Effective Immunity to Dental Caries: Passive Transfer to Rats of Antibodies to *Streptococcus mutans* Elicits Protection

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Rat dams, given intravenous injections of heat-killed *Streptococcus mutans* 6715, mutant C211 demonstrated significant agglutinin activity to the homologous *S. mutans* in colostrum, milk, and serum. This antibody activity was associated with the immunoglobulin G (IgG) class. High titers of anti-*S. mutans* antibody associated with the IgG class were also exhibited in the sera and saliva of the offspring that suckled these dams. After challenge with the homologous, live *S. mutans*, these offspring developed significantly fewer caries on all molar surfaces than did nonimmunized infected controls. A secretory immune response (manifested by the presence of specific IgA antibody to *S. mutans* in colostrum and milk) was elicited (i) in rat dams locally injected, in the region of the mammary gland, with heat-killed *S. mutans* antigen, and (ii) in other rat dams that were provided formalin-killed *S. mutans* in their drinking water. Offspring suckling these dams were challenged with virulent *S. mutans* before weaning and developed significantly fewer caries than did their infected controls. These findings clearly suggest that passively derived IgG or IgA antibodies to *S. mutans* are protective against dental caries.

The bacterial disease dental caries is perhaps the most prevalent disease affecting man today (27). The ability of *Streptococcus mutans,* in the presence of sucrose, to adhere to and produce acid at the tooth surface is currently thought to be among the major determinants of the virulence of this bacterium in causing dental caries (9). The possibility of controlling this disease by immunization is currently being extensively investigated (3). Dental caries in man develops in the presence of the oral secretion saliva, which contains locally produced immunoglobulin A (IgA) as the predominant antibody (11). Recent studies have demonstrated that injection of *S. mutans* into the salivary-gland region or direct instillation into the parotid duct stimulated a salivary antibody response (6, 19, 32). Other studies have indicated that specific antibody in the IgA class can be induced in salivary- and mammary-gland secretions by oral administration of *S. mutans* antigen (22). In most of these studies, the salivary immune response could be correlated with a reduced incidence of dental caries (19, 22, 32).

Other investigators have suggested the importance of milk antibody in protecting infants against enteric infections, such as *Escherichia*

coli-induced diarrhea (12, 16). Recent experimental animal studies support the concept that colostral antibodies to *E. coli* or *Vibrio cholerae* are passively transferred and protect offspring (24, 30, 34). From these and other findings, it was proposed that protective IgA antibodies may function by: (i) immobilizing the bacteria, as was recently suggested in the case of *V. cholerae* (5, 10); (ii) preventing adherence of bacteria to mucosal surfaces (7, 8); and (iii) inducing aggregation of bacteria (2, 28, 29). Preliminary studies in this laboratory have suggested that caries immunity can be passively derived in rat pups suckling dams that were injected intravenously (i.v.) with *S. mutans* (18). However, the antibody class responsible for protection was not determined. We report that the rat dams immunized through different routes with *S. mutans* exhibited specific colostral and milk antibodies of either the IgA or IgG class. The offspring of these dams were protected against *S. mutans*-induced dental caries.

MATERIALS AND METHODS

Animals. Two strains of rats were used: (i) antibiotic-suppressed (AS), Sprague-Dawley-derived COBS/CD; and (ii) gnotobiotic (GN) Fischer CD F(344)GN (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). The AS rats were treated with selected antibiotics and maintained under sterile

housing conditions, as previously described (21). The GN rats were maintained in Trexler plastic isolators (22, 33) until the day of parturition. At this time, GN dams and their offspring were removed from the isolators and subsequently maintained under housing conditions similar to those for AS rats.

Microbiology and immunogen preparation. *S. mutans* 6715, mutant C211 was used in this study. This mutant, in comparison with the wild-type strain (23), exhibited greater virulence with respect to glucosyltransferase activity, adherence, and caries-promoting ability. Stock cultures were maintained at 4°C in brain heart infusion agar stabs that contained excess calcium carbonate. An 18-h culture of this bacterium, grown in brain heart infusion broth at 37°C in an atmosphere of 5% carbon dioxide and 95% nitrogen, was used to infect animals (described below) and prepare immunogen.

For antigen preparation, cultures of *S. mutans* 6715, mutant C211 were grown in a tryptose-dialyzed medium, as previously described (19). One portion of washed cells was adjusted to 5×10^8 colony-forming units (CFU) per ml and heated at 60°C for 30 min. With this heat-killed antigen preparation, rats were injected through either i.v. or intramammary routes (described below). The remaining washed cells were suspended in 0.5% Formalin-saline. The killed cells were washed, suspended in 0.1% Formalin-saline (2×10^9 CFU/ml), and used as antigen for oral immunization of rats as previously described (22).

Experimental design. The AS dams in groups A and C were given i.v. injections in the lateral tail vein of heat-killed *S. mutans* 6715, mutant C211 (5×10^8 CFU/ml) for 3 consecutive days in each of 4 successive weeks (Fig. 1). The antigen dosage during week 1 was 0.5 ml daily. Dosage was increased by 0.5 ml for each of the 3 successive weeks, and a final, single injection (2.5 ml) was administered 4 days before parturition. The AS dams in group D were given intramammary injections of heat-killed *S. mutans* 6715, mutant C211 (5×10^8 CFU/ml; without adjuvant) twice weekly for 2 weeks and at weekly intervals after conception and before parturition. Week 1 injections (0.5 ml) were administered in five different sites in the region of the mammary-gland tissue. Subsequent injections (1 ml) were administered in 10 different sites in this tissue. The AS dams in groups B and E served as nonimmunized controls.

The GN dams in group F were provided Formalin-killed *S. mutans* 6715, mutant C211 in drinking water (10^8 CFU/ml) (22). Rat dams in groups F and G were monoinfected previously (age 45 days) with *S. mutans* 6715, mutant C211 and were provided defined caries-promoting diet no. 305 (25). All rat dams (15 per group) were bred (Fig. 1), and, after parturition, litters were reduced to 8 pups per dam. Samples of colostrum, milk, and serum were collected (described below). When weaned (age 20 days), all pups (groups A to G) were provided purified diet no. 305. At age 21 days, pups in groups A and B were challenged orally
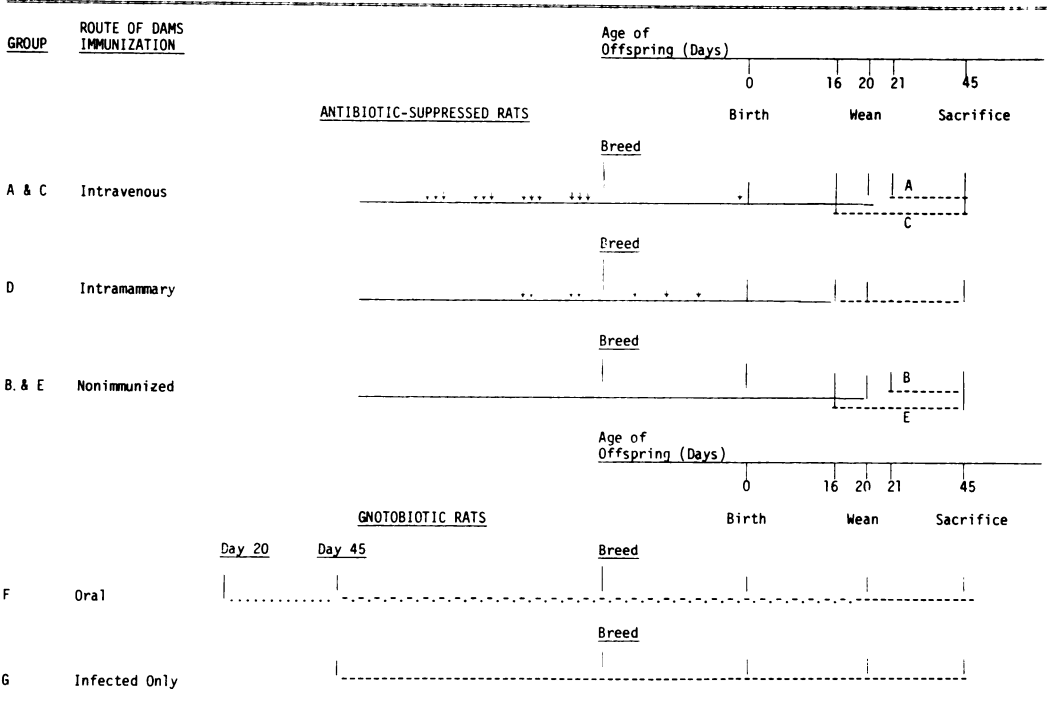

FIG. 1. *Experimental design employed in passive immunity studies. Symbols: ($\downarrow$) rats injected with S. mutans 6715, mutant C211; (----) rats infected with known inocula of S. mutans 6715, mutant C211 and provided diet no. 305; ($\cdots$) rats provided S. mutans 6715, C211 antigen (10^8 CFU/ml) in drinking water.*

with a 50-μl inoculum of an 18-h culture of mutant C211 (5.5×10^6 to 6.0×10^6 CFU). Pups in groups C, D, and E received similar treatment 4 days before weaning (at age 16 days, when molars begin to erupt) and were provided diet no. 305. In groups F and G, natural colonization of *S. mutans* 6715, mutant C211 occurred in all offspring by infection derived from their mothers. Before sacrifice (age 45 days), serum and saliva were collected from individual animals and stored ($-20°C$) until used. After sacrifice and removal of mandibles, caries were scored by the method of Keyes (13).

Collection of rat colostrum, milk, saliva, and serum. Colostrum, milk, and serum were collected from each rat dam at 2- to 5-day intervals throughout lactation (17). Colostrum and milk were centrifuged at $20,000 \times g$ for 2 h at $4°C$, and the whey was collected from between the insoluble material and lipid. Whey and serum samples were stored at $-20°C$ until assayed. Serum samples were collected from offspring of i.v.-injected mothers (groups A and C) each time samples were obtained from the dams. Additional serum and saliva samples were collected from these pups on days 25, 30, 37, and 45 (sacrifice). Serum and saliva were collected from all rats (groups A to G) at age 45 days. All samples were stored ($-20°C$) until assayed.

Chromatographic fractionation. After decaseination (20), whey samples (1.0 ml) were chromatographed on Sephadex G-200 columns (1.6 by 100 cm; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). Peak fractions were pooled and concentrated by negative-pressure dialysis to the original sample volume (1.0 ml). Similar chromatographic procedures were employed on a limited number of serum and saliva samples from rat offspring. The level of immunoglobulin (sensitivity of 0.20 mg/100 ml of IgG, 0.20 mg/100 ml of IgM, and 0.40 mg/100 ml of IgA) in each peak fraction was determined by the radial immunodiffusion technique, using antisera to rat γ, μ, and α immunoglobulins (17).

Antibody assays. All whey, serum, and saliva samples as well as column fractions were assayed in duplicate for agglutinin activity to *S. mutans* 6715, mutant C211 by the microtitration technique (1). Plates were incubated at $37°C$ (2 h) and then incubated overnight at $4°C$, after which the agglutinin pattern was recorded. In separate experiments, the agglutinin activity in selected samples was determined before and after enhancement with optimum concentrations of either anti-rat α or anti-rat γ heavy-chain sera (17, 19).

Caries evaluation. At age 45 days (sacrifice), individual mandibles from all rats were removed, cleaned, and stained; then the buccal, sulcal, and proximal molar surfaces were evaluated for caries by the method of Keyes (13). The caries scores from each group of animals 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 (4).

RESULTS

Antibody response to *S. mutans* in i.v.-injected rat dams and their offspring. Rat dams given i.v. injections of *S. mutans* 6715, mutant C211 (groups A and C, Fig. 1) exhibited high levels of serum agglutinin activity (Table 1). Throughout lactation, colostrum and milk whey samples collected from these dams also exhibited significant antibody levels; but, during this same period, the level of antibody tended to decrease slightly in both serum and whey. However, levels greater than 512 ($\log_2 = 9$) for serum and 64 ($\log_2 = 6$) for whey were observed late in lactation. On the other hand, in the offspring, levels of serum antibody to *S. mutans* steadily increased during this period; newborn pups had levels of less than 4 ($\log_2 = 2$), indicating that very little antibody was transferred across the placenta. However, significant agglutinin activity occurred during suckling; by day 15, high titers ($\log_2 = 8$) were observed. After weaning, the level of serum antibodies steadily decreased, but significant titers ($\log_2 = 5$) were detected as late as age 45 days (sacrifice). Of even greater importance from the standpoint of caries immunity was the observation that significant levels of salivary agglutinins to *S. mutans* occurred in these offspring.

Characterization of immunoglobulin agglutinin class in i.v.-immunized dams and

TABLE 1. *Mean agglutinin titers ($\log_2$) to S. mutans 6715, mutant C211 in colostrum, milk, and sera of i.v.-injected rat dams and in sera and saliva of their offspring*[a]

Group	Sample	Mean agglutinin titers ($\log_2$)								
		Day of lactation					Age of offspring (day)			
		2	5	10	15	19 to 20	25	30	37	45
Dams	Serum	11.5 ± 0.4	11.6 ± 0.8	9.8 ± 0.3	8.4 ± 0.4	9.1 ± 0.9				
	Whey	7.7 ± 0.3	7.9 ± 0.5	6.4 ± 0.4	6.8 ± 0.4	6.3 ± 0.6				
Offspring	Serum[b]	1.8 ± 0.2	4.3 ± 0.7	6.1 ± 0.5	8.0 ± 0.4	6.9 ± 0.5	6.5 ± 0.3	6.1 ± 0.7	5.2 ± 0.5	5.0 ± 0.4
	Saliva	—[c]	—	—	—	3.9 ± 0.6	3.1 ± 0.5	2.6 ± 0.3	2.1 ± 0.4	2.3 ± 0.2

[a] Values represent mean ± standard error of 10 rat dams and 32 offspring.

[b] Mean agglutinin titer of 1.9 ± 0.4 (range, 1.0 to 3.0) was observed in pup serum immediately after birth (16 pups from a total of 8 litters were sampled).

[c] —, No sample collected.

their offspring. To determine the class of antibody to *S. mutans* that was present in whey and was passively derived by offspring, samples of whey and of pup serum and saliva were fractionated by column chromatography (Fig. 2A). Most of the agglutinin activity in the whey of i.v.-immunized dams was associated with the second peak from G-200, a fraction rich in IgG.
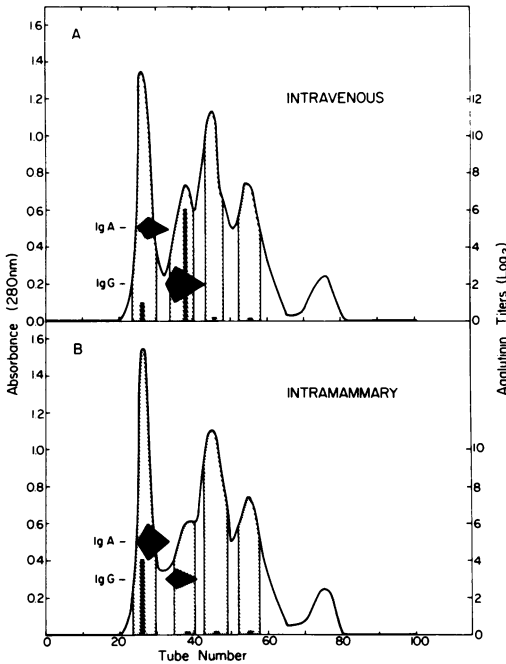

FIG. 2. *Sephadex G-200 profiles of whey from rat dams immunized by either i.v. (A) or intramammary (B) routes. Immunoglobulins in fractions determined by radial immunodiffusion with monospecific antisera (solid triangles). Agglutinin titers (solid bars) to S. mutans 6715, mutant C211 were determined in pooled peak fractions (diagonal-lined areas) by the microtitration technique. Representative of four whey samples from each group.*

This agglutinin activity was significantly enhanced after the addition of anti-rat γ heavy-chain sera, although no augmentation occurred after the addition of anti-rat α serum (Table 2). That the antibody to *S. mutans* in whey was of the IgG class and was responsible for passively derived antibody in offspring was suggested when serum from these offspring displayed its greatest activity in the second peak from G-200. The agglutinin activity was augmented with anti-rat γ serum, thus suggesting its IgG nature. Saliva from these offspring contained significant levels of antibody to *S. mutans* that was associated with the IgG class.

Intramammary and oral immunization studies. Local application of *S. mutans* whole-cell antigen in the region of the mammary tissue resulted in the production of significant levels of antibody in colostrum and milk (Table 3). Addition of anti-rat α heavy-chain serum significantly enhanced the agglutinin titer, suggesting that the antibody was in the IgA class, a fact further confirmed by the demonstration that almost all the agglutinin activity was associated with the first peak of whey fractionated through G-200 (Fig. 2B) and was significantly enhanced by the addition of anti-rat α serum. No augmentation of activity was observed subsequently to the addition of anti-rat γ serum, nor could any antibody activity be associated with the IgG-rich second peak from G-200 (Fig. 2 and Table 3). It was also observed that less IgG was present in colostrum of dams locally injected as compared with dams immunized by the i.v. route (Fig. 2).

The GN rats (group F) that were provided *S. mutans* antigen in their drinking water had high titers ($\log_2 = 8$; Table 3) of antibody in their milk. As previously shown, this activity was associated with the IgA-rich first-peak fraction from G-200 and was significantly enhanced after the addition of optimum concentrations of anti-rat α serum. It should be pointed out that milk

TABLE 2. *Agglutinin titers ($\log_2$) to S. mutans 6715, mutant C211 in whole, and Sephadex G-200 fractions of, pooled whey samples from i.v.-immunized dams and pooled sera and saliva from their offspring[a]*

Group	Sample	Whole	G-200 fraction[b]	
			Peak one	Peak two
Dams	Whey	7 (6, 10)[c]	1 (1, 2)	6 (6, 9)
Offspring (age, 21 days)	Serum	7 (7, 11)[d]	2 (1, 2)	5 (5, 8)
	Saliva	4 (2, 7)	0 (0, 1)	4 (3, 6)

[a] Mean serum agglutinin titer ($\log_2$) in 10 rat dams was 11.9 ± 0.7. Low to undetectable agglutinin activity was observed in samples from control animals.

[b] Values obtained from four whey, five sera, and five saliva chromatographed samples.

[c] Agglutinin titers after enhancement with optimum concentrations of anti-rat α and anti-rat γ sera, respectively. No IgM could be detected in whey from these dams, as determined by radial immunodiffusion (17).

[d] No enhancement was observed after the addition of optimum concentrations of anti-μ heavy-chain serum.

samples (days 16 to 20 of lactation) from both orally and intramammary-immunized dams contained significant levels of IgA antibody to *S. mutans* ($\log_2 = 4$ and 7, respectively), whereas low to undetectable agglutinin activity was demonstrated in sera and saliva from their offspring.

Immune protection by passively derived antibody. When i.v.-immunized mothers displayed high levels of IgG antibodies in colostrum and milk, their offspring were protected from caries formation when challenged with the live, virulent organism (Table 4). Pups challenged at age 16 days exhibited significantly less ($P < 0.01$) caries activity than controls (group D) and in some cases, significantly fewer caries than similarly derived offspring infected at age 21 days (1 day postweaning). The latter off-

spring exhibited significantly fewer caries than their controls. These results strongly suggest that the IgG antibodies derived from milk were protective in this system. The demonstration of specific IgG antibodies in serum and saliva of offspring in group A further suggests, but does not prove, that passively derived milk antibodies were absorbed by the pups and reached high levels in their serum and, in turn, transduded into saliva, where they exerted a biological protection against caries formation. On the other hand, rat dams exhibiting significant colostral and milk IgA antibody to *S. mutans* (groups D and F) conferred significant caries protection to their offspring. These findings clearly support the important role of secretory IgA in caries immunity.

DISCUSSION

Previous investigations suggested that passively acquired milk antibodies in diarrheal enteropathies caused by *E. coli* (24, 30) or *V. cholerae* (30) may be protective. Nevertheless, definitive studies that associate antibodies in milk with infant protection have yet to be demonstrated. The present investigation suggests that colostral antibodies in both the IgA and IgG class protect offspring against challenge with a highly cariogenic strain of *S. mutans*.

From the experiments reported here, it was apparent that the i.v.-immunization regimen induced the production of high levels of specific IgG antibodies in serum, colostrum, and milk of rat dams; these levels were sustained through lactation. It was also evident that offspring suckling the i.v.-immunized mothers absorbed significant quantities of milk antibody; these antibodies reached peak concentration in the pups' circulation at approximately 15 days of age. The antibody in these offspring was associ-

TABLE 3. *Agglutinin titers (log₂) to S. mutans 6715, mutant C211 in whole, and Sephadex G-200 fractions of, pooled whey from orally and intramammary-immunized rat dams*

Group[a]	Whole milk whey	G-200 fraction[b]	
		Peak one	Peak two
Intramammary	6 (10, 6)[c]	4 (6, 3)	0 (0, 0)
Control	0 (0, 0)	0 (0, 0)	0 (0, 0)
Oral	8 (11, 7)	7 (12, 6)	1 (0, 0)
Control	2 (2, 1)	2 (2, 1)	0 (0, 0)

[a] Intramammary-immunized and control rat dams were antibiotic-suppressed, conventional animals. Orally immunized and control rat dams were gnotobiotic animals infected with *S. mutans* 6715, mutant C211. Each group contained 10 to 12 dams.

[b] Values obtained from four whey fractionations per group.

[c] Agglutinin titers after enhancement with optimum concentrations of anti-rat α and anti-rat γ sera, respectively. No IgM could be detected.

TABLE 4. *Mean caries scores of 45-day-old rat offspring from immunized and control rat dams*

Group	Route of immunization	Day (age) of challenge[a]	Mean caries score[b]					
			Buccal		Sulcal		Proximal	
			Enamel	Dentinal (slight)	Dentinal (slight)	Dentinal (moderate)	Enamel	Dentinal (slight)
A	Intravenous	21	8.7 ± 0.5	4.8 ± 0.6	11.0 ± 1.1	5.1 ± 0.8	2.4 ± 0.5	2.1 ± 0.4
B	Nonimmunized	21	19.7 ± 0.7	17.3 ± 0.7	15.2 ± 0.5	9.8 ± 0.6	5.1 ± 0.2	3.2 ± 0.3
C	Intravenous	16	5.9 ± 0.6	3.3 ± 0.4	9.1 ± 0.9	3.1 ± 0.6	1.5 ± 0.6	1.0 ± 0.5
D	Intramammary	16	8.3 ± 0.7	4.4 ± 1.0	11.8 ± 1.3	4.5 ± 1.2	2.2 ± 0.8	0.6 ± 0.3
E	Nonimmunized	16	21.1 ± 0.8	18.6 ± 0.5	17.0 ± 0.7	10.6 ± 0.5	6.5 ± 0.4	4.4 ± 0.4
F	Oral	Natural	10.8 ± 0.4	7.3 ± 0.5	10.3 ± 0.3	5.3 ± 0.3	2.2 ± 0.3	0.9 ± 0.2
G	Infected only	Natural	22.4 ± 0.7	18.9 ± 0.8	17.8 ± 0.9	11.6 ± 0.9	7.0 ± 0.6	5.4 ± 0.5

[a] Offspring in groups A to E challenged with *S. mutans* 6715, mutant C211. Infection of offspring in groups F and G was derived from their mothers; *S. mutans* 6715, mutant C211 could be recovered from each rat offspring.

[b] Evaluated by the Keyes procedure (13). Values represent mean ± standard error of 32 to 40 rat offspring per group. Mean caries scores of offspring from immunized mothers are significantly lower ($P \leq 0.01$) than scores of offspring from nonimmunized mothers.

ated with the IgG class. Previous studies have also demonstrated that rat pups derive high levels of circulating IgG during the suckling period (18). In fact, the increase observed in passively derived antibodies correlated directly with the increase in IgG. Furthermore, rat offspring, exhibiting significant levels of IgC̄ antibodies in their serum, had agglutinin activity (in the IgG class) to *S. mutans* and were protected against challenge by virulent *S. mutans*. These findings support the observations of Lehner and others (14, 15, 26) that primates immunized against *S. mutans* by peripheral routes exhibited serum IgG antibodies to this organism and were protected against caries formation. The results of these studies further suggest that serum IgG antibodies can transude into saliva via the crevicular fluid and perhaps effect protection against *S. mutans*-induced dental caries.

Recent experimental animal studies clearly indicate that the induction of a salivary IgA immune response to *S. mutans* correlates with caries immunity (6, 19, 22, 32). Such a concept is supported by findings of the present investigation. In the earlier studies, *S. mutans* antigen was either injected in the vicinity of the salivary gland (19, 32) or instilled directly into the parotid duct (6); both routes induced the formation of salivary IgA antibody to *S. mutans*. In the present study, local application of *S. mutans* antigen in the region of the mammary tissue induced a secretory immune response, as manifested by the presence of specific IgA antibodies in colostrum and milk. This response was similar to that reported previously in rabbits (31). The presence of these antibodies can be correlated with the protection of suckling offspring against challenge with virulent *S. mutans*.

Earlier studies indicated that ingestion of *S. mutans* antigen elicits a secretory immune response, as evidenced by the presence of IgA antibody in saliva and milk, but not in serum; these antibodies correlated with protection against *S. mutans*-induced dental caries (22). This work was extended by the present experiments, wherein rat dams fed antigen exhibited IgA antibody in their colostrum and milk and conferred protection against *S. mutans* infection on their offspring. It is noteworthy that both immunized and control rat offspring from dams infected with *S. mutans* became infected by bacteria the mother harbored. Nevertheless, greater resistance to caries formation was exhibited by pups that suckled dams with colostral IgA antibodies. This did not stem from a lesser challenge being conveyed to the pups by immunized mothers, since offspring challenged artificially at age 16 days also developed signifi-

cantly fewer caries (data not shown). Because greater amounts of IgA antibodies occur in colostrum than in other accessible secretions of the rat, this particular model of passsive immunity should make possible more detailed exploration into the mechanisms by which IgA antibodies inhibit the pathogenesis of *S. mutans*-induced caries.

The present study clearly indicates that passive transfer of antibody of either the IgA or the IgG class renders protection to rat offspring. Thus, reliable models are now available for evaluation of the antigens that are of importance in the virulence of *S. mutans*. It should therefore be possible to induce different classes of antibody to *S. mutans* (through different routes of immunization) and to evaluate these antibodies in terms of immune protection.

Passive immunity to dental caries could be important to man, since the disease principally afflicts the young from the ages of 6 to 16 years, a period when large quantities of milk are usually consumed. One could envision the use of bovine milk supplemented with antibody or milk from cows previously immunized with selected strains of cariogenic bacteria. This latter form of immunity, although not new, could provide one approach to prevention of a disease as widespread and prevalent as dental caries.

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