Effective Immunity to Dental Caries: Protection of Malnourished Rats by Local Injection of \textit{Streptococcus mutans}

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When rats from dams fed a low-protein diet were injected with whole, killed \textit{Streptococcus mutans} 6715 cells in the region of the submandibular gland, they developed serum and salivary agglutinins to this microorganism. Titers of agglutinins in malnourished rats were similar to those observed in rats from dams fed a nutritionally adequate diet that were locally injected with \textit{S. mutans}. Furthermore, both groups of immunized rats subsequently infected with cariogenic \textit{S. mutans} 6715 had lower mean caries scores than infected, nonimmunized rats. This reduced incidence of caries was evident on all molar surfaces. The mean body weights of immunized and nonimmunized, protein-deficient rats were not significantly different; likewise, both immunized and nonimmunized normally nourished rats exhibited similar weight gains. Malnourished rats, not immunized but infected with \textit{S. mutans}, had significantly more caries than normal, nonimmunized infected rats. Both dietary groups of noninfected rats had very few carious lesions. These results suggest that carious lesions observed in these rats resulted from \textit{S. mutans} 6715 infection. Furthermore, protein-malnourished rats, injected in the region of the submandibular gland with whole, killed \textit{S. mutans} elicit an immune response and are protected against \textit{S. mutans}-induced caries.

Protein deficiency of varying degrees of severity afflicts most of the world's population. Furthermore, it is clear that malnutrition predisposes the host to a higher incidence of infection (32–34).

Several studies have been directed toward explaining this higher incidence of infection in conditions of malnourishment in terms of an impaired host immune response. In a study of 57 malnourished survivors from a German concentration camp, Gell (10) noted that these people responded very poorly to tobacco mosaic virus and avian erythrocytes, in contrast to the normal response obtained in 16 healthy adults. In a carefully controlled study, Wohl et al. (44) noted that malnourished patients responded poorly to typhoid vaccine; however, after protein supplementation these same patients gave a much higher antibody response. Significantly higher titers to \textit{Salmonella} TAB vaccine were observed in a group of children suffering from protein-calorie malnutrition (PCM) who were fed a diet supplemented with 50 g of protein per day in contrast to a group fed 30 g/day (31). Chandra (4) reported reduced antibody responses to tetanus toxoid in PCM children. He noted that some children who exhibited no apparent infection had significantly lower serum immunoglobulin G (IgG) levels than normal children. Recently Sirisinha and co-workers (35) reported that the local immune system of children suffering PCM is severely affected. Mean concentrations of immunoglobulin A (IgA) in nasal washings of PCM children were significantly lower than the mean IgA concentration in normal children. This was specific for secretory IgA (s-IgA), since IgG and albumin concentrations were within the normal range (35). Studies in undernourished animals have corroborated these findings in humans. Protein malnutrition resulted in decreased immunoglobulin production (1, 19, 20). Furthermore, it has been shown previously that rats reared on dams fed a low-protein diet develop significantly more dental caries than pups reared on dams fed a normal protein diet (26).

Dental caries has been described as an infectious disease in which certain oral streptococci, most notably \textit{Streptococcus mutans}, have been considered to be the principal etiologic agents. This microorganism produces the enzyme dextran sucrase, which synthesizes high-molecular-weight glucans when sucrose serves as substrate (14, 15, 45). These glucans have been considered to contribute to plaque by forming large, sticky accumulations that facilitate ad-

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herence of S. mutans and other bacteria to teeth (7, 11–13, 16, 24). Fermentation of fructose and glucose moieties of sucrose and the resultant accumulation of organic acid has been suggested to be the major contributor to decalcification and decay (5, 6, 22).

Attempts to immunize animals with S. mutans have resulted in protection in some instances (2, 23, 37–39, 41) and failure in others (9, 17, 36). Recent studies have clearly demonstrated that injection of immunogen into the region of the salivary gland resulted in local salivary antibody formation (25, 39). In these experiments, significant caries reduction was observed in immunized animals.

The purpose of this study was to determine the effect of injection of S. mutans into the region of the submandibular gland of nutritionally compromised and normal rats on protection against subsequent challenge with the homologous, cariogenic S. mutans.

**MATERIALS AND METHODS**

Rats and colony maintenance. Sprague-Dawley-derived rats (COBS/CD, Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were employed in this study. All rats were housed in a room cleaned daily with Lysol and maintained at constant temperature (72 ± 3 F [25 ± 2 C]), 50% relative humidity, and a 12-h light cycle. Adult rats were mated and, on the day of conception, each dam was placed in a hooded cage. All cages, bedding, cage tops, water bottles, water, and hoods were autoclaved and maintained under sterile conditions until used. Cages and bedding were changed three times weekly, and water and purified gel diet (described below) were provided ad libitum. On the day of parturition, all litters were reduced to eight pups per dam. At weaning (day 19) all pups were maintained under the same housing conditions and provided purified caries-promoting diet no. 305.

Microbiology. The methods employed for antibiotic control of the indigenous flora of the rat are the subject of another report (S. M. Michalek and J. R. McGhee, manuscript in preparation). Briefly, at conception and at weekly intervals thereafter, oral swabs and fecal samples were collected from each rat and cultured on blood, Mitis Salivarius (MS), and Rogosa agar plates. Antibiotic sensitivity disks were applied to duplicate sets of plates to determine which antibiotics were most effective in controlling the rats’ microbial flora. Plates were incubated under aerobic and anaerobic conditions at 37 C. In COBS/CD rats, chloramphenicol, cephalothin (CF), ampicillin (AM), and carbenicillin were the most effective. These were supplemented in combinations of three in the gel diet at concentrations of 1 g of each antibiotic/kg of diet.

Streptomycin-resistant (10 mg/ml) S. mutans 6715 was employed as the cariogenic microorganism in this study. This strain has been previously shown to be highly cariogenic in gnotobiotic rats fed a defined, 5% sucrose diet (25, 28). Stock cultures were maintained in brain heart infusion agar stabs containing excess calcium carbonate.

For preparation of cells for specific immunization, S. mutans 6715 was grown in dialyzed tryptose medium containing 1% sucrose (3), and the cells were harvested by centrifugation at 4000 x g, washed five times with 0.1 M phosphate-buffered saline (pH 7.0), and suspended in 0.5% Formalin in saline. The killed cells were adjusted in 0.1% Formalin to a concentration of 5 x 10^6 equivalent colony-forming units as reported previously (25).

In microbial challenge experiments, S. mutans 6715 was grown in brain heart infusion broth, and 50 μl of a log-phase culture (18-h growth) was introduced into the oral cavity of each 25-day-old rat with the aid of a micropipette. Duplicate plating of aliquots on MS and blood agar determined the size of the bacterial challenge (5.8 x 10^3 colony-forming units in this experiment).

Colonization of S. mutans 6715 in rats offspring was verified the day after challenge (day 26), and continued colonization was confirmed at weekly intervals until day of sacrifice (day 45). Oral swabs and fecal samples were collected and cultured on blood, MS, and MS plus streptomycin (1 mg/ml) agar plates.

Diets. Twenty-one days before gestation, dams were divided into two groups (Fig. 1). One group of rats was provided low-protein, purified gel diet no. 408, which contains 8% casein (30), whereas the other group received a normal protein purified diet no. 425, which contains 25% casein (Table 1). Seven days before gestation until day 19 of lactation, dams were fed this same diet supplemented with CF, AM, and CB (1 g of each drug/kg of diet).

All pups were weaned at 19 days of age and fed purified caries-promoting diet no. 305 (26) supplemented with CF, AM, and CB (1 g of each drug/kg of diet). On day 23, all pups were offered diet no. 305 supplemented with streptomycin alone (1 g/kg of diet). Pups were fed this diet until day of sacrifice (day 45).

Immunization regimen. On the day of delivery, dams and litters on each dietary regimen were divided into three groups (Fig. 1). Immunization was initiated in 14-day-old suckling pups (groups A and D) with subcutaneous, bilateral injections (total volume, 0.2 ml) of a suspension of antigen (5 x 10^3 S. mutans/ml) into the region of the submandibular glands (hereafter referred to as local injection). Injections were repeated daily until day 15, the last injection being given in complete Freund adjuvant (CFA). Additional injections (0.4 ml) in CFA were given at weekly intervals (days 23, 30, and 37). Animals subsequently infected (groups B and E) and noninfected controls (groups C and F) were injected at the same time intervals with Formalin in saline or in saline plus CFA.

Collection of rat saliva and serum. On day 45, each rat was weighed and injected with pilocarpine (0.75 mg/100 g of body weight) while under Nembu
tal sedation. The animal was placed in the decubital position on a board at a 30° angle, and approximately 1.0 ml of whole saliva was collected over a 20-
The individual saliva samples were clarified by centrifugation at 2,800 × g for 30 min and stored at -20°C until used. After saliva collection, blood was obtained by cardiac puncture. The blood was allowed to clot at room temperature and incubated overnight at 4°C. Serum was separated by centrifugation at 4,400 × g for 15 min at 4°C and collected, and individual serum samples were stored at -20°C until used.

**Serum and saliva antibody assay.** Antibacterial antibody in rat saliva and serum was determined on individual samples by agglutination employing 2.5 × 10⁵ Formalin-treated *S. mutans* 6715 per ml. Two-fold dilutions of samples were made in saline containing 0.1 mg of bovine serum albumin per ml. Bacteria were added to each dilution, followed by incubation at 37°C for 2 h and 4°C for 16 and 40 h. Agglutinating patterns were recorded after each incubation period. The levels of serum and saliva agglutinins were expressed as the reciprocal titer of the last dilution exhibiting 2+ agglutination. Inhibition of salivary agglutination was carried out using excess anti-α or anti-γ2a as described previously (25).

**Caries scoring.** Rats were killed by decapitation, and the heads were autoclaved for 5 min to loosen soft tissue. Mandibles were dissected, defleshed, and stained with 0.4% murexide in 70% alcohol. After staining, the molars were hemisectioned with a dental drill, and the buccal, sulcal, and proximal caries were scored by the Keyes procedure (27). 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 analysis of variance and multiple mean comparisons with the Duncan test (8).

**RESULTS**

Both dams and offspring were monitored throughout lactation to ensure that antibiotic therapy was effective and that continued suppression of the flora was being accomplished, as evidenced by absence of growth on blood and MS agar. This antibiotic regimen effectively maintained suppression of the indigenous flora; however, it did not interfere with the colonization of streptomycin-resistant *S.*
TABLE 1. Composition of gel diets fed to dams during gestation and lactation

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Diet no. 408 (8% protein diet)</th>
<th>Diet no. 425 (25% protein diet)</th>
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</thead>
<tbody>
<tr>
<td>Casein</td>
<td>480</td>
<td>1,500</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Dextrose</td>
<td>1,000</td>
<td>1,000</td>
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<tr>
<td>Sucrose</td>
<td>1,002</td>
<td>1,002</td>
</tr>
<tr>
<td>Dextrin</td>
<td>1,000</td>
<td>1,000</td>
</tr>
<tr>
<td>Corn starch</td>
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<td>58</td>
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<td>Corn oil</td>
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<td>900</td>
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<tr>
<td>Salt mix (Harper)</td>
<td>240</td>
<td>240</td>
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<tr>
<td>Vitamin mix*</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Choline</td>
<td>24</td>
<td>24</td>
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<tr>
<td>Agar</td>
<td>210</td>
<td>210</td>
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<tr>
<td>Distilled water (liters)</td>
<td>6</td>
<td>6</td>
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*As developed by Navia et al. (30).

Composed of the following (in grams): vitamin A acetate and D₂, 1.00 (325,000 USP IU A/32,500 D₂); vitamin E acetate (25%), 40.00; vitamin K, 0.50; thiamine hydrochloride, 1.00; riboflavin, 2.00; niacin, 5.00; vitamin C, 20.00; pyridoxine, 1.00; p-aminobenzoic acid, 10.00; biotin, 0.050; calcium pantothenate, 5.00; folic acid, 0.20; inositol, 20.00; vitamin B₁₂ (0.1%), 5.00; and sucrose, 890.27.

mutans 6715 in offspring. This microorganism could be detected in each infected rat throughout the experimental period.

Both protein-malnourished and normally nourished rats, locally injected in the submandibular region with killed S. mutans 6715, exhibited similar agglutinin titers in serum (Fig. 2). On the other hand, infected, nonimmunized rats (groups B and E) showed low but detectable agglutinins, whereas uninfected, nonimmunized rats (groups C and F) had negligible agglutinins. These results indicate that protein-deficient rats can elicit a humoral immune response to S. mutans 6715 that is comparable to that observed in normal rats of the same age.

The salivary agglutinins in titers in both groups of immunized rats were higher than those observed in nonimmunized rats infected with S. mutans (Fig. 2). Uninfected, nonimmunized rats had no salivary antibacterial antibody. It appears that most of the salivary agglutinins are of the IgA class, since excess anti-α blocked most of the antibody activity in both groups of immunized rats, whereas anti-γ2a had no inhibitory effect on the agglutinin titers (Table 2). These results demonstrate that nutritionally compromised rats have agglutinins in saliva to S. mutans similar to normal rats treated in the same manner. Thus protein malnutrition did not appear to affect synthesis of serum or salivary agglutinins after local injection with killed S. mutans 6715.

Table 3 presents the mean caries scores of buccal, sulcal, and proximal surfaces in offspring from dams fed diet no. 408 and from dams fed diet no. 425. Nonimmunized, malnourished rats infected with S. mutans 6715 had more smooth-surface (buccal and proximal) carious lesions and exhibited lower body weights when compared with infected, normally nourished animals. These results confirm previous observations that pups from dams fed diet no. 408 exhibit marginal protein malnutrition and develop more caries than normally nourished pups (26). Both groups of uninfected rats had very few carious lesions, indicating that the indigenous microorganisms were not contributing to the caries observed. This observation clearly suggests that carious lesions observed in nonimmunized rats infected with S. mutans 6715 resulted from infection with this microorganism.

Both protein-malnourished and normally nourished rats injected with S. mutans 6715...
exhibited protection against *S. mutans* on all smooth surfaces (Table 3). The level of carious lesions on buccal and proximal surfaces of immunized animals (groups A and D) were significantly lower (*P* < 0.01) than the level observed in nonimmunized, infected rats (groups B and E, respectively). Fewer sulcal caries were observed in both groups of immunized rats when compared with nonimmunized, infected controls; however, the differences were not statistically significant at the 0.01 level. No differences were observed in mean body weights of rats from dams fed diet no. 408 or in rats from dams fed diet no. 425 (Table 3). These results clearly demonstrate that protein-malnourished rats injected in the region of the submandibular glands with *S. mutans* 6715 are protected against *S. mutans*-induced caries.

**DISCUSSION**

The indigenous microbial flora of dams and their offspring employed in this study were controlled with selected antibiotics as reported elsewhere (Michalek and McGhee, manuscript in preparation). These antibiotics were evaluated by use of antibiotic disks on cultures from oral swabs. When dams were provided with either diet no. 408 or diet no. 425 supplemented with these antibiotics, the indigenous flora were usually suppressed within 1 week after treatment.

Uninfected control rats exhibited very low caries scores, whereas *S. mutans* 6715 could be isolated from each infected rat from the day after challenge until the day of sacrifice; therefore it was concluded that carious lesions observed in infected rats resulted from *S. mutans* 6715 pathogenesis.

It was of considerable interest that local injection with killed *S. mutans* cells resulted in significant anti-*S. mutans* agglutinin titers in both serum and saliva of normally nourished and protein-malnourished rats. Of even greater importance was the reduced level of carious lesions in both malnourished and normal rats injected with *S. mutans* when compared with infected animals. The mean caries score ob-

**TABLE 2. *S. mutans* 6715 saliva agglutinin titers in 45-day-old protein-malnourished and normal rats after reaction with excess antiglobulin**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Group’</th>
<th>Mean agglutinin titerb</th>
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<td></td>
<td></td>
<td>Anti-α</td>
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<td>Anti-γ2a</td>
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<td>Untreated</td>
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<tr>
<td>0.08</td>
<td>A (immunized, infected)</td>
<td>35.0 ± 3.6</td>
<td>6.9 ± 5.1</td>
<td>28.3 ± 4.7</td>
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<tr>
<td>0.08</td>
<td>B (infected only)</td>
<td>4.0 ± 2.3</td>
<td>3.5 ± 3.2</td>
<td>4.5 ± 3.1</td>
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<tr>
<td>0.08</td>
<td>C (control – no infection)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
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<tr>
<td>0.08</td>
<td>D (immunized, infected)</td>
<td>40.0 ± 3.8</td>
<td>7.0 ± 3.9</td>
<td>33.1 ± 6.8</td>
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<td>0.08</td>
<td>E (infected only)</td>
<td>5.0 ± 2.8</td>
<td>2.0 ± 1.8</td>
<td>5.8 ± 3.1</td>
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<td>0.08</td>
<td>F (control – no infection)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
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’ As outlined in Fig. 1.

b Expressed as reciprocal agglutinin titer ± standard error.

**TABLE 3. Mean caries scores: effect of immunization with *S. mutans* in protein-malnourished and normal rats**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Group’</th>
<th>Mean caries scoresb</th>
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<tr>
<td></td>
<td></td>
<td>Buccal</td>
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<td></td>
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<td>Enamel</td>
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<td>Dm</td>
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</tr>
<tr>
<td>0.08</td>
<td>A (immunized, infected)</td>
<td>3.9 ± 0.8</td>
<td>0.1 ± 0.3</td>
<td>104.0 ± 2.6</td>
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<tr>
<td>0.08</td>
<td>B (infected only)</td>
<td>8.6 ± 0.6</td>
<td>2.4 ± 0.3</td>
<td>200.5 ± 3.2</td>
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<tr>
<td>0.08</td>
<td>C (control – no infection)</td>
<td>0.8 ± 0.5</td>
<td>0.3 ± 0.1</td>
<td>101.7 ± 5.0</td>
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<tr>
<td>0.08</td>
<td>D (immunized, infected)</td>
<td>1.2 ± 0.4</td>
<td>0.6 ± 0.3</td>
<td>144.6 ± 3.9</td>
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<tr>
<td>0.08</td>
<td>E (infected only)</td>
<td>4.0 ± 0.9</td>
<td>2.1 ± 0.6</td>
<td>154.5 ± 6.5</td>
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<tr>
<td>0.08</td>
<td>F (control – no infection)</td>
<td>0.7 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>142.8 ± 5.0</td>
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’ As outlined in Fig. 1. Rat pups in groups A, B, D, and E challenged with 5.6 × 10⁸ viable *S. mutans* 6715 in 50 μl.

b Evaluated by the Keyes procedure (21); E, penetration into enamel; Ds, slight penetration into dentin; Dm, moderate penetration into dentin. Score ± standard error (represents 31 [A], 22 [B], 15 [C], 23 [D], 16 [E], and 23 [F] rats).

c Expressed as grams ± standard error.

d Values significantly lower than infected controls (*P* < 0.01).
served in immunized, malnourished rats was similar to the score obtained in nonimmunized, normally nourished animals challenged with S. mutans. Apparently, although nutritionally compromised, protein-malnourished rats injected with S. mutans can elicit a protective immune response. This protection was comparable to the innate, natural immunity that accrues in normally nourished animals.

During the past few years, several studies have been directed toward induction of caries immunity in experimental animals. Although some reports demonstrate protection against dental caries (25, 37–39), a number of investigators report variability in their findings (9, 17, 36). In studies of anticaries immunity, several factors are important and must be considered. First, s-IgA is the principal antibody in external secretions, including saliva (40), and recent studies have shown that s-IgA antibody is formed to S. mutans in animals demonstrating caries immunity (25, 39); therefore, it is probable that earlier unsuccessful experiments did not effect a local immune response. In studies employing peripheral routes of antigen administration (9, 16, 36), systemic immunity would develop; however, the local immune response, as manifested by s-IgA antibody, could be low. Although serum antibodies could transudate across the gingival crevice, the level of the host's local immune protection might be insufficient to withstand challenge with a cariogenic microorganism. In this regard, several studies have clearly shown that maximum s-IgA is induced after local administration of antigen (18, 25, 42, 43). A second important factor involves the specificity of the experimental design. To evaluate immune protection against a specific cariogenic bacterium, the host must be free of other potentially cariogenic bacteria. If a condition of complete specificity is not established, local immunity to the test cariogen may be operative; however, other cariogens that differ antigenically from the test microorganism would be responsible for resultant caries and lack of immune protection. Finally, in studies concerned with caries immunity, attention should be given to the size of the microbial challenge. If the host is repeatedly infected with the cariogen in the presence of sucrose, a condition favoring the establishment of the parasite would occur, and the host's defense would be overcome despite existing local immunity.

Some of these problems in studies of caries immunity have been obviated by use of gnotobiotic rats. The problem of specificity of microbial infection is overcome, because no competing flora exist. Furthermore, studies have shown that gnotobiotic rats injected in the salivary gland region with S. mutans elicit a local immune response protective against S. mutans infection (25, 39). By careful control of the challenging dose of S. mutans, very significant caries reductions have been effected in young gnotobiotic rats utilizing an immunization regimen and experimental design almost identical to that reported here (25).

Previous studies with malnourished rats have shown decreased levels of serum immunoglobulins when compared with normally nourished rats (20). In this regard, we have observed considerable reduction of serum IgG in protein malnourished rats; however, no differences were found in the levels of serum immunoglobulin M or IgA between malnourished and normal rats (29). Similarly, the levels of salivary immunoglobulins do not differ between malnourished and normal rat offspring (29); however, malnourished rats secrete less saliva than normal rats (27). Therefore, although malnourished rats can elicit an immune response similar to normal animals as determined by the level of salivary agglutinins, the extent of protection against S. mutans-induced caries may not be as effective as in normal animals. The findings reported here corroborate earlier work suggesting that protein malnutrition results in increased caries susceptibility (26). Further, this work lends support to the well-documented observation that protein malnutrition contributes to an increased incidence of infectious diseases (32–34). However, from this study it is clear that a nutritionally compromised host can elicit an immune response that reduces the severity of S. mutans-induced caries.

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LITERATURE CITED

