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Virulence of *Streptococcus mutans*: Cariogenicity of *S. mutans* in Adult Gnotobiotic Rats

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Gnotobiotic rats infected with *Streptococcus mutans* 6715, mutant C211 at 45 days of age and provided a purified diet containing 5% sucrose developed carious lesions on buccal, sulcal, and proximal molar surfaces within 15 days (60 days of age). The level of caries increased significantly ($P \leq 0.01$) within the next 15 days (by day 75), and extensive decay was observed on all three molar surfaces of 90-day-old infected rats (45 days after challenge). Mutant C211 was previously shown to exhibit increased glucosyltransferase activity and greater adherence and virulence than *S. mutans* 6715 wild type (wt). Gnotobiotic rats (90 days of age) infected with either *S. mutans* AHT or *S. mutans* 6715 (wt) at 45 days of age developed significantly ($P \leq 0.01$) fewer caries on all molar surfaces than rats of the same age that were infected with *S. mutans* 6715, mutant C211. The level of plaque increased 2-fold, and the number of viable *S. mutans* in plaque increased 10-fold between days 60 and 90 in rats infected with *S. mutans* 6715, mutant C211. Ninety-day-old rats infected with either *S. mutans* AHT or *S. mutans* 6715 (wt) had similar levels of plaque and numbers of *S. mutans* in plaque; however, these values were two- to fourfold lower than those observed in rats of the same age that were infected with *S. mutans* 6715, mutant C211.

Streptococcus mutans has been implicated as a principal microbial agent in the pathogenesis of dental caries in man and experimental animals (14, 20, 21, 23). The ability of *S. mutans* to produce disease is related to its capacity to adhere to the tooth surface (13, 17) and produce organic acid (2, 3, 20) when sucrose serves as substrate. The ability of *S. mutans* to adhere to hard surfaces has been correlated primarily with the presence of a cell-associated glucosyltransferase (14, 18). This enzyme synthesizes high-molecular-weight, insoluble glucans as well as low-molecular-weight oligosaccharides that contribute to plaque formation and facilitate the adherence of *S. mutans* to teeth (11, 12, 13, 17, 24). Results of investigations employing mutants of *S. mutans* also support the concept that the ability of this microorganism to synthesize cell-associated glucans and adhere to hard surfaces are among the determinants of virulence (5, 29, 33). Mutants of *S. mutans* that could not form adherent masses on hard surfaces or synthesize cell-associated glucans in vitro were less virulent than the wild type (wt) in monoinfected gnotobiotic rats (5, 33). Other mutants of *S. mutans* that exhibited either more or less glucosyltransferase activity and adherence in vitro than the wt parent strain were greater or less virulent, respectively, than the wt in gnotobiotic rats (29).

The germfree rat has been widely employed in studies to determine the caries-inducing potential of microorganisms (15). These studies usually entailed mono-infecting rodents with a selected oral bacterium and providing a caries-promoting diet for various periods of time (9, 11, 15, 28, 33). Because the young rat is more susceptible to a caries attack, notably during the period of tooth maturation (22), maximum caries develop when infection with *S. mutans* occurs prior to 25 days of age (24, 28).

The purpose of this study was to develop an adult gnotobiotic rat model to study the virulence of *S. mutans*. This model system would employ hypercariogenic strains of *S. mutans* for studies of virulence factors of *S. mutans* and subsequently allow investigation of immunity to *S. mutans*-induced dental caries in a young adult host.

MATERIALS AND METHODS

Microorganisms and growth conditions. *Streptococcus mutans* AHT (serotype *a*), *S. mutans* 6715 (wt) (originally classified as type *d* but more recently as type *g*; 1,4), and *S. mutans* 6715, mutant C211 (29) were employed in this study. *S. mutans* 6715, mutant C211 was isolated after treatment of *S. mutans* 6715 (wt) with *N'*-nitro-*N*-nitrosoguanidine and previously shown to exhibit higher glucosyltransferase activity, greater adherence in vitro, and greater cariogenicity in 35-day-old gnotobiotic rats

that were infected at 19 days of age and provided a caries-promoting diet (no. 305) than did the wt strain (29). Stock cultures of each strain were maintained at 4°C in brain heart infusion agar stabs containing excess calcium carbonate. For animal infectivity, cultures of each test bacterium were grown at 37°C in an atmosphere of 5% carbon dioxide and 95% nitrogen in capped tubes containing brain heart infusion broth. An 18-h broth culture of the appropriate *S. mutans* strain was introduced into the isolator just prior to infection (described below).

Gnotobiotic rats and experimental design. Germfree Fischer rats [CD F(344) GN, original breeding stock obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass.] were employed in this study. All rats were housed in plastic box cages containing hardwood laboratory bedding (Northeastern Products, Corp., Warrensbury, N.Y.) and maintained in Trexler plastic isolators (28, 34). Adult breeding rats were provided Wayne Lab-Blox sterilizable rat chow (Allied Mills Inc., Chicago, Ill.) and sterile drinking water ad libitum. After parturition, eight to nine pups were assigned to each nursing dam. The overall experimental design employed in this study is presented in Fig. 1. At weaning (20 days of age), equal numbers of male and female offspring were transferred to separate experimental isolators and provided a sterile cariogenic diet (no. 305; 28) and drinking water ad libitum.

At 45 days of age, each rat was challenged orally with a 50-μl inoculum of a culture (2.5×10^8 to 4.0×10^8 colony-forming units/ml) of the appropriate *S. mutans* strain with the aid of a micropipette. On the day after challenge and at weekly intervals thereafter, fecal samples and oral swabs were collected from individual animals and cultured on blood agar (with brain heart infusion base) and Mitis-Salivarius agar. Cultures were incubated at 37°C under anaerobic conditions to verify colonization of the infecting *S. mutans* and insure the absence of other microbial contaminants.

Rats infected with mutant C211 were removed from isolators at either 60, 75, or 90 days of age (days 15, 30, or 45), whereas all noninfected controls and rats infected with either *S. mutans* AHT or *S. mutans* 6715 (wt) were removed from the isolators at 90 days of age (day 45). All rats were weighed prior to sacrifice performed by cardiac exsanguination.

Plaque analysis. On the day of sacrifice, both mandibles from each rat were aseptically removed and defleshed with the aid of a sterile scalpel. The right mandible was stained with safranin (2%) for 3 min, and the level of plaque on molar surfaces was recorded by drawing the plaque pattern on score sheets, with pictures used to depict the buccal, morsal, and lingual molar surfaces. The degree of the molar surfaces covered with plaque was scored by a modification of the Keyes procedure for caries evaluation (19). Each molar surface was divided into three regions, and the amount of plaque in that region was evaluated using the following scores: 1+, plaque on less than half the region; 2+, plaque on more than half the region; and 3+, entire region covered with plaque. A maximum score for one molar surface would be 9, and the maximum score per mandible would be 27. Total plaque was collected from the molar surfaces of the left mandible with the aid of a sterile dental explorer and tranferred into sterile 0.067 M phosphate buffer (pH 7.2). Plaque samples were sonically treated (sonifier cell disruptor; Branson, Plainview, N.Y.) for 15 s at a setting of 30. Samples were diluted, plated on blood agar and Mitis-Salivarius agar, and incubated at 37°C in an atmosphere of 5% carbon dioxide and 95% nitrogen.

The group specificity of the *S. mutans* isolates obtained from individual animals was determined by sugar fermentation (7, 16, 32) and serological testing with type-specific antisera. These antisera were prepared in rabbits by daily intravenous administration of heat-killed *S. mutans* whole cells (2.5×10^8 colony-forming units) for 3 consecutive days (1). This schedule was repeated for 2 additional weeks with twice the original dosage, and the animals were test bled 7 days after the last injection. Subsequent injections were given as required for

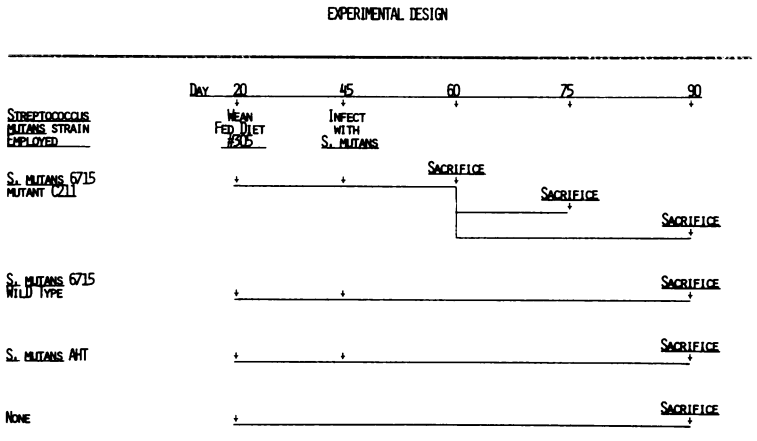

FIG. 1. *Experimental design employed to evaluate the cariogenicity of S. mutans AHT, 6715 (wt) and 6715, mutant C211 in adult gnotobiotic rats.*

maximum precipitating antibody titer when tested by Ouchterlony analysis with Lancefield antigen preparations of the appropriate *S. mutans* strain. Ouchterlony analysis of antisera for Lancefield preparations of *S. mutans* AHT (serotype *a*) yielded two bands, one of which was cross-reactive with extracts of *S. mutans* 6715 (serotype *d*). This latter band was removed by adsorption with whole *S. mutans* 6715 cells, and the adsorbed antiserum was specific for Lancefield preparations of serotype *a*. All antisera to *S. mutans* 6715 were adsorbed with *S. mutans* AHT cells and yielded a monospecific serotype *d* antiserum.

Caries scoring. Individual mandibles were stained with murexide, molars were hemisectioned, and buccal, sulcal, and proximal molar surfaces were scored for caries by using the Keyes procedure (19). The caries scores from each group of rats were statistically reduced by computing means, standard deviations, and standard errors. Differences among means were evaluated by an analysis of variance and by multiple mean comparisons using the Duncan test (6).

RESULTS

Studies with *S. mutans* 6715, mutant C211. In the first series of experiments, rats were infected with mutant C211 when they were 45 days of age, and groups of animals were sacrificed at 60, 75, and 90 days of age (days 15, 30, and 45 after challenge). After only 15 days of infection with this bacterium, animals developed moderate numbers of carious lesions (Table 1). The number of lesions was significantly increased ($P \leq 0.01$) on the buccal, sulcal, and proximal molar surfaces in 30 days, and extensive decay was observed in 45 days in rats challenged with this mutant of *S. mutans* (Table 1). These results support earlier findings that mutant C211 is highly cariogenic in gnotobiotic rats (29). Furthermore, these results suggest that, within 45 days, this mutant can cause extensive lesions on buccal, sulcal, and proximal molar surfaces in young adult rats fed a diet containing 5% sucrose.

Studies with *S. mutans* AHT and *S. mutans* 6715 (wt). Because 45-day-old rats infected with mutant C211 developed extensive lesions on the three molar surfaces in 45 days when fed diet no. 305, the cariogenic potential of *S. mutans* strains AHT and 6715 (wt) were determined under the same experimental conditions (Fig. 1). Rats infected at 45 days of age with either *S. mutans* AHT or *S. mutans* 6715 (wt) had moderate enamel, dentinal buccal and sulcal lesions, and essentially no enamel or dentinal lesions on the proximal surfaces in 45 days (Table 2). Both infected and noninfected rats exhibited similar weight gains.

The extent of decay in rats infected with either *S. mutans* AHT or *S. mutans* 6715 (wt) was significantly lower ($P \leq 0.01$) than the

TABLE 1. *Development of caries on the mandibular molar surfaces of gnotobiotic rats infected with S. mutans 6715 mutant C211 at 45 days of age and fed diet no. 305*

Age at sacrifice (days)	No. of rats/ group	Mean caries scores[a]					
		Buccal		Sulcal		Proximal	
		Enamel	Dentinal (slight)	Dentinal (slight)	Dentinal (moderate)	Enamel	Dentinal (slight)
60	17	5.7 ± 0.5	3.5 ± 0.5	10.9 ± 0.8	4.2 ± 0.8	2.2 ± 0.5	1.2 ± 0.3
75	16	12.4 ± 1.0	10.1 ± 1.0	16.7 ± 0.4	8.1 ± 0.5	5.7 ± 0.5	4.2 ± 0.5
90	24	23.6 ± 0.5	18.5 ± 0.8	21.8 ± 0.7	17.9 ± 0.7	7.5 ± 0.2	7.1 ± 0.2

[a] Evaluated by the Keyes procedure (19). Values represent means ± standard error.

TABLE 2. *Comparison of virulence of S. mutans strains in 90-day-old gnotobiotic rats infected at 45 days of age and fed diet no. 305*

S. mutans strain	No. of rats/ group	Mean caries scores[a]						Mean body wt[b]
		Buccal		Sulcal		Proximal		
		Enamel	Dentinal (slight)	Dentinal (slight)	Dentinal (moderate)	Enamel	Dentinal (slight)	
AHT	15	8.1 ± 0.3	5.3 ± 0.5	8.0 ± 1.1	4.2 ± 0.5	0.0	0.0	139.6 ± 5.1
6715 (wt)	22	7.0 ± 0.3	4.4 ± 0.3	8.0 ± 0.4	3.5 ± 0.4	0.6 ± 0.2	0.2 ± 0.1	138.5 ± 5.7
6715, Mutant C211	24	23.6 ± 0.5	18.5 ± 0.8	21.8 ± 0.7	17.9 ± 0.7	7.5 ± 0.2	7.1 ± 0.2	141.5 ± 3.6
Noninfected	16	0.0	0.0	0.0	0.0	0.0	0.0	143.7 ± 4.1

[a] Evaluated by the Keyes procedure (19). Values represent means ± standard error of caries on the mandibular molar surfaces.

[b] Expressed in grams ± standard error.

level of caries observed in molars of rats that were infected with mutant C211. The severity of decay on the buccal molar surfaces of 90-day-old rats infected with mutant C211 (45 days after challenge) is illustrated in Fig. 2A. This is in contrast to that observed on the molar surfaces of rats of the same age that were infected with *S. mutans* 6715 (wt) (Fig. 2B), which is also typical of the pattern observed in rats infected with *S. mutans* AHT. Control, noninfected rats fed diet no. 305 were caries-free (Fig. 2C). These findings clearly demonstrate that rats infected at 45 days of age with *S. mutans* AHT and *S. mutans* 6715 (wt) which were previously shown to be highly virulent in young, weanling gnotobiotic rats fed diet no. 305 (28, 29), develop only moderate carious lesions. On the other hand, *S. mutans* 6715, mutant C211 is highly virulent in rats infected at either day of weaning (29) or at 45 days of age when provided a caries-promoting diet (no. 305) containing 5% sucrose.

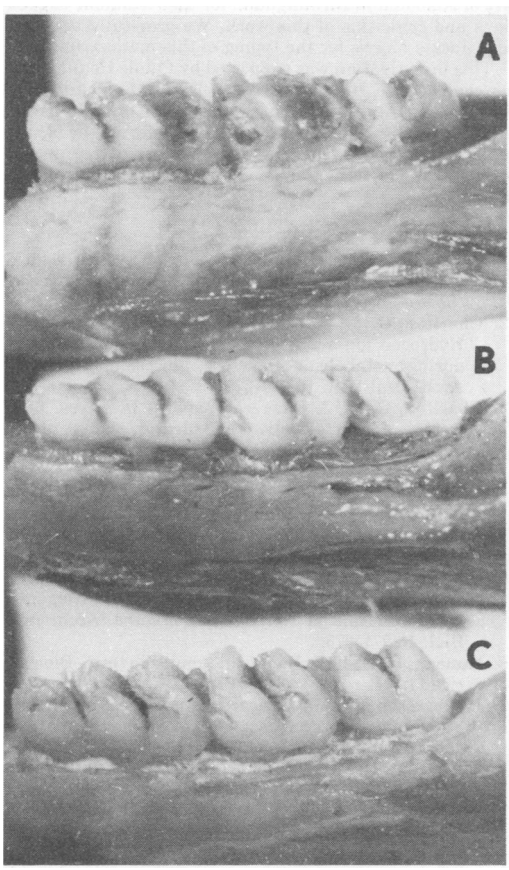

FIG. 2. *Carious lesions in 90-day-old gnotobiotic rats fed caries-promoting diet (no. 305) (×12.5). (A) Infected with S. mutans 6715, mutant C211. (B) Infected with S. mutans 6715 (wt). (C) Noninfected controls.*

Plaque evaluation in young adult gnotobiotic rats. In an attempt to correlate cariogenicity with the extent of plaque accumulation, the amount of smooth surface plaque and the number of viable *S. mutans* present in this material from each infected rat were assessed. Between days 15 and 45 after challenge, rats infected with mutant C211 demonstrated a 2-fold increase in their plaque score (5.5 and 12.3, respectively), a 10-fold increase in the number of viable *S. mutans* in plaque (1.3×10^5 and 1.2×10^6 colony-forming units per mandible, respectively), and a 2- to 5-fold increase in caries (Table 1). Similar amounts of plaque, numbers of viable *S. mutans* (Table 3), and levels of caries lesions (Table 2) were observed in 90-day-old rats infected with either *S. mutans* AHT or *S. mutans* 6715 (wt). However, these values were approximately two- to fourfold lower than those obtained from rats infected with mutant C211. *S. mutans* could be isolated from each infected rat, and these isolates always demonstrated morphological, biochemical, and serological characteristics identical with the original infecting strain of *S. mutans*. These results suggest that the amount of smooth surface plaque and the number of viable *S. mutans* present on the molars of rats infected with *S. mutans* and provided a caries-promoting diet (no. 305) correlates with caries activity.

DISCUSSION

Studies of immunity to *S. mutans*-induced dental caries in rodent models have been limited, because the rat must receive the cariogenic challenge early in life, prior to immunological maturity, if maximum carious lesions are to develop (22, 24, 25, 28). Because the first and second molars of the rat erupt between 16 and 21 days of age, the animals are usually challenged with cariogenic bacteria between 19

TABLE 3. *Comparison of the amount of smooth surface plaque and the number of viable S. mutans in plaque of 90-day-old gnotobiotic rats*

S. mutans strain	Plaque score[a]	No. of viable *S. mutans* (CFU)[b]
AHT	3.0 ± 0.2	4.8×10^5
6715 (wt)	5.6 ± 0.4	5.2×10^5
6715, Mutant C211	12.3 ± 0.3	12.0×10^5

[a] Represents mean value of smooth surface plaque on one mandible per rat per group ± standard error.

[b] Represents mean number of colony-forming units (CFU) in plaque from one mandible per rat per group as determined on blood and Mitis-Salivarius agar.

and 24 days of age (8, 24, 28, 29, 33). After eruption of the molars, mineralization and maturation occurs, and the teeth become increasingly resistant to carious attack (15, 22, 24). If typical strains of *S. mutans* are used as challenge and the animals are fed a caries-promoting diet, the rats will develop lesions within 3 to 10 weeks, depending upon the strain of rat employed and other experimental conditions (5, 8, 22, 28, 29, 33).

Various alterations in experimental design were made to increase the susceptibility of the rat to caries attack (22). The most common method involves use of purified diets that may contain as much as 67% sucrose (5, 8, 30, 33). However, it was recently found that high levels of sucrose are not always necessary to promote the development of caries in gnotobiotic rats, because a purified diet containing only 5% sucrose promotes the development of carious lesions in gnotobiotic rats infected with *S. mutans* (26, 28, 29). These studies were performed in young, weanling rats, and the experiments were terminated within 2 to 4 weeks.

The results of this study clearly show that mutant C211 can colonize molar surfaces of 45-day-old rats and induce significant levels of carious activity within 2 weeks. It should be pointed out that animals infected with this mutant develop a similar amount of decay during this 2-week period as do rats infected with either *S. mutans* AHT or *S. mutans* 6715 (wt) develop in 45 days. On the other hand, rats infected at 45 days of age with *S. mutans* 6715, mutant C211 developed moderate lesions on all molar surfaces within 30 days and rampant decay within 45 days.

It is of interest that plaque scores and recoverable numbers of *S. mutans* from plaque correlated with the severity of the caries lesions. The plaque scores and the level of caries in 60-day-old rats infected with *S. mutans* 6715, mutant C211 (day 15) were similar to the scores obtained for 90-day-old rats infected with either *S. mutans* AHT or *S. mutans* 6715 (wt) (day 45). The level of plaque, the number of *S. mutans* in plaque, and the level of caries in mandibular molars increased markedly between days 15 and 45 in rats infected with mutant C211. These findings further support the evidence that this mutant is highly cariogenic in this model and that a positive correlation exists between *S. mutans* plaque and caries activity. It should be pointed out, however, that one must exercise caution in utilizing bacterial mutants that exhibit enhanced virulence when making comparisons to the more standard *S. mutans* isolates until a more clear understanding of all factors responsible for virulence have been delineated.

We report here a sensitive, gnotobiotic rat model for studies of *S. mutans* pathogenesis in a mature host. This model could be useful in several aspects of caries research. Because mutants of *S. mutans* have more in vitro biochemical activity, such as glycosyltransferase production (31), and adherence (29) than the wt strain, this microorganism could allow characterization of factors important in their virulence. Studies of immunity to *S. mutans* can be performed in a sensitive, immunologically mature gnotobiotic rat model, and this should allow an evaluation of the host response to challenge with this bacterium.

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