Effect of Neonatal Thymectomy on Immune Responses of Rats to Streptococcus mutans

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The effect of neonatal thymectomy on secretory and systemic antibody responses in rats was studied. Groups of normal or thymectomized (Tx) rats were infected or immunized and infected with Streptococcus mutans 6715. Tx rats exhibited a significantly lower level of salivary immunoglobulin A (IgA) antibody to S. mutans after a 45- to 65-day infection. Similarly, after multiple local injections of formalinized S. mutans, Tx rats showed a delay in the appearance and lower levels of salivary IgA antibody to S. mutans. Serum IgG antibody levels were also decreased in Tx rats with both experimental protocols. In contrast, salivary IgG and serum IgM anti-S. mutans activity in Tx and normal rats were similar during the experiments. These results demonstrated that thymus deprivation at birth produces profound effects on the ability of rats to manifest secretory IgA antibody responses to the pathogenic microorganism S. mutans.

The importance of secretory antibodies, in particular secretory immunoglobulin A (IgA), in the protection of animals (5, 6, 9, 15) and humans (4, 14, 24) from a variety of pathogens has been well documented. Local injection of nonviable Streptococcus mutans in the vicinity of the major salivary glands has been utilized to induce salivary IgA antibodies that are associated with a diminished pathogenic effect after infection of immunized rats with S. mutans (21, 30). We have been investigating the function of T cells in the secretary immune system and have shown that neonatally thymectomized (Tx) rats exhibit substantially decreased levels of secretory IgA in saliva (11). Also, these Tx rats are unable to respond to a locally injected T-dependent antigen (12). Similarly, oral ingestion of killed S. mutans or Vibrio cholerae by athymic mice did not elicit salivary IgA agglutinins (R. R. Arnold, J. R. McGhee, and T. Shiota, Fed. Proc. 37:1389, 1978). In the present study, we investigated the influence of T cells on serum and secretory antibody responses to S. mutans. The following two protocols were used to examine the ability of T cell-depleted (neonatally Tx) rats to respond to an S. mutans antigenic challenge: (i) local injection of nonviable S. mutans and (ii) experimental infection with live microorganisms. The results obtained in this investigation indicated that the thymus (and presumably the thymus-derived lymphocyte) is an important component of secretory IgA responses to natural challenge or active immunization with bacterial antigens.

MATERIALS AND METHODS

Rats. Male and female Sprague-Dawley CD-1 rats were used in experiments A1, A3, A4, B1, and B2, and inbred Fischer CDF(F-344)/CrlBR rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were used in experiment A2. Litters from multiparous females were Tx within 24 h postparturition, sham-Tx, or untreated (N) as described previously (11). At the termination of the experiment, each Tx animal was sacrificed and examined for thymic rudiments under a stereomicroscope. Spleen cells were prepared from N and Tx rats at sacrifice and examined for T cells by direct immunofluorescence with a specific anti-rat T lymphocyte serum (29). Rats with thymic tissue remaining in the mediastinum (confirmed by histological studies) or having >15% positive T lymphocytes in the spleen were excluded from the study. Less than 5% of neonatally Tx rats showed these characteristics.

Bacteria. S. mutans 6715 was grown and formalinized for use in the immunization regimen as previously described (30).

Experimental protocol. Two experiments were performed (A1 and A2) in which rats without indigenous S. mutans were used (16). In experiment A1, 12 N rats (group IV) and 5 Tx rats (group II) were used. There were 10 sham-Tx (group IV) and 6 Tx (group II) rats in experiment A2. All rats were weaned and infected with S. mutans 6715 on day 20 after birth in both experiments (Fig. 1).

Rats in experiments A3, A4, B1, and B2 were not monitored before initiation of the experiments for indigenous S. mutans. However, before infection with S. mutans 6715, serum from these rats had no detectable antibody to S. mutans, suggesting a lack of this organism in their indigenous flora. In experiments A3 and B1, 41 pathogen-free rats were divided into the following five groups: group I rats were Tx and
immunized (n = 7), group II rats were Tx and sham-immunized (n = 5), group III rats were normal and sham-immunized (n = 9), and group V rats were normal and unimmunized (n = 7). Immunized rats were injected subcutaneously in the vicinity of the major salivary glands with 10^9 formalinized S. mutans organisms emulsified in complete Freund adjuvant (CFA) (28). Groups I and III were injected four times, beginning on day 17 after birth and at weekly intervals before infection. Sham-immunized rats were injected in a manner identical to that used for the immunized groups, except equal volumes of phosphate-buffered saline and CFA were mixed for the injections. The rats were also injected one more time approximately 30 to 35 days after infection with S. mutans 6715 (Fig. 1). One week before infection (days 46 to 48), experimental rats were given cariogenic diet 2000 until termination of the experiment.

Experiments A4 and B2 followed essentially the same protocol as that used for experiments A3 and B1. The rats from the four groups used, groups I (n = 9), II (n = 9), III (n = 10), and IV (n = 11), were treated identically to rats from comparable groups used in experiment A3 and infected with S. mutans on days 43 to 45 (Fig. 1).

Infection. Rats in all experiments were infected with an overnight culture of S. mutans 6715 on 3 consecutive days approximately 1 week after being placed on cariogenic diet 2000. Infection of all rats was confirmed by swabbing between 6 and 21 days postinfection (16; Fig. 1).

Collection of specimens. Blood from the retroorbital sinus and stimulated whole saliva ( pilocarpine nitrate, 1 mg/100 g of body weight) were collected from ether-anesthetized rats (13). Saliva and sera were stored at -20°C until analyzed.

Antisera. Rabbit anti-rat IgA was prepared by subcutaneous injection of 100 μg of IgA obtained from ascitic fluid (IR461; obtained from H. Bazin, Brussels, Belgium) emulsified in CFA. The IgA was isolated by DEAE-cellulose chromatography (3) and elution from an anti-a-chain specific immunoadsorbent (34). Thirty days later, the rabbit was boosted intramuscularly with 100 μg of IR461 IgA in CFA. The animal was exsanguinated 14 days later by cardiac puncture, and the serum was obtained by centrifugation. Globulins were prepared by salt fractionation with 50% (NH₄)₂SO₄, and the final precipitate was dissolved in two-thirds of the original volume of phosphate-buffered saline. This antiglobulin preparation was made specific for a-chain by adsorption with insolubilized, 7-day-old rat serum as described previously (11). The specificity of the anti-a-globulin was shown by immunoelectrophoresis and immunodiffusion analyses. When tested in an immunoelectrophoresis assay against rat saliva, milk, and serum, the antiserum formed a single band in each case with the electrophoretic mobility of rat IgA (5). In an immunodiffusion test, anti-γ, anti-μ, and anti-α sera exhibited bands of nonidentity against rat serum.

Rabbit anti-rat IgG was prepared by injecting Fc-rich fractions of rat serum IgG prepared by DEAE-cellulose chromatography, (NH₄)₂SO₄ precipitation, and papain digestion (31). The specificity of this antiglobulin has been reported (31), and it reacted with both IgG1 and IgG2a myeloma proteins (H. Bazin).

Rabbit anti-rat IgM was prepared by injecting 105 μg of purified rat IgM emulsified in CFA. The IgM was prepared from whole rat serum by sequential binding and elution of the material from an immunoadsorbent composed of goat anti-rat IgM (H. Bazin) coupled to Sepharose 6B with cyanogen bromide (34). The anti-globulins obtained were made monospecific as de-
TABLE 1. Salivary antibody levels in Tx and Control rats after challenge with viable S. mutans

<table>
<thead>
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<th>Expt</th>
<th>Treatment (group)</th>
<th>No.</th>
<th>Mean antibody activity ± SEM in:</th>
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<td></td>
<td></td>
<td></td>
<td>IgA</td>
</tr>
<tr>
<td>A1</td>
<td>Tx (II)</td>
<td>5</td>
<td>5.1 ± 3.9c-e</td>
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<tr>
<td></td>
<td>N (IV)</td>
<td>12</td>
<td>13.8 ± 2.6</td>
</tr>
<tr>
<td>A2</td>
<td>Tx (II)</td>
<td>6</td>
<td>4.6 ± 1.5f</td>
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<tr>
<td></td>
<td>Sham Tx (IV)</td>
<td>10</td>
<td>10.3 ± 2.9</td>
</tr>
<tr>
<td>A3</td>
<td>Tx (II)</td>
<td>5</td>
<td>8.7 ± 0.8f</td>
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<tr>
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<td>N (IV)</td>
<td>9</td>
<td>14.9 ± 2.8</td>
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<tr>
<td></td>
<td>N (V)</td>
<td>7</td>
<td>14.4 ± 2.6</td>
</tr>
<tr>
<td>A4</td>
<td>Tx (II)</td>
<td>9</td>
<td>6.5 ± 0.9g</td>
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<tr>
<td></td>
<td>N (IV)</td>
<td>11</td>
<td>9.2 ± 1.1</td>
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</table>

* Salivary IgA and IgG antibody activity (in EU) was measured at a 1:5 dilution of the saliva collected at the termination of the experiments. IgA and IgG antibody levels in saliva are not necessarily comparable, because each assay was developed to provide optimal detection capabilities for each isotype.

* Serum IgG antibody activity (in EU) was measured at a 1:5 dilution of serum, and IgM was measured at 1:10 dilution. IgG and IgM antibody levels in serum are not necessarily comparable, because each assay was developed to provide optimal detection capabilities for each isotype.

* Preinfection background levels of activity in uninfected Tx and N rats (n = 30) were 3.6 ± 0.4 EU for IgA and 1.9 ± 0.2 EU for IgG.

* Preinfection background levels of activity in uninfected Tx and N rats (n = 40) were 0.8 ± 0.1 EU for IgG and 0.02 ± 0.02 EU for IgM.

* P < 0.05; statistically significant difference between antibody levels in Tx versus N rats.

* P < 0.01.

* P < 0.025.

scribed previously (13). This anti-μ-chain globulin reacted with a single band (characteristic of rat IgM) when examined against rat serum in an immunoelctrophoresis assay. The anti-μ serum also showed a band of identity when reacted with rat serum and a goat anti-rat IgM serum in an immunodiffusion assay and showed a band of nonidentity against rat serum as compared with previously described anti-α and anti-γ globulins.

**Antibody analysis.** Antibodies in sera and saliva were assayed by a modified enzyme-linked immunosorbent assay (13). To compare the enzyme-linked immunosorbent assay results among experiments, serial dilutions of a reference serum and saliva were included in the studies. Plotting optical density (OD) at 400 nm versus log_{10} of the reciprocal dilution resulted in linear reference curves. For the experiments, the following ODs of the reference standards were assigned an ELISA unit (EU) value of 100 at a similar dilution: saliva, 0.796 (anti-IgA) and 1.203 (anti-IgG); serum, 1.662 (anti-IgG) and 1.680 (anti-IgM). Although the levels of antibody activity among the salivas and sera can be related within each individual isotype, antibody levels among isotypes are not necessarily comparable, because different developing reagents were used in the enzyme-linked immunosorbent assay procedure, and the assays were optimized for each isotype of immunoglobulin in sera and saliva. The relative antibody activities of experimental sera and saliva were then compared with the reference curves described by the following linear equations: saliva, OD = [0.807 (log_{10}EU) - 0.640] (IgA) and OD = [1.703 (log_{10}EU) - 2.032] (IgG); serum, OD = [0.975 (log_{10}EU)] - 0.499 (IgG) and OD = [1.350 (log_{10}EU)] - 1.092 (IgM).

**Statistical analyses.** Differences in the data were determined by the analysis of variance test and Student's t test.

**RESULTS**

Salivary and serum antibody responses of Tx rats to S. mutans infection. Absolute volumes of saliva were determined in all experiments (A1 through A4, B1, and B2), and no significant differences were noted between the control and Tx rats. Although the Tx rats gave slightly less saliva in most experiments, previous findings have shown that the salivary protein level in the Tx rats is within normal limits (32).

Table 1 shows the salivary antibody response in the control and Tx rats 45 (experiment A1) or 65 (experiment A2) days after challenge with viable S. mutans 6715. In both experiments in which rats were infected (A1 and A2), salivary IgA antibody levels in the Tx rats were significantly lower than those in comparably infected normal animals (P < 0.05 or P < 0.01, respectively). Sham-immunized or nonimmunized groups in experiments A3 and A4 exhibited negligible IgA antibody activity in saliva before infection. After a 65-day infection interval, the Tx group and both N groups (IV and V) showed salivary IgA antibody, with the normal levels
FIG. 2. Salivary IgA (A) and IgG (B) antibody levels during and after S. mutans immunization regimen in experiments A3 and B1. Points represent the group mean (five to six rats), and the brackets enclose standard errors of the mean. Symbols: ●, N, immunized rats; ○, N, sham-immunized rats; □, Tx, sham-immunized rats; ▲, Tx, sham-immunized rats; ○, nonimmunized, normal rats; △, injection times of S. mutans in CFA, CFA alone, or phosphate-buffered saline; *, all rats in experiments A3 and B1 infected with S. mutans 6715 on days 48 to 50. IgA and IgG levels in saliva are not necessarily comparable, because each assay was developed to provide optimal detection capabilities for individual isotypes.

being significantly higher ($P < 0.025$) than those of the Tx group in both experiments (Table 1). Salivary IgG antibody responses in Tx rats were slightly decreased in the experiments; however, the overall effect of neonatal thymectomy on IgG antibody levels in saliva was minimal after S. mutans infection. Sham-immunized Tx rats challenged with S. mutans (Table 1, experiments A3 and A4) showed only 7 to 16% less salivary IgG antibody compared with the N groups (IV and V) in each experiment. Thus, the results of experiments A1 through A4 indicate that infection of rats for 45 to 65 days elicits a salivary IgA and IgG antibody response to S. mutans. However, in rats that have been T cell deprived (neonatally Tx), the ability to synthesize IgA antibody in saliva is significantly diminished.

Serum antibody levels were also examined in the rats after infection (Table 1). In each experiment, little or no IgG antibody was detected. Although there did appear to be somewhat lower levels in the Tx versus the N rats, these changes were not statistically significant. The primary systemic immune response to the infection was IgM, which increased by approximately 20-fold. The IgM antibody was unaffected in the Tx rats as compared with control animals (Table 1). These findings suggest that the major antibody response in serum may be directed to T-independent antigens on the microorganism.

Dynamics of salivary and serum antibody re-
sponses after local immunization of Tx rats. In experiments A3 and B1, we examined the production of salivary and serum antibodies in Tx rats to *S. mutans* during a regimen consisting of multiple local immunizations followed by challenge with viable *S. mutans*. Five days after the first, second, and fourth injections, as well as at the termination of the experiment, the rats were bled, saliva was collected, and antibody levels in the fluids were determined. Even after one injection (Fig. 2A), the levels of salivary IgA antibody were significantly higher in the normal rats (18.6 ± 1.1 EU) than in the Tx rats (6.8 ± 0.4 EU). These levels remained higher in the normal rats throughout the injection regimen and at the termination of the experiment. Unimmunized groups IV (N, sham-immunized) and V (N, nonimmunized) rats exhibited an increase in salivary IgA antibodies after infectious challenge (day 49) and subsequent immunization (day 83) that was 50% greater than the response observed in Tx, sham-immunized rats (Fig. 2A). No significant differences in IgG antibody levels in saliva were noted between group III (N, immunized) and I (Tx, immunized) rats until four injections were administered (Fig. 2B). After the fourth injection and at the termination of the experiment, IgG levels were 56 and 53% lower (P < 0.01) in Tx, immunized rats (group I) as compared with N, immunized rats (group III). In contrast to salivary IgA antibody levels, a similar increase in salivary IgG antibody levels was noted among the N and Tx rats that received no active immunization and an infectious challenge with *S. mutans* (Fig. 2).

Serum IgG antibody levels were greater at all time periods in the immunized, normal rats (group III) as compared with the Tx, immunized rats (group I) (Fig. 3B). However, the pattern of IgM serum antibody levels increased after infection alone and were similar between N and Tx animals.

Effect of neonatal thymectomy on salivary and serum antibody responses to local immunization and infectious challenge with *S. mutans*. In experiments B1 and B2, the ability of Tx rats to produce serum and salivary antibodies after five injections of *S. mutans* was examined (Table 2). N rats had greater concentrations of salivary IgA antibodies than similarly injected Tx rats in both experiment B1 (78 ± 7.3 versus 46 ± 22.2 EU; P < 0.06) and experiment B2 (81 ± 12.0 versus 57 ± 14.4 EU; P < 0.01) (Table 2). During a subsequent infection and a fifth injection, the IgA antibody levels decreased in Tx rats, whereas in the normal group (N, postinfection), salivary antibody levels remained significantly higher than those in the Tx group in both experiments (P < 0.001) (Table 2). Salivary IgG antibody levels were lower in the Tx, immunized (preinfection) group as compared with the N group in both experiments B1 and B2 (Table 2). Similar results were noted in comparing Tx and N groups that received a combination of immunization and infection (postinfection) (Table 2).

Serum IgG antibody levels were in general lower (P < 0.05) in the Tx group (I) than in the N group (III) after immunization with *S. mutans* or immunization and challenge with *S. mutans* (Table 3). Immunization with *S. mutans* antigens also appeared to increase the differences in serum IgG antibody levels between the Tx and N rats, as compared with the results obtained with only an infectious challenge of these animals.

### DISCUSSION

Infection of rats with *S. mutans* for 45 or 65 days resulted in an increase in salivary IgA and

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<th>Expt</th>
<th>Treatment (group)</th>
<th>No.</th>
<th>Mean antibody activity ± SEM in rats a</th>
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<td></td>
<td></td>
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<td>Immunized IgA</td>
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<td></td>
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<td>preinfection</td>
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<td>preinfection</td>
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<td></td>
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<td></td>
<td>Immunized-infected postinfection</td>
<td></td>
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<tr>
<td>B1</td>
<td>Tx (I)</td>
<td>7</td>
<td>46.0 ± 22.2b,c</td>
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<td></td>
<td>N (III)</td>
<td>10</td>
<td>78.0 ± 7.3</td>
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<tr>
<td>B2</td>
<td>Tx (I)</td>
<td>9</td>
<td>57.9 ± 1.4d</td>
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<tr>
<td></td>
<td>N (III)</td>
<td>10</td>
<td>81.2 ± 12.0</td>
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a Salivary IgA and IgG antibody activity (in EU) was determined at a 1:5 dilution.
b P < 0.001; statistically significant difference between antibody levels in Tx versus N rats.
c P < 0.01.
IgG antibody levels in both control and Tx rats; however, the levels of IgA antibody were significantly less in the Tx rats in each experiment. Previous findings from our laboratory have shown that, although no salivary IgA antibody is produced in Tx rats after a local injection of a T-dependent antigen, detectable amounts of IgA antibodies were elicited by a T-independent antigen (12). An intact microorganism presents both T-dependent and T-independent determinant antigens. Thus, the finding of significantly decreased but detectable secretory IgA antibody levels in Tx rats may represent a limited response to only the T-independent antigens on the microorganisms. Salivary IgG antibody activity was similar in the Tx and N rats in each experiment. The finding that neonatal thymectomy of rats had a minimal effect on salivary IgG antibody is in keeping with the apparent isotype-specific T dependency of primarily IgG1 antibodies (18, 22, 27, 33) and the reports that IgG2 (relatively T independent) is the predominant subclass in rodent saliva (10, 20).

Infection with *S. mutans* elicited a minimal systemic response, whereas secretory IgA antibody levels were consistently elevated. Recently, it has been suggested that deposition of antigen in the gut may induce systemic suppression of antibody synthesis (19, 23). Challacombe and Tomasi (7) have also shown that, although oral immunization can elicit secretory (salivary) IgA antibodies, an accompanying systemic tolerance to *S. mutans* is found. Therefore, it appears that oral infection with this organism may elicit a host response similar to intragastric immunization.

Local injection of nonviable *S. mutans* in the vicinity of the major salivary glands resulted in a pronounced increase in salivary IgA antibody levels in normal rats within 5 days after two weekly injections. The levels of IgA antibody in the saliva of normal rats also increased after subsequent injections. Similarly treated Tx rats did not show increased IgA antibody levels until day 43 after initiating a regimen of four local injections. IgG antibody levels in saliva were increased in both N and Tx rats after two injections; however, the N rats exhibited higher levels of IgG antibody than comparable Tx rats after further antigen administration. These results suggest that local infections of *S. mutans* elicit increased levels of IgA and IgG antibody in the saliva of both N and Tx rats. However, the IgA and IgG antibody responses were delayed in Tx animals (Fig. 2). These findings may be explained on the basis of the necessity for cooperation between antigen-activated T and B cells in T-dependent immune responses (17). We have previously shown that both T and B cells are present in salivary glands of rats (13). Immunization in the salivary gland vicinity may stimulate salivary antibody in situ by local immune cell cooperation. Also, the T cells in the glands presumably represent a random population of circulating T cells that lodge in the salivary glands either with (25) or without (26) the necessity for antigen deposition. Therefore, the lag in production of T-dependent salivary antibodies could result from a quantitative diminution in the frequency of T cells migrating to (through) the salivary glands, and thus the cumulative cooperative effects would take longer to reach a threshold in production of detectable salivary antibody.

Challenge of previously immunized rats with live antigen for 65 days resulted in minimal changes in salivary IgA antibody levels in N rats. However, in both experiments B1 and B2, salivary IgA antibody levels decreased in immunized Tx rats after infection. These postinfection
results may reflect the type of salivary secretion that is being collected from the rats. Since whole saliva was used as the secretion, the actual antibody level detected represented the total amount of antibody secreted minus the amount which was adsorbed to bacteria in the oral cavity (2). Thus, the N rats appeared to be synthesizing and secreting levels of IgA anti-S. mutans that were being collected from saliva. This amount is being collected from which results are being obtained (VOL. 37, 1982).

Thus, as Taubman and D. J. Smith submitted for publication. In both experiments B1 and B2, serum and salivary IgG antibody levels increased in immunized Tx and N rats after infection with S. mutans. The explanation for this finding is not clear; however, it could possibly be related to a serum-derived IgG component in the saliva (13, 23), an inherently greater capability of Tx rats to synthesize salivary IgG antibody (12; Ebersole et al., submitted for publication), or a combination of these factors.

These results demonstrate that thymus deprivation at birth produces profound effects on the ability of rats to manifest secretory IgA antibody responses after local immunization with S. mutans. Similar immune deficiencies in humans have been shown to increase the incidence and severity of dental caries (1, 8). We have subsequently utilized these immunologically deprived rats to examine the functional importance of T lymphocytes in host resistance to S. mutans infection (Ebersole et al., submitted for publication). The results of another study suggest that T cell diminution results in significantly decreased resistance of these immunodeficient rats to S. mutans-induced dental caries (Ebersole et al., submitted for publication).

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LITERATURE CITED


