those found for controls. One other HFI subject was also heavily colonized with *S. mutans*, but protein determinations were not performed on any of these samples. In making these comparisons we analyzed only data from those HFI and control subjects whose interproximal plaque harbored *S. mutans*, excluding many samples (mainly from HFI subjects) in which *S. mutans* could not be detected. We did not consider it valid to use zero values to calculate the mean proportions of *S. mutans* in plaque samples. Had all samples from HFI subjects been used, the differences between the two groups in proportions of *S. mutans* would have appeared much larger.

The distribution and incidence of various serotypes of *S. mutans* are of interest. We randomly selected 308 *S. mutans* isolates (from both HFI and control subjects) and assigned them to biotypes (Table 4). Biotype I (serotypes c, e, and f) was the most prevalent and could be found in all but one *S. mutans*-positive subject. Biotypes II (serotype b) and III (serotype a) were not isolated from any of the subjects. The distribution of biotype V (melibiose-negative serotype e), which was isolated from 23.1% of control subjects and 18.2% of HFI subjects, was not markedly different. However, control subjects appeared to be colonized with biotype IV (serotypes d and g) to a greater extent than HFI subjects (control, 38.5%; HFI, 18.2%).

Chemical composition of dental plaque. The wet weight chemical composition of dental plaque from HFI subjects and control subjects was similar in most respects (Table 5). The protein content of dental plaque from both groups was similar, but 1.5 to 2 times larger than

FIG. 1. Isolation frequency of *S. sanguis*, *S. mutans*, and *Lactobacillus* from supragingival dental plaque of 17 HFI and 14 control subjects. Numbers in parentheses: numerator = number of isolations, denominator = number of plaques sampled.

TABLE 2. Total CFU per microgram of protein of supragingival dental plaque from HFI and control subjects^a

Population	Total CFU/ μ g of protein
HFI	$4.5 \times 10^6 \pm 1.3 \times 10^6$
Control	$4.3 \times 10^6 \pm 0.7 \times 10^6$

^a Mean $\pm$ standard error for 50 interproximal plaque samples from 13 HFI subjects (range, 8.8×10^5 to 2.6×10^6) and 59 interproximal plaque samples from 14 control subjects (range, 1.3×10^6 to 2.2×10^6).

TABLE 3. *S. mutans* CFU per microgram of protein of supragingival dental plaque from HFI and control subjects

Population	<i>S. mutans</i> CFU/ μ g of protein
HFI ^a	$7.0 \times 10^1 \pm 5.9 \times 10^1$
Control ^b	$3.0 \times 10^4 \pm 1.3 \times 10^4$
One HFI subject ^c	$4.0 \times 10^4 \pm 2.7 \times 10^4$

^a Mean $\pm$ standard error for nine interproximal plaque samples from seven HFI subjects (range, 6.0×10^{-1} to 1.6×10^5).

^b Mean $\pm$ standard error for 28 interproximal plaque samples from 13 control subjects (range, 3.0×10^1 to 3.0×10^5).

^c Mean $\pm$ standard error for six interproximal plaque samples from one HFI subject (range, 6.0×10^1 to 1.6×10^5).

literature values (1, 2, 5, 33). This may be attributed to the use of different protein assay methods or standards. Control plaques contained more carbohydrate and less calcium than HFI plaques, but similar amounts of magnesium and phosphate. The carbohydrate content of individual plaque samples from HFI subjects was generally lower than that from control subjects, but the group means were not significantly different ($P > 0.1$). Our values for the carbohydrate content of plaque compare well with literature values (18, 33, 52). The calcium, magnesium, and phosphate contents of plaque from HFI and control subjects were also not significantly different (calcium, $P > 0.1$; magnesium, $P > 0.05$; phosphate, $P > 0.7$). The concentrations of calcium and magnesium we found are generally comparable with those found in other studies (1, 18, 21); however, a wide range of values have been reported. The phosphate content determined in this study is two to four times greater than expected from prior reports (1, 2, 18). The use of micromethods or different techniques might account for these discrepancies.

DISCUSSION

Because dietary sucrose is an important factor in the etiology of dental caries (22, 25, 40, 44), we studied a population with a rare genetic

enzyme deficiency who drastically restrict their intake of sucrose. Sucrose serves not only for acid production but also as a substrate for glucan synthesis by certain streptococci, thereby augmenting the ability of these bacteria to accumulate in dental plaque (10, 19). Our HFI subjects consumed less than $\frac{1}{20}$ as much sucrose as controls and experienced about $\frac{1}{10}$ as many caries (Newbrun et al., submitted for publication). No differences in plaque or oral hygiene index could be found between the two groups. We therefore compared the chemical and microbiological composition of their dental plaque to see whether differences in composition could account for the differences in caries.

Previous studies claim that in vivo exposure of dental plaque to sucrose increases the concentration of carbohydrate and reduces the levels of calcium and phosphate (2; R. F. Wilson, J. Dent. Res. 52[Special Suppl.]:942, 1973, abstr. no. 63) and that caries activity is directly related to the carbohydrate content and inversely related to the calcium and phosphate content of dental plaque (2, 52; I. Kleinberg, R. Chatterjee, C. R. Castalde, and F. Chebib, J. Dent. Res. 48[Special Issue A]:199, abstr. no. 635).

We were unable to confirm these findings. No significant differences were detected in the chemical composition of individually pooled dental plaque from HFI and control subjects. Differences in the age and location of plaque and the length of time since exposure to dietary substrate may influence the chemical composition (2; F. P. Ashley, J. Dent. Res. 51[Special Suppl.]:1234, 1972, abstr. no. 3). We were unable to control these factors in this study.

Numerous epidemiological studies show a positive correlation between dental caries and the presence/prevalence of *S. mutans* (5, 13, 29, 32, 46). The reduced isolation frequency and lower proportions of *S. mutans* found in HFI subjects

TABLE 4. Isolation frequency of various biochemical types of *S. mutans* from dental plaque of HFI and control subjects^a

Population	Isolation frequency (%)		
	Biotype I (serotypes c, e, f)	Biotype IV (serotypes d, g)	Biotype V (serotype e, melibi- ose nega- tive)
Control	92.3 (12/13) ^b	38.5 (5/13)	23.1 (3/13)
HFI	100 (11/11)	18.2 (2/11)	18.2 (2/11)

^a Data for *S. mutans*-positive subjects only.

^b Ratio of number of individuals positive for a given biotype to the number of *S. mutans*-positive individuals.

TABLE 5. Chemical composition^a of dental plaque from HFI and control subjects

Population	Protein ($\mu\text{g}/\text{mg}$)	Carbohydrate ($\mu\text{g}/\text{mg}$)	Calcium ($\mu\text{g}/\text{mg}$)	Magnesium ($\mu\text{g}/\text{mg}$)	Phosphate ($\mu\text{g}/\text{mg}$)
HFI ($n = 17$)	144.3 $\pm$ 8.6	18.1 $\pm$ 2.3	3.0 $\pm$ 0.9	0.3 $\pm$ 0.05	10.2 $\pm$ 1.2
Control ($n = 14$)	141.7 $\pm$ 5.4	23.8 $\pm$ 3.1	1.5 $\pm$ 0.2	0.4 $\pm$ 0.03	8.4 $\pm$ 0.5

^a Average of two to four analyses for each patient; micrograms per milligram (wet weight) $\pm$ standard error; n = number of individuals.

are consistent with their minimal caries experience. *S. mutans* is widespread in human populations and is present in 60% of sucrose-deficient children (51) and 70% of our HFI subjects. Potentially cariogenic microorganisms are present in almost all people, even those who have severely limited the amount of sucrose in their diet for their entire lives. Thus, restriction of sucrose intake neither totally prevents colonization by *S. mutans* nor completely eliminates it from the oral cavity. But, although sucrose is not essential for colonization of dental plaque by *S. mutans*, it greatly facilitates their colonization on the teeth and increases their proportions in plaque (48, 49). These observations suggest that *S. mutans* is indigenous to the oral cavity and that excessive sucrose consumption is the prime cause of dental caries.

Quantitative recovery (30 to 80%) of *S. sanguis*, *S. mutans*, and *Lactobacillus* may be accomplished from dental plaque for up to 72 h after collection (27). Selective media (mitis-salivarius, bacitracin agar) afford less recovery of *S. mutans* CFU than do nonselective media (34). However, because *S. mutans* often constitutes a small percentage of total CFU (especially in HFI subjects), the use of selective media increases detection (isolation frequency). Plaque samples from both groups were examined under identical culture conditions. The limit of detection for the various organisms is difficult to define for anything but in vitro-grown stock cultures and may have little bearing on the detection limit of wild-type organisms from dental plaque. To argue that potentially cariogenic microorganisms would escape detection because they are present in lower numbers in HFI subjects than in controls would be tautological.

S. mutans comprises a serologically and biochemically heterogeneous group of microorganisms. Bratthall (6, 8) characterized five serological types of *S. mutans* (*a*, *b*, *c*, *d*, *e*). Perch et al. (42) found two additional serotypes (*f* and *g*). Shklair and Keene (45) have devised a biochemical scheme that separates *S. mutans* into five biotypes and correlates them with the recognized serotypes. In early reports, when fluorescent-antibody techniques were used to recognize and enumerate *S. mutans* serotypes directly in plaque smears, serotypes *a* and *b* were most

commonly found (7, 15, 20). Because of immunological cross-reactivity or salivary masking of antigens or both, these reports were probably erroneous. Recent studies (9, 24, 42) indicate that serotype *c* predominates in most populations. Our results (Table 4) are consistent with these studies in that biotype I (which includes serotype *c*) was the most prevalent and was present in 11/11 *S. mutans*-positive HFI subjects and 13/14 *S. mutans*-positive control subjects.

Several studies have postulated that various biotypes of *S. mutans* may preferentially cause smooth surface caries or be more cariogenic than other biotypes (30, 36, 38, 48; D. S. Harper and W. J. Loesche, *J. Dent. Res.* 57[Special Issue A]:142, 1977, abstr. no. 269; W. H. van Palenstein Helderman, P. Sampaio Camaergo, and J. H. J. Huis In't Veld, *J. Dent. Res.* 57[Special Issue A]:142, 1977, abstr. no. 271). We isolated biotype IV more often from control subjects (5/14) than from HFI subjects (2/11). Four of the five control subjects who were colonized with biotype IV had greater caries scores than the mean for the control group. One HFI subject, who exhibited the highest caries scores, the highest isolation frequency of *S. mutans*, and the highest proportion of *S. mutans* of any HFI subject, also was heavily colonized with biotype IV. Detailed studies with gnotobiotic rats infected with multiple strains of each biotype are required before any definitive correlations between biotype and cariogenic potential can be made. This information is important for the design and production of an anticaries vaccine. The infrequent isolation of biotype IV from HFI subjects might be explained by the observation that biotype IV *S. mutans* requires sucrose to colonize and remain on teeth of gnotobiotic rats (49). The habitually low sugar intake of HFI subjects could minimize colonization by biotype IV.

Our results support the specific plaque hypothesis of caries formation (35) which states that owing to the presence of specific pathogens or relative increases in the levels of certain indigenous plaque organisms, only certain plaques can cause infection. Dietary sucrose plays a selective role in facilitating colonization and multiplication of specific cariogenic microorganisms

in dental plaque. Caries activity occurs when the sucrose content of the diet and the ingestion frequency exceed a certain level.

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