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Virulence-Related Physiological Changes and Antigenic Variation in Populations of *Streptococcus mutans* Colonizing Gnotobiotic Rats

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The purpose of this study was to determine if populations of *Streptococcus mutans* which were undergoing antigenic variation while colonizing gnotobiotic rats concomitantly became altered in physiological characteristics which affected their virulence. *S. mutans* strain JBP (serotype c), which was freshly isolated from a carious lesion in a 6-year old child, was used to inoculate gnotobiotic rats; uninfected animals served as controls. Substrains were isolated from animals 1, 2, 3, 4, and 12 weeks after infection; samples of pilocarpine-stimulated saliva were also obtained from representative animals for antibody analyses. Isolates derived from stock cultures of strain JBP proved to be homogeneous with respect to all of the physiological characteristics monitored. However, substrains isolated from the animals within 4 weeks after infection were altered with respect to their ability to agglutinate in the presence of sucrose, their ability to form adherent growth in sucrose broth, and the terminal pH attained in glucose broth. Some isolates obtained 12 weeks after infection no longer synthesized detectable levels of c antigen or intracellular glycogen, and they formed atypical smooth colonies on mitis salivarius agar. With an enzyme-linked immunosorbent assay, low levels of immunoglobulin A (IgA) antibodies reactive with whole JBP cells were detected in saliva samples of uninfected control animals at each sampling period; these evidently were induced to antigens contained in the diet of the animals. Significantly higher levels of IgA antibodies were present in saliva samples from animals infected with strain JBP for 3 weeks or longer. Thus, the emergence of antigenic and physiological variants of *S. mutans* in the animals was paralleled by increased levels of salivary IgA antibodies. The reactivity of salivary IgG with JBP cells was low, and it fluctuated in both groups of animals. No antibodies of the IgM class were detected. When tested in gnotobiotic rats, several variants, including strains which no longer formed typical rough colonies or adherent growth in sucrose broth, proved much less virulent than parental strain JBP in inducing carious lesions. Prior oral immunization, which resulted in higher levels of salivary and serum IgA antibodies reactive with strain JBP, was found to accelerate the emergence of smooth-colony variants in the animals; it was also associated with decreased streptococcal population levels on the teeth and in feces of the rats. It is suggested that part of the mechanism by which artificial immunization leads to a reduction in dental caries development in experimental animals is due to the earlier selection of less virulent streptococcal populations.

Often present in the sera (35, 39) and secretions (2, 5, 11, 27, 48) of humans are antibodies which react with indigenous bacteria; however, it is not clear how these microbial populations are affected. Many organisms appear to persist in the mouths of humans throughout life. For example, strains of *Streptococcus salivarius* can be isolated from the tongue dorsum of humans from infancy on (18). Similarly, strains of *Streptococcus mutans* can usually be recovered from the teeth of children and adults (18). The apparent ability of these indigenous species to colonize persistently could be partially due to antigenic variation which enables them to evade the immune response of the host.

Antigenic variation is well known to occur in populations of viruses (12), protozoa (20), and certain pathogenic bacteria (3, 7, 8, 40, 49). For example, in relapsing fever, *Borrelia* species increase in numbers in the blood and elicit a fever; they then disappear coincident with the appearance of antibodies (8). However, organisms of a different antigenic makeup emerge after a few days due to the immune selection pressure. Antigenic variation occurs with other bacteria, but it has not been especially well studied. Sack and

Miller (40) demonstrated that populations of *Vibrio cholerae* change serotype while colonizing gnotobiotic mice, and Corbeil, Schurig, and co-workers (7, 42) showed that populations of *Campylobacter fetus* undergo antigenic changes during natural infections in cattle. Strains of hemolytic streptococci have also been reported to exhibit antigenic changes during repeated mouse passage (28, 49), and populations of *S. mutans* (4) and *S. salivarius* (23) have recently been observed to exhibit antigenic changes while colonizing gnotobiotic rats.

It was noted in the latter studies that antigenic variants of *S. salivarius* often attached in much lower numbers than the parental strain to buccal epithelial cells derived from either humans or rats (23). Furthermore, strains of *Escherichia coli* associated with asymptomatic urinary tract infections seem to undergo antigenic variation, and they attach poorly to epithelial cells in comparison with strains isolated from acute urinary tract infections (22, 43). We have also observed that isolates of *Actinomyces viscosus* and *Actinomyces naeslundii* derived from gnotobiotic rats 3 months after infection adsorbed more variably to hydroxyapatite pretreated with human saliva than did isolates derived from laboratory stock cultures (Gibbons and Qureshi, unpublished data). Collectively, these observations suggest that bacterial populations which are undergoing antigenic variation may also exhibit alterations in components which affect their attachment to host tissues and hence their colonization and virulence. The purpose of the present study was to determine whether populations of *S. mutans* in experimental animals became altered in physiological as well as antigenic properties which could affect their virulence.

MATERIALS AND METHODS

Cultures and cultural conditions. In an effort to start with a highly virulent strain, an isolate of *S. mutans* was obtained from a carious lesion affecting the first permanent molar of a 6-year-old child with extensive dental decay. The isolate (designated strain JBP) was restreaked to insure purity, transferred to Trypticase soy (T. soy) broth (BBL Microbiology Systems, Cockeysville, Md.), and samples were preserved by freezing; these were used to inoculate additional broth cultures as needed. Subcultures were characterized biochemically to confirm identification; analyses of hot saline antigen extracts of the organism by immunodiffusion indicated that it belonged to serotype c.

Antiserum preparation. Washed cells of *S. mutans* JBP were prepared from an 18-h T. soy broth culture. The organisms were killed by suspension in formalinized saline overnight and then washed and suspended in saline at a concentration of 10^8 streptococci per ml. An equal volume of the streptococcal suspension was mixed with Freund complete adjuvant (Difco Laboratories, Detroit, Mich.), and the mixture was inoculated subcutaneously into rabbits as previously described (37). The animals were boosted 2 weeks later and bled by cardiac puncture at the end of 4 weeks. The antiserum produced a strong precipitin band with Rantz-Randall (38) antigen extracts of strain JBP, which corresponded to the c antigen when analyzed by either immunodiffusion or immunoelectrophoresis as previously described (23, 37).

Emergence of physiological and antigenic variants in gnotobiotic rats. A group of 27 germfree Sprague Dawley rats were fed diet 2000 and maintained in one isolator as previously described (4). The animals were inoculated when 22 days old by swabbing their teeth with an 18-h T. soy broth culture of strain JBP derived from the preserved frozen stocks. A group of uninfected animals was maintained in a separate isolator to serve as controls. Three animals from each group were sampled by swabbing their teeth using Calgiswabs (Inolex, Inc., Glenwood, Ill.) at 1, 2, 3, 4, and 12 weeks after infection. The swabs were cultured on mitis salivarius (MS) agar (Difco) for the isolation of substrains of *S. mutans* and on blood agar plates for monitoring contamination. Thirty substrains were obtained by selecting every colony growing on a portion of a plate, irrespective of their morphology, at each sampling period. These were restreaked to insure purity and grown in T. soy broth; samples were frozen at −70°C until analyzed further.

Five animals were also removed from the isolators at each sampling period. Samples of pilocarpine-stimulated saliva were collected from them as previously described (1). The samples were pooled, heat inactivated at 56°C for 30 min, and frozen until assayed for antibodies.

Analysis of potential virulence-related properties. Isolates obtained from stock cultures of parental strain JBP and substrains derived from the gnotobiotic rats were analyzed for several physiological properties which could affect their virulence. Alterations in colonial morphology on MS agar were sought by using a dissecting microscope; these could reflect alterations in the synthesis of extracellular glucans from sucrose. The synthesis of intracellular glycogen was determined by flooding colonies on T. soy agar supplemented with 1% glucose with 0.2% I_2 in 2% KI as previously described (17). Ability to form adherent streptococcal deposits on culture vessels was tested by using Trypticase basal broth supplemented with 5% sucrose (15). The ability of washed T. soy broth-grown streptococci in phosphate-buffered saline (pH 7) to agglutinate after addition of 1 mg of sucrose per ml was assayed as previously described (16, 21). The terminal pH produced in T. soy broth supplemented with 1% glucose was determined after 48 h of anaerobic incubation with glass electrodes and a Beckman model SS-3 pH meter. The acid end products present in such cultures of parental strain JBP and selected variants were determined by gas-liquid chromatography of butyl esters (41). Uninoculated culture medium was run as a control, and the quantities of acids present were subtracted from the values for the cultures. The isolates were also examined for the production of detect-

able levels of serotype c antigen. Antigen extracts were prepared from carefully standardized washed bacterial suspensions as previously described (23, 38). All extracts from a given sampling period were prepared simultaneously to reduce variation. The extracts were analyzed by immunoelectrophoresis with 1.2% agarose in Veronal buffer (pH 9.6) and the anti-*S. mutans* JBP serum as previously described (23). Preparations of c antigen isolated by the method of Linzer et al. (30) were analyzed in a comparable manner to serve as controls.

Cariogenicity of *S. mutans* strain JBP and selected variants. The ability of parental strain JBP and selected animal isolates to induce dental caries in gnotobiotic rats was determined as previously described (15). Overnight T. soy broth cultures of the organisms were adjusted to contain 2×10^8 streptococci per ml. Calgiswabs soaked in the cultures were rubbed into the mouths of groups of 10 weanling germfree rats (21 days old) maintained on diet 2000. Implantation of each strain was monitored the next day and at weekly intervals by culturing fecal samples on MS agar and on blood agar plates. At 90 days after infection, the animals were sacrificed and decapitated; their heads were defleshed by Dermestid beetles and evaluated for carious lesions by the method of Keyes (26).

Antibody analysis of saliva samples. The presence of antibodies in pooled saliva samples collected from the animals was determined by an indirect enzyme-linked immunosorbent assay (ELISA) (10). Whole *S. mutans* JBP cells harvested from overnight Todd-Hewitt broth (Difco) cultures served as antigen; the organisms were heated at 56°C for 30 min to inactivate endogenous phosphatase. The streptococci were suspended in 0.1 M Na_2CO_3 buffer (pH 9.6) at a concentration of 2×10^8 cells per ml and used for coating wells in polystyrene microtiter plates. Antigen-free control wells were treated only with carbonate buffer. All plates were stored at 4°C; they were washed 3 times with saline containing 0.05% Tween 20 before use.

Samples of the rat saliva were diluted in phosphate-buffered saline containing 0.05% Tween 20 and 0.02% NaN_3 at pH 7.2. The reagents used were rabbit anti-rat IgG, rabbit anti-rat IgM, and goat anti-rat IgA purchased from Miles Laboratories, Inc., Kankakee, Ill. The gamma globulin fractions of goat anti-rabbit immunoglobulin G (IgG) and rabbit anti-goat IgG (Miles) were conjugated to alkaline phosphatase (type VII; Sigma Chemical Co., St. Louis, Mo.) (10) and used for the indirect technique as previously described (2). The goat anti-rabbit IgG phosphatase conjugate was used for the detection of IgG and IgM antibodies, whereas the rabbit anti-goat IgG phosphatase conjugate was used for the detection of IgA antibodies. All reagents were absorbed with heat-inactivated, formalinized *S. mutans* JBP cells to reduce nonspecific binding.

The optimal dilutions of the anti-rat immunoglobulins and the enzyme-labeled conjugates were found to be 1:200 and 1:500 by checkerboard titration. Dilutions of saliva were assayed to establish the range of linearity. Controls which contained no saliva or no antigen were performed in the same plate to check for nonspecific binding and to reduce plate-to-plate variation. All samples were assayed in triplicate. The absorbance at 400 nm due to the release of *p*-nitrophenol by bound phosphatase from *p*-nitrophenyl phosphate (Sigma) was measured using a Gilford Stasar II spectrophotometer. ELISA values were considered to be the absorbance times $60/t$, where t represents the termination time of the reaction in minutes. All ELISA values were corrected for nonspecific binding as determined from the controls; these values were usually around 0.4.

Effect of oral immunization on the emergence of smooth-colony variants of *S. mutans* JBP. Oral vaccines were prepared using *S. mutans* JBP cells harvested from overnight T. soy broth cultures. The streptococci were washed with sterile saline and killed by suspension in saline containing 0.5% Formalin overnight at 4°C. The organisms were washed twice with sterile saline, and samples were plated on blood agar and on MS agar plates for sterility testing.

Two groups of 10 21-day-old weanling germfree rats were fed diet 2000. The Formalin-killed *S. mutans* cells were administered to one group for a period of 21 days by adding them to the drinking water at a concentration of 10^8 cells per ml. This concentration and time were previously determined to be optimal for induction of a secretory antibody response in gnotobiotic rats (33). The second group of animals served as controls.

After the immunization period, approximately 5×10^6 *S. mutans* JBP cells derived from an overnight T. soy broth culture were introduced into the mouth of each rat. Oral swabbings taken 1 day later were cultured on MS agar to confirm establishment. Smooth-colony variants of strain JBP were enumerated in oral swabbings and in fecal samples obtained from the animals 3, 7, 11, and 14 days after infection. The samples were diluted in 0.1% Trypticase, and samples were spread in duplicate on MS agar plates which were incubated anaerobically in Brewer jars for 48 h. Approximately 1,375 colonies were examined from each sample, and the proportion of smooth variants was determined.

The experiment was terminated 21 days after infection. The animals were sacrificed, and the 6 molar teeth on one side of their mouth were extracted. These were ground with 0.1% Trypticase with a glass tissue grinder, and appropriate dilutions of the suspension were plated in duplicate on MS agar plates for enumeration of the total number of colony-forming units of *S. mutans* cells present. Freshly collected fecal pellets were also weighed and dispersed by grinding in 0.1% Trypticase broth; these were cultured in a comparable manner to determine the total number of colony-forming units of *S. mutans* per gram of feces. The proportion of smooth-colony variants on the plates was also determined.

Samples of pilocarpine-stimulated saliva and serum were collected from five animals in each group. The samples were pooled, heated at 56°C for 30 min, and stored at −70°C. They were analyzed for IgA antibodies which reacted with whole *S. mutans* JBP cells by the ELISA assay described above.

RESULTS

Emergence of physiological and antigenic variants of *S. mutans* JBP in gnotobiotic rats. Thirty substrains derived from stock cultures of *S. mutans* JBP were homogeneous with respect to all of the characteristics studied (Tables 1 and 2). Isolates derived from monoinfected animals up to 4 weeks after infection were also homogenous and similar to parental strain JBP with respect to the presence of c antigen, the formation of rough colonies on MS agar, and the synthesis of intracellular glycogen. However, many isolates obtained from the rats 12 weeks after infection were altered in these characteristics (Table 1). Thus, of 60 isolates tested, only 37 formed detectable levels of c antigen, 49 formed intracellular glycogen, and half formed atypical smooth colonies on MS agar (Fig. 1). Almost all smooth colony-type strains also failed to produce adherent accumulations in sucrose broth (Table 1); both of these characteristics probably reflect an alteration in the synthesis of extracellular glucans from sucrose.

Washed streptococcal suspensions prepared from 30 isolates derived from stock cultures of JBP agglutinated after addition of sucrose, but a large number of isolates derived from the animals at every sampling period varied with respect to this characteristic (Table 1). In fact, none of the isolates obtained 2 and 4 weeks after infection agglutinated in the presence of sucrose.

Terminal pH and acid end products formed by *S. mutans* strain JBP and isolates derived from gnotobiotic rats. The terminal pH attained by 30 isolates derived from a stock culture of *S. mutans* JBP after 48 h of growth in T. soy-1% glucose broth ranged between pH 4.2 and 4.4 with a mean of pH 4.3; none was more that ±0.1 pH unit from the mean (Table 2). However, isolates derived from monoinfected rats were more variable (Table 2). This was indicated by their broader pH range and by the number whose terminal pH was greater than ±0.1 pH unit from the mean.

The reproducibility of the terminal pH measurements was assessed for strain JBP and 8 selected animal isolates in a second experiment. As indicated in Table 3, the pH measurements of triplicate subcultures were similar for all strains. Strains such as C29, C226R, and C8 which formed a higher terminal pH than parental strain JBP in experiment 1 also formed a higher terminal pH upon retesting in experiment 2 (Table 3); however, some variation was noted in the pH values attained by some strains.

In some instances, a high terminal pH was associated with decreased growth as judged by the culture optical density (i.e., strains 157, C22, C29, and C8). However, other strains grew to similar or higher cell densities but still did not produce a low pH, (i.e., strains C222S and C226R).

The acid end products in the spent culture liquors of parental strain JBP and selected animal isolates were determined by gas chromatography. The lower terminal pH attained by strain JBP was paralleled by the formation of higher concentrations of lactic, acetic, and formic acids compared with the four variants tested (Table 4).

Salivary antibodies reactive with *S. mutans* JBP. The presence of IgA, IgG, and IgM antibodies reactive with whole cells of *S. mutans* JBP was determined in pooled saliva samples collected at each sampling period by ELISA. Saliva from uninfected animals at all sampling periods contained low levels of IgA antibodies which reacted with strain JBP (Table 5). However, significantly higher levels of IgA antibodies were present in saliva obtained from animals which had been infected with *S. mutans* JBP for 3 weeks or longer (Table 5). The reactivity of IgG with JBP cells was generally low and tended to fluctuate in both groups of animals (Table 5). No IgM antibodies were detected which reacted with strain JBP.

TABLE 1. *Characteristics of isolates of S. mutans JBP derived from stock cultures and from gnotobiotic rats*

Strain derived from:	No. of isolates with characteristic/total no. of isolates				
	Detectable levels of c antigen	Intracellular glycogen	Smooth colonies on MS agar	Adherent growth in sucrose broth	Agglutination in presence of sucrose
Stock culture	30/30	30/30	0/30	30/30	30/30
Rats (1 wk)	30/30	30/30	0/30	30/30	19/30
Rats (2 wk)	30/30	30/30	0/30	30/30	0/30
Rats (3 wk)	30/30	30/30	0/30	29/30	3/30
Rats (4 wk)	30/30	30/30	0/30	29/30	0/30
Rats (12 wk)	37/60	49/60	30/60	29/60	18/60

Cariogenicity of *S. mutans* JBP and selected variants in gnotobiotic rats. Two separate experiments were performed to assess the cariogenic potential of *S. mutans* JBP and selected variants. In the first experiment, the cariogenic potential of strain JBP was compared with that of strains C29, 157, and C22. These strains varied with respect to colonial morphology, the formation of adherent growth in sucrose broth, the ability to agglutinate upon addition of sucrose, the formation of detectable levels of c antigen, and the terminal pH formed in T. soy-1% glucose broth (Table 6). Parental strain JBP proved highly cariogenic (Table 7) as judged by the number of carious lesions induced in the animals and by the extent of the lesions, especially those which affected the buccal-lingual and sulcal surfaces. In contrast, strains C29, 157, and C22 were much less virulent (Table 7).

TABLE 2. *pH of glucose broth cultures of isolates of S. mutans strain JBP derived from cultures and from gnotobiotic rats*

Strain derived from:	Terminal pH after 48 h in T. soy-1% glucose broth		No. of isolates more than ±0.1 from mean
	Range	Mean	
Stock culture	4.2–4.4	4.3	0/30
Rats (1 wk)	4.3–4.8	4.6	3/30
Rats (2 wk)	4.2–5.1	4.6	7/30
Rats (3 wk)	4.1–4.4	4.4	1/30
Rats (4 wk)	4.2–4.8	4.3	8/30
Rats (12 wk)	4.2–5.6	4.5	13/60

Because all three variants were less active than parental strain JBP, a second experiment was performed in which the cariogenic potential of strains JBP and C29 was retested and compared with two additional variants (strains 164 and 1571R) which had properties more similar to those of strain JBP (Table 6). Strain JBP again exhibited a high cariogenic potential, whereas variant strain C29 was much less active (Table 7). The moderate differences in caries scores on sulcal and proximal surfaces of animals infected with these strains between experiments could be due to chance variation in the rates of emergence of physiological variants in the streptococcal populations or to other experimental

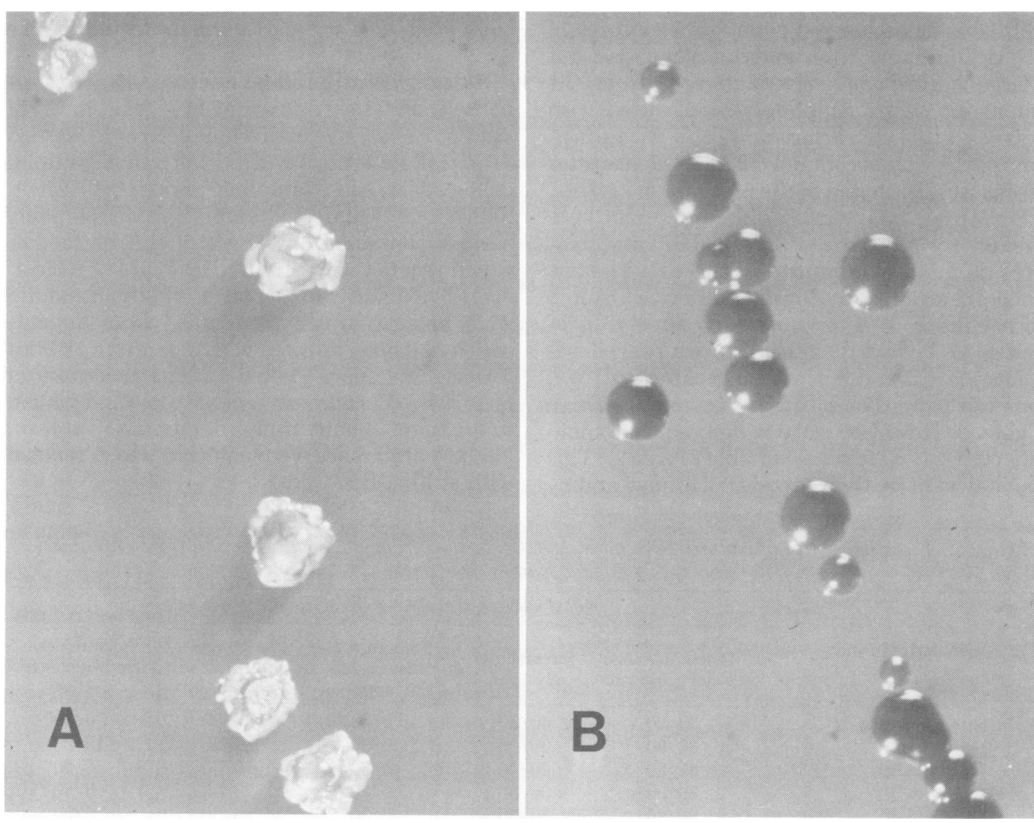

FIG. 1. *Typical rough colonies of S. mutans strain JBP (A) and atypical smooth colonies of variant strain C22 (B) on MS agar.*

variables. The virulence of strains 164 and 1571R appeared to be equal to or greater than that of strain JBP.

Cultural analyses indicated that mixtures of rough and smooth colony types were present in oral swabbings and in fecal samples taken from all animals at the termination of the experiment, irrespective of whether the animals had initially been inoculated with a smooth strain (C22, C29) or a rough strain (JBP, 157, 164, 1571R). Thus, not only do rough colony-type strains yield smooth variants, but smooth strains also readily give rise to rough revertants in the animals. However, the relative reproducibility of the caries scores induced by strains JBP and C29 suggests that the initial colonizing populations largely determined the extent of dental caries which developed over the experimental period utilized.

Effect of immunization on the emergence of smooth-colony variants of *S. mutans* JBP in gnotobiotic rats. We previously observed that prior immunization of gnotobiotic rats accelerated the emergence of antigenic variants of *S. mutans* (4) and *S. salivarius* (23). Therefore, it was of interest to determine whether immunization also accelerated the emergence of physiological variants which could be of altered virulence. Variants forming smooth colonies on MS agar were selected for study because they could be easily enumerated.

Smooth-colony variants were detected in fecal pellets from both immunized and control animals 7 days after infection (Table 8). Their proportions increased with time in the immunized and control animals, but statistically significantly higher percentages were present in fecal samples and in oral swabbings of the immunized animals 14 days after infection (Table 8).

The animals were sacrificed 21 days after infection. At that time, the orally immunized animals had significantly higher levels of IgA antibodies in their saliva and serum which reacted with strain JBP than did the nonimmunized controls (Table 9). In addition, the number of colony-forming units of *S. mutans* recovered from the molar teeth and feces of the immunized animals was markedly lower, whereas the proportions of smooth-colony variants were significantly higher than in control animals (Table 9).

TABLE 3. *pH in T. soy-1% glucose broth cultures of S. mutans JBP and selected variants after 48 h of incubation*

Strain	Terminal pH in expt 1	Terminal pH of triplicate cultures in expt 2			Mean optical density at 550 nm of cultures in expt 2
		1	2	3	
JBP	4.4	4.5	4.5	4.5	1.9
157	4.6	5.2	4.9	4.9	1.5
C22	4.6	4.9	4.9	4.8	1.5
C29	5.5	5.2	5.3	5.4	1.6
164	4.4	4.5	4.7	4.5	2.0
C226R	5.4	5.2	5.4	5.4	2.4
C8	5.6	5.4	5.4	5.3	1.5
1571R	4.5	4.5	4.5	4.6	1.9
C222S	4.5	5.2	5.2	5.3	2.2

TABLE 4. *Growth and acid production by S. mutans JBP and selected variants in T. soy-1% glucose broth*

Strain	Culture turbidity[a]	Terminal pH	μM concn of acid per ml		
			Lactic	Acetic	Formic
JBP	1.9	4.5	18.8	4.8	6.4
157	1.5	5.0	9.0	2.8	4.8
C22	1.5	4.9	12.2	3.2	5.0
C29	1.6	5.3	14.0	3.2	2.6
C222S	2.2	5.2	10.2	3.3	0.0

[a] Optical density at 550 nm.

TABLE 5. *ELISA values of antibodies present in pooled saliva which reacted with S. mutans JBP*

Weeks after infection	ELISA values[a]			
	Uninfected rat saliva[b]		*S. mutans*-infected rat saliva[b]	
	IgA	IgG	IgA	IgG
0	0.84 ± 0.07	0.01 ± 0.01	—	—
1	0.95 ± 0.05	0.10 ± 0.03	0.12 ± 0.07	0.63 ± 0.1
2	0.84 ± 0.03	0.15 ± 0.04	0.73 ± 0.04	0.00 ± 0.01
3	0.80 ± 0.08	0.14 ± 0.06	2.43 ± 0.10	0.16 ± 0.02
4	0.88 ± 0.01	0.01 ± 0.01	2.51 ± 0.01	0.41 ± 0.01
12	0.97 ± 0.01	0.15 ± 0.01	4.53 ± 0.06	0.02 ± 0.02

[a] ELISA values presented as optical density at 400 nM × (60/*t*).

[b] One saliva pool from five animals in each group was tested at a 1:20 dilution; no IgM antibodies were detected which reacted with JBP cells. Results expressed as mean ± standard error of mean. —, Not determined.

DISCUSSION

Populations of viruses (12), protozoa (20), and bacteria (3, 4, 7, 8, 23, 40, 49) have been observed to undergo antigenic variation during the course of infecting mammalian hosts. These population changes are thought to be due to the selection and emergence of antigenically altered mutants when the parental population becomes affected by the immune response of the host (4, 7, 23, 42). Antigenic variation may therefore increase the duration of host-parasite interactions.

TABLE 6. *Characteristics of S. mutans strains tested for cariogenicity*

Strain	Colony type[a]	Intracellular glycogen	Adherent growth in sucrose broth	Agglutination in presence of sucrose	Detectable levels of c antigen	Terminal pH in 1% glucose broth
JBP	R	+	+	+	+	4.4
157	R	+	+	+	+	4.6
C22	S	+	−	−	+	4.6
C29	S	+	−	−	−	5.5
164	R	+	+	−	−	4.7
157 1R	R	+	+	+	+	4.6

[a] R, Rough colony formation; S, smooth colony formation.

TABLE 7. *Dental caries induced in gnotobiotic rats by parental S. mutans strain JBP and isolates derived from animals*

Expt. no.	*S. mutans* strain	No. of animals	No. of lesions	Caries score:			
				Buccal lingual	Morsal	Proximal	Sulcal
1	JBP	10	32 ± 2[a]	104 ± 12	15 ± 5	23 ± 3	75 ± 3
	C29	10	12 ± 2	20 ± 4	3 ± 1	4 ± 1	21 ± 6
	157	10	12 ± 2	21 ± 7	5 ± 2	4 ± 1	20 ± 6
	C22	10	17 ± 2	45 ± 8	4 ± 2	19 ± 3	23 ± 3
2	JBP	10	27 ± 2	104 ± 19	2 ± 1	12 ± 2	48 ± 8
	C29	8	14 ± 2	10 ± 4	5 ± 2	6 ± 2	33 ± 7
	164	9	29 ± 2	130 ± 20	3 ± 2	6 ± 2	63 ± 10
	1571R	10	30 ± 2	153 ± 18	1 ± 1	10 ± 1	73 ± 5

[a] Results expressed as mean ± standard error of mean.

TABLE 8. *Effect of oral immunization on emergence of smooth variants of S. mutans JBP in gnotobiotic rats*

Rats tested (no.)	Days after infection	% Smooth variants detected in[a]:	
		Oral swabs	Fecal pellets
Controls (10)	3	0	0
	7	0	0.42 ± 0.07
	11	ND	0.98 ± 0.19
	14	0.22 ± 0.06	2.41 ± 0.53
Immunized (10)	3	0	0.01
	7	0.1 ± 0.04	0.34 ± 0.05
	11	ND	1.59 ± 0.21
	14	1.08 ± 0.48[b]	5.82 ± 0.40[c]

[a] Approximately 1,375 colonies were examined per sample per animal. Results expressed as mean ± standard error of mean. ND, Not done.
[b] $P < 0.05$.
[c] $P < 0.001$.

Although antigenic changes have been previously monitored in bacterial populations, it is surprising that few other properties of the microorganisms have been examined. We have found that populations of *S. mutans* become altered with respect to a number of physiological as well as antigenic characteristics while colonizing gnotobiotic rats. Since variants were not detected among isolates derived from laboratory stock cultures, it appeared that a selective pressure which led to their emergence was operative in the animals. This could be due in part to the presence of different growth conditions in various portions of the alimentary canal which favored the selection of streptococcal variants best adapted to proliferate in each site. However, it is also likely that the immune response of the animals was at least partially responsible. This was strongly suggested by the observation that prior immunization, which resulted in elevated levels of IgA antibodies in serum and in saliva, accelerated the emergence of smooth-colony variants of *S. mutans* JBP. Moreover, even in non-artifically immunized animals, the emergence of variants was paralleled by an increase in salivary IgA antibodies reactive with parent strain JBP. It is interesting to note that lower, though potentially important levels of IgA antibodies were also detected in the saliva of uninfected germ-free rats which reacted with strain JBP, though their quantity did not increase with time. These antibodies probably were induced to cross-reactive antigens such as lipoteichoic and wall teichoic acids, glucans, etc. present in the diet of the animals. Nevertheless, their presence could affect *S. mutans* cells as soon as colonization was initiated. This may explain why variants are sometimes detected only a few days after infection (23).

TABLE 9. *Variants, IgA antibodies, and S. mutans recovered from gnotobiotic rats 21 days after infection*

Rats tested (no.)	No. of CFU[a] recovered ($\times 10^9$)		% Smooth-colony variants		IgA antibodies[b]	
	6 Molar teeth	Per g of feces	6 Molar teeth	Fecal pellets	Saliva (1:10)	Serum (1:10)
Controls (10)	4.2 ± 0.17[c]	49.0 ± 4.1	0.28 ± 0.12	0.56 ± 0.20	1.03 ± 0.03	1.12 ± 0.03
Immunized (10)	0.039 ± 0.011[d]	0.72 ± 0.4[d]	3.72 ± 0.87[d]	3.20 ± 0.80[d]	1.65 ± 0.02[d]	2.79 ± 0.03[d]

[a] CFU, Colony-forming units of *S. mutans*.
[b] As determined by ELISA.
[c] Results expressed as mean ± standard error of mean.
[d] $P < 0.01$.

The physiological properties selected for study were those which could be expected to affect the virulence of *S. mutans* for initiating dental caries. The production of rough colonies on sucrose-containing media, the formation of adherent streptococcal accumulations in sucrose broth, and the ability to agglutinate in the presence of sucrose all reflect the synthesis of extracellular glucans from sucrose by glucosyltransferases elaborated by the organism (15, 16, 21). In addition, sucrose-induced agglutination is also thought to be dependent upon the presence of a glucan-binding lectin on the surface of the organisms (32). These parameters relate to the ability of *S. mutans* to accumulate on teeth so as to generate sufficient quantities of acid from carbohydrate fermentation to cause enamel demineralization. Alterations were also sought in the synthesis of intracellular glycogen, which affects the rate of endogenous and exogenous acid production (17, 47), and in the terminal pH attained in glucose broth.

Readily isolated from monoinfected animals were variants which were altered in each of the virulence-related properties, and several proved much less cariogenic than parental strain JBP. This loss in virulence is of interest. Intravenous or intraperitoneal passage of avirulent strains of group A streptococci through laboratory animals often results in the selection of organisms with increased virulence (36). Therefore, in an effort to maintain virulence of *S. mutans* some investigators have maintained strains (e.g., 6715) in the mouths of laboratory animals for many generations. However, the data obtained in the present study indicate that pathogenic *S. mutans* populations often lose virulence while colonizing animals and undergoing antigenic and physiological variation. Consequently, the passage of noninvasive organisms which do not have to cope with systemic host defenses in laboratory animals does not necessarily promote, maintain, or ensure virulence.

Many of the isolates obtained from the animals 12 weeks after infection produced atypical smooth colonies, and they failed to form adherent deposits in sucrose broth. Two such strains (C22 and C29) tested proved much less cariogenic than parental strain JBP. These naturally emerging smooth-colony variants appear to be similar, if not identical, to artificially induced mutants studied previously by Freedman and Tanzer (14), Tanzer et al. (44), and by Michalek and co-workers (34) which also failed to form adherent accumulations in sucrose broth, and which possessed diminished cariogenicity. Ikeda and co-workers (24) recently observed that such smooth-colony mutants could revert to rough forms in vitro and in gnotobiotic rats, and the revertants concomitantly regained the ability to form adherent accumulations in sucrose broth. Thus, these appear to be pleiotropic characteristics. Smooth-colony variants were also observed to revert to rough types while colonizing animals in the present study. However, despite their variation, the extent of dental caries which developed appeared to reflect the initial colonizing populations. It is also of interest to note that few of the traits studied appeared to be directly associated with high virulence. For example, all strains tested for cariogenicity synthesized intracellular glycogen irrespective of their virulence. Also, both virulent and avirulent strains could either possess or not possess c antigen, or agglutinate or not agglutinate in the presence of sucrose; this suggests that these characteristics are not essential determinants of cariogenicity. Furthermore, strain 157, of low to moderate virulence, had properties which were qualitatively similar to those of more virulent strains JBP and 1571R (Tables 6 and 7). These observations emphasize that the cariogenic properties of an organism depend upon a complex set of properties.

The question arises as to whether physiological and antigenic variants also emerge in animals and in humans who harbor complex mixtures of potentially competing organisms. Several observations suggest that they do. For example, different reisolates of "tagged" strains of *S. mutans* serially passed in conventional rodents may vary in their cariogenicity (45). In humans, the pop-

ulations of *S. mutans* and *S. salivarius* colonizing discrete surfaces within the oral cavity are antigenically heterogeneous and represent a number of antigenic subtypes which appear to fluctuate over time (23). It has also recently been shown that both the prevalence and the distribution of the major serotypes of *S. mutans* present in plaque repeatedly fluctuate (31). In addition, such streptococcal populations are often comprised of strains of varying bacteriocin types (25). These observations suggest either that the bacterial populations are undergoing variation or that humans are continuously being infected from exogenous sources. Also, strains of *S. mutans* isolated from humans may differ in the extent of caries which they induce in laboratory animals (9, 13); this indicates that organisms of different virulence exist in vivo. Furthermore, *S. mutans* strains of the same serotype can exhibit significant differences in their abilities to attach to saliva-treated hydroxyapatite surfaces (6) and in their minimum dose required to initiate infection in laboratory animals (46). Thus, it is clear that *S. mutans* strains can vary considerably in their physiological and colonizing properties. Finally, it should be noted that Edwardsson (9), in fact, isolated smooth-colony varients from 7% of human saliva samples examined. This estimate is probably low because he used MS agar which is not highly selective for this organism. Also, smooth-colony variants would not be recognized as *S. mutans* in most surveys. In this regard, we noted that all of eight strains which formed smooth colonies on MS agar produced rough colonies when grown on the highly selective MS-bacitracin medium (19) which contains 20% sucrose. Thus, this medium, which allows much greater sensitivity for detecting the organism, does not always permit expression of the smooth-colony trait, presumably because of its very high sucrose content.

Recognition of the antigenic and physiological variation which occurs in populations of *S. mutans* colonizing laboratory animals is important for investigations aimed at developing vaccines for reducing dental caries. Antigenic variation helps to account for the variable experiences which several investigators have reported (29, 45) regarding the efficacy of *S. mutans* vaccines in experimental animal models. The populations of this organism in the oral cavity and intestinal canal of animals a few weeks after infection would only partially resemble the antigenic and physiological makeup of the strain used for making the vaccine. This would be especially so in previously immunized animals, since this procedure accelerates the emergence of variants (4, 23, 40). The present study has also shown that prior immunization accelerates the selection of smooth-colony variants which have an impaired ability to accumulate on solid surfaces and are less virulent. Therefore, part of the mechanism responsible for the reduction in dental caries achieved by immunization would seem to be due to the earlier selection of a less cariogenic streptococcal population. If most of the caries reduction resulting from immunization is due to the selection of less virulent organisms, then its effectiveness would be of only short duration, for variant populations arise naturally without artificial immunization, though at a slower rate.

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