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Local Active Gingival Immunization by a 3,800-Molecular-Weight Streptococcal Antigen in Protection against Dental Caries

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Local gingival immunization was attempted in an effort to confine the immune response to the oral cavity and bypass the systemic immune response. A low-molecular-weight (3.8K) streptococcal antigen (SA) I/II was applied 10 times over a period of 1 year to the gingival crevices of rhesus monkeys. The antigen was maintained in situ by means of silicone rubber appliances. Serial examinations over a period of 1 year showed that topical gingival immunization with the 3.8K SA results in a significantly lower incidence of dental caries and colonization of *Streptococcus mutans* compared with that of the sham-immunized controls. This was associated with an increase in gingival crevicular immunoglobulin G and salivary immunoglobulin A anti-SA I/II antibodies, whereas no change occurred in serum antibodies to SA I/II. The immune mechanism which prevents the colonization of *S. mutans* and the development of caries may involve antibodies that prevent the adherence of *S. mutans* to the teeth and facilitate phagocytosis and killing by the local neutrophils. This novel route of local immunization is noninvasive, does not cause side effects, and bypasses systemic immunization.

The route of immunization is an important factor in eliciting different types of cellular immune responses, antibody class, immunological memory, and potential side effects. In general, six routes have been used in active immunization with *Streptococcus mutans* to prevent colonization by this organism and the occurrence of dental caries in animals. The oral route, in which *S. mutans* is swallowed, has been successfully used in germfree rats (25) and elicits predominantly a secretory immunoglobulin A (IgA) response. Injecting *S. mutans* near the major salivary glands of rats also results in salivary IgA antibodies and a reduction in caries (37). An increase in salivary IgA antibodies was also induced in humans in some (24) but not other experiments (9) by having them swallow capsules containing *S. mutans*. Although salivary IgA antibodies were also elicited in rhesus monkeys by oral immunization (6), a reduction in the colonization of *S. mutans* or in caries was not observed. Oral immunization in irus monkeys (39) failed to induce salivary antibodies or to protect against caries. The direct immunization of the parotid salivary glands in monkeys was also attempted by the repeated introduction of a vaccine into the salivary ducts (8); this induced elevated levels of salivary IgA antibodies, but any changes in caries were not reported.

The systemic route by subcutaneous immunization was used successfully in monkeys and elicits predominantly serum antibodies (2, 16). A combination of the systemic and oral routes has also been used sequentially by either first immunizing orally followed by the subcutaneous route or by the reverse sequence without any obvious advantage (18). Furthermore, a topical application of a vaccine to the oral mucosa, attempted in rodents to induce salivary IgA antibodies in the minor salivary glands, had limited success in preventing the colonization of *S. mutans* (12).

The systemic route of immunization by *S. mutans* elicits serum IgG, IgM, and IgA antibodies which find their way into the oral cavity via the gingival crevicular fluid (34) and at least one of them, IgG, is protective against dental caries (19, 21). This has been confirmed in passive immunization by the systemic (intravenous) route (22) and recently by local passive immunization, i.e., by applying monoclonal IgG antibodies directly to the teeth (15).

Investigations of the biology of the gingiva suggest that it contains a fast-migrating neutrophil population (30–33), as well as a much less mobile mononuclear cell population, i.e., monocytes, T and B lymphocytes, and plasma cells (4, 27, 40). The gingiva is therefore a potential site for local immunization. Indeed, direct injection of lysozyme into rabbit gingiva elicited local antibody-forming cells to lysozyme (3), and culture of mononuclear cells from gingival tissue showed lymphoproliferative responses to mitogens and antigens (11). However, attempts at direct immunization by brushing live *S. mutans* onto the gingiva in rhesus monkeys failed to induce antibodies or to prevent the development of caries (18). In a pilot study, gingival immunization was attempted by injecting streptococcal antigen (SA) directly into the gingivae of one half of the jaws and saline into the gingivae of the other half (T. Lehner, unpublished data). A significant decrease in caries was found in the teeth of the immunized halves compared with those of the sham-immunized halves.

Our objectives were to attempt local gingival immunization in order to bypass the systemic immune response and prevent any associated side effects. We applied a low-molecular-weight 3.8K SA (10) to the gingival crevices of one group, a high-molecular-weight 185K SA (29) to a second group, and saline to a third group of monkeys. The animals immunized with the 3.8K SA showed a lower caries score than those of the sham-immunized animals. Protection against caries was associated with a decreased number of *S. mutans* colonizing the teeth and an increased level of gingival fluid IgG and salivary IgA antibodies to SA.

MATERIALS AND METHODS

Experimental animals, examination for caries, and collection of fluids. A total of 12 rhesus monkeys (*Macacca mulatta*) born in Britain were used. After they were weaned, they were

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TABLE 1. Topical gingival immunization schedule in three groups of monkeys[a]

Group (no. of monkeys)	Immunizing prepn	SA dose (μg)
1 (4)	3.8K SA + DMSO	5
2 (5)	Saline	
3 (3)	185K SA + DMSO	5

[a] Times of immunization (in weeks): 0, 0, 1, 2, 12, 13, 14, 21, 32, 44 for all three groups.

maintained on a primate diet (Mazuri; Special Diet Services Ltd., Witham, Essex, England) containing 45 ppm of fluoride. The fluoride content of the drinking water was 0.06 to 0.24 ppm. The monkeys were transferred to our care when they had reached a weight of between 1.5 and 2.5 kg. All of the deciduous teeth were fully erupted, but none of the first permanent molars had fully erupted. About 3 weeks after immunization, the monkeys were offered a human type of diet containing about 15% sucrose (17) with no added fluoride; the drinking water contained 0.025 ppm of fluoride. The teeth of rhesus monkeys are naturally colonized by *S. mutans* (serotype c) under the influence of the human type of diet (5, 14, 16, 17, 19, 21), so there is no need to infect them. Before immunization and subsequently at intervals of 1 to 2 months, the animals were examined for caries both with a dental probe and mirror and radiographically. Whole saliva and blood were also collected on each occasion (17). Saliva was collected by injecting 0.5 mg of pilocarpine per kg subcutaneously and then collecting the saliva in petri dishes. Gingival crevicular fluid was collected by repeated washings with a Hamilton syringe, as described elsewhere (34).

Culture of *S. mutans*. Plaque was collected at 2- to 3-month intervals with sterile probes from (i) smooth surfaces of the upper central incisors, (ii) fissures of the upper-left second deciduous molar, and (iii) fissures of the upper-left first permanent molar after it had erupted. These sites were chosen to standardize sequential sampling from smooth surfaces and fissures of the most cariogenic sites of monkey teeth (17). The samples were placed into 5 ml of transport medium (1) and grown on tryptone–yeast extract–L-cystine (TYC) medium (7). The number of CFU of *S. mutans* was determined as described previously (5) and expressed as a percentage of the total colony count on TYC medium.

Preparation of the SAs. Both the high- and low-molecular-weight antigens were prepared from *S. mutans*, serotype c (Guy's strain). The 185K SA I/II was prepared as described previously (28, 29). The 3.8K SA I/II was prepared by gel filtration of a predominantly 185K SA I/II preparation on Sephacryl S-200 (Pharmacia, Uppsala, Sweden) in the presence of sodium dodecyl sulfate. This preparation yielded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis a single band in the 3.8K region (10) and reacted with specific antibodies to SA I/II, but not to SA III, on single radial immunodiffusion and radioassay.

Immunization. The monkeys were divided into three groups (Table 1) and matched for sex; there were three female and two male animals in the sham-immunized group and four female and three male animals in the immunized groups. The state of eruption of the dentition was comparable in all animals; all of their deciduous teeth had fully erupted whereas their first permanent molars had not erupted.

(i) Group 1. In this group, four monkeys were immunized by 10 topical applications of the 3.8K SA in 50% dimethyl sulfoxide (DMSO) to the gingival crevices. Samples (5 μg) in about 75 μl of distilled water with DMSO were applied at the intervals indicated in Table 1. To retain the antigen in situ, individually prepared silicone rubber appliances made with Optosil (Beyer Dental, Federal Republic of Germany) were fitted to the teeth and gums and maintained with slight digital pressure for 5 min. The DMSO was used to increase the permeability of the gingival crevicular epithelium.

(ii) Group 2. In this group, five monkeys were sham immunized by applying saline to the teeth, with or without the silicone rubber appliance, at the same intervals as those described for Group 1.

(iii) Group 3. In this group, three monkeys were immunized as in Group 1, but the 185K SA was used in 50% DMSO.

Radioassay for serum, salivary, and gingival fluid antibodies. Serum IgG and IgA and salivary IgA antibodies to SA I/II were tested by a solid-phase radioassay (34). Briefly, polystyrene wells (Remova-U wells; Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 1 μg of 185K SA (dry weight) per ml of phosphate-buffered saline. The wells were then treated with 0.5% bovine serum albumin, washed, and incubated with serially diluted monkey sera in duplicate. IgG antibodies were assayed by incubation with ^{125}I-labeled affinity-purified rabbit anti-monkey IgG (Fc; Nordic pharmaceuticals, Ltd.), at a concentration of 0.1 μg/ml. IgA and IgM antibodies were assayed by an indirect method, using rabbit anti-monkey IgA and IgM (Fc; Nordic) at concentrations of 5 and 1 μg/ml, respectively. After being washed, the wells were incubated with 1 μg of ^{125}I-labeled anti-rabbit IgG per ml (Tago, Inc., San Francisco, Calif.). The bound ^{125}I-labeled antiserum was counted in a gamma counter (Hydragamma 16; Innotron). The results were expressed as the mean (± standard error) counts per minute, after the base-line value (i.e., the count before immunization) was subtracted. Controls included in each experiment were antigen-coated wells without added serum or with reference immune and control sera. The specificity of the assay has been established by competitive inhibition (34).

RESULTS

Dental caries. Serial examination of caries over a period of 52 weeks (Fig. 1) showed a greatly reduced caries score in the teeth of monkeys which had topical gingival immunization with the 3.8K SA (caries score, 0.25 ± 0.25; one fissure

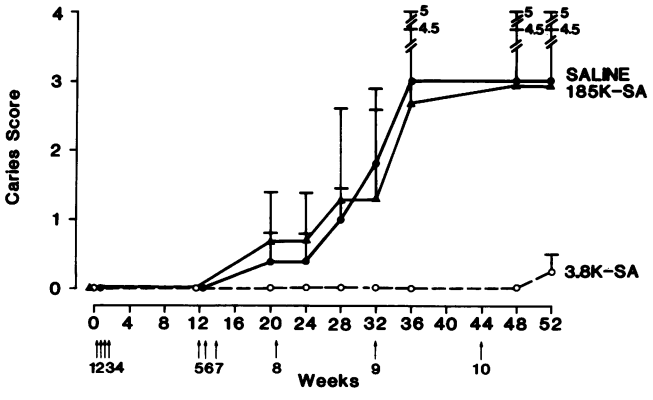

FIG. 1. Sequential caries score after topical gingival application of the 3.8K SA in DMSO (○), 185K SA in DMSO (▲), or saline (●) on 10 occasions at intervals indicated by the arrows. The results are expressed as the mean ± standard error.

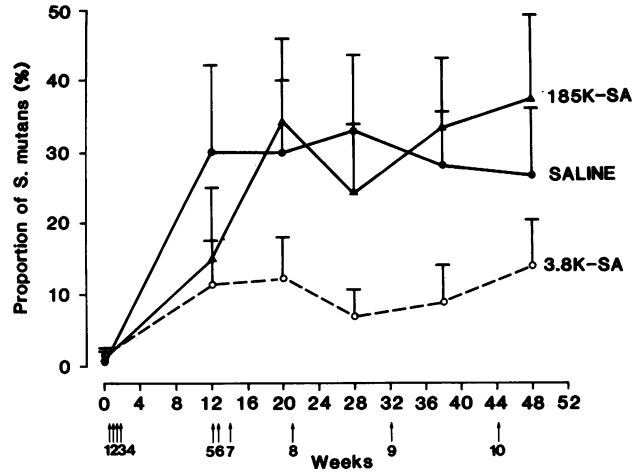

FIG. 2. Sequential proportion of *S. mutans* expressed as mean (± standard error) percent of plaque removed from the smooth surfaces and fissures of teeth from animals which had topical gingival applications of the 3.8K SA (○), 185K SA (▲), or saline (●) at intervals indicated by the arrows.

lesion) compared with the sham-immunized animals (caries score, 3.0 ± 2.0; seven fissure and eight smooth-surface lesions). However, topical gingival immunization with the 185K SA failed to reduce the caries score (3.0 ± 1.7; six fissure and three smooth-surface lesions). All carious lesions affected deciduous teeth except one fissure lesion in the first permanent molar in each of Groups 2 and 3.

Recovery of *S. mutans*. Topical gingival immunization with the 3.8K SA showed consistently lower proportions of *S. mutans* (Fig. 2) than did the sham-immunized animals. The difference was greater with the plaque from smooth surfaces than that from fissures. The mean of the total of five sequential counts of *S. mutans*, taken between 12 and 52 weeks, was 33.6 ± 2.6% for smooth-surface plaque in sham-immunized animals compared with 8.4 ± 1.5% in the plaque from immunized animals; the corresponding values for fissure plaque were 24.3 ± 2.1% and 14.5 ± 2.7%, respectively. Topical gingival administration of the 185K SA (Group 3) failed to yield a lower proportion of *S. mutans* than that found in sham-immunized animals (Fig. 2).

Serum antibodies. Topical gingival immunization with the 3.8K SA failed to induce higher serum IgG, IgA, or IgM antibody titers than those found in sham-immunized animals (Fig. 3; results for IgM are not shown). This was also observed with topical gingival immunization with the 185K SA. For comparison, the high serum anti-SA antibody titers elicited by subcutaneous immunization with the 3.8K SA in aluminum hydroxide and published previously (14) are also shown. Hence, gingival immunization failed to elicit a greater serum antibody response than did sham immunization.

Salivary antibodies. Topical gingival immunization with the 3.8K SA elicited a slightly higher salivary IgA level than that found in the corresponding sham-immunized animals, though the antibody titer decreased after about 16 weeks (Fig. 4). There was, however, little change in the salivary IgA antibodies when the 185K SA was used in topical immunization. A slight increase in the salivary IgA antibodies in the sham-immunized animals can be ascribed to a natural immune response caused by colonization by *S. mutans*.

Gingival crevicular fluid antibodies. Gingival fluid IgG antibodies to *S. mutans* were detected by 4 weeks in the animals immunized by the topical gingival route with the 3.8K SA, and this was maintained throughout the period of investigation (Fig. 4). A much smaller increase in antibody levels was detected in the sham-immunized animals, probably because of a natural immune response to the *S. mutans* in plaque. However, a rather irregular increase in the number of IgG antibodies was also detected in those animals given the 185K SA by the topical gingival route. The titer of

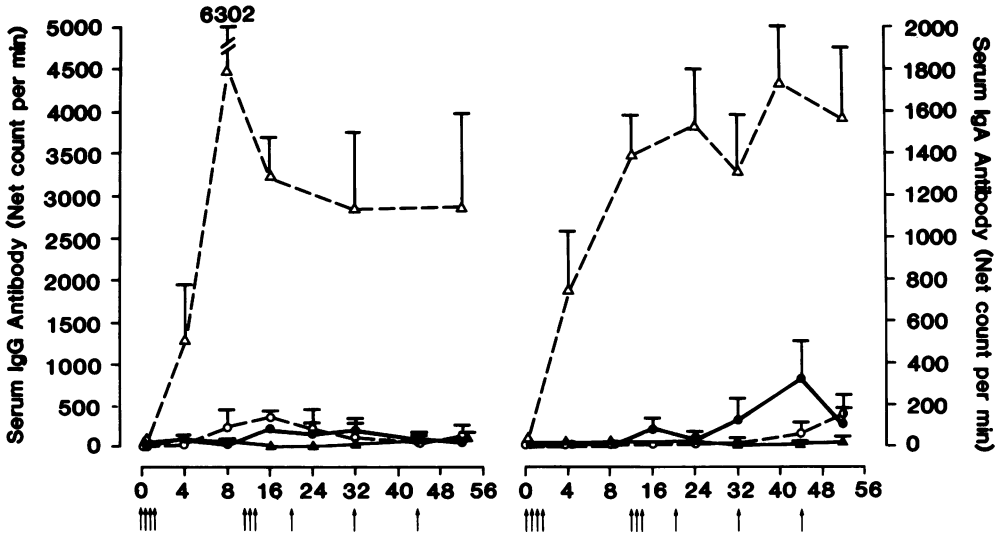

FIG. 3. Sequential serum IgG and IgA antibodies expressed as mean (± standard error) of the net count per minute in topical gingival immunization with the 3.8K SA in DMSO, 185K SA in DMSO, or in sham immunization, as indicated by the arrows. The data is compared with that for corresponding IgG and IgA antibodies obtained after subcutaneous immunization with the 3.8K SA in Alhydrogel as published previously (14). Symbols: ○, gingival immunization with 3.8K SA, ●, sham immunization; △, subcutaneous immunization with 3.8K SA; ▲, gingival immunization with 185K SA.

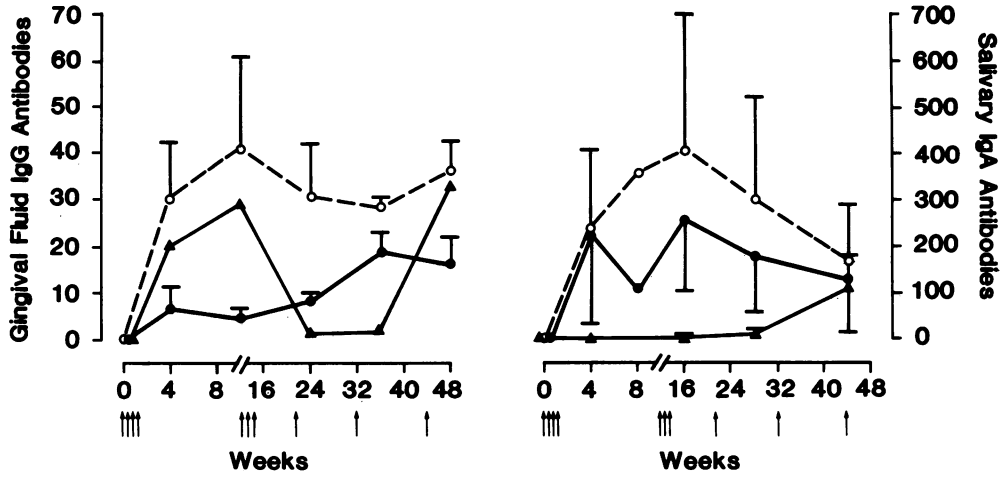

FIG. 4. Sequential gingival fluid IgG and salivary IgA antibodies in topical gingival immunization with the 3.8K SA, 185K SA, and sham immunization at intervals indicated by the arrows. The results are expressed as mean (± standard error) of the net count per minute.

gingival fluid antibodies was rather low. This was expected since the dilution of the fluid inherent in the collection technique is about 1 in 100.

DISCUSSION

In a preliminary investigation, the 185K SA was injected into the interdental gingivae of one half of each jaw and saline into the other half of each jaw of young rhesus monkeys (Lehner, unpublished data). A marked decrease in the incidence of caries and in the proportion of *S. mutans* was detected on the teeth of the immunized halves of jaws compared with those of the sham-immunized halves. The protective effect of intragingival immunization in the absence of serum IgG antibodies suggested that local immunization may be effective in preventing the development of dental caries. In this investigation, we attempted topical gingival immunization via the gingival crevicular epithelium by using the low-molecular-weight 3.8K SA. The antigen was applied in DMSO to increase epithelial permeability and facilitate the penetration of the SA through the gingival tissue. Indeed, only one fissure caries developed in the immunized animals (mean caries score of 0.25 ± 0.25) compared with seven fissure and eight smooth-surface caries (score of 3.0 ± 2.0) in the sham-immunized aniamls (Fig. 1). This difference in caries was associated with a consistently lower proportion of *S. mutans* in the plaque from immunized animals than in that from sham-immunized animals (Fig. 2). The difference in *S. mutans* was greater in the smooth-surface plaque than in the fissure plaque.

Protection against caries was achieved in the absence of significant serum antibodies (Fig. 3). However, an increase in gingival crevicular fluid IgG antibodies to SA I/II was detected in the immunized compared with the sham-immunized monkeys (Fig. 4). This was consistent with the rationale for gingival immunization, namely, to induce a local gingival immune reponse without systemic immunization. There was also an increase in the number of salivary IgA anti-SA-I/II antibodies, and this was more marked in the immunized animals than in the sham-immunized animals (Fig. 4). Since only whole saliva (oral fluid) was examined, the source of the additional IgA antibodies is not clear. The possibility that the IgA antibodies were also produced locally by the gingival lymphoid cells will be investigated. However, the most likely source of salivary IgA antibodies is either the major or minor salivary glands as a response to the direct entry of SA either via the salivary ducts in the oral cavity or through swallowing some of the SA applied to the gingival crevice. This raises the interesting possibility that topical gingival immunization may elicit gingival IgG and salivary IgA antibodies and both classes of antibodies may be involved in preventing colonization of *S. mutans* and the development of dental caries.

The greatly improved results from topical immunization with the 3.8K SA in DMSO (one carious lesion) compared with that of the 185K SA in DMSO (nine carious lesions) might be due to the size of the antigen. The 3.8K SA may penetrate the crevicular epithelial barrier with greater ease than the 50-times-larger 185K SA. There is some evidence that permeability through the oral epithelium might be related to the size of the molecule (36, 38). The DMSO was not responsible for the protective effect when applied with the 3.8K SA since it was also used with the 185K SA. An alternative or additional interpretation is that whereas the 185K SA induces both helper and suppressor activities against the SA (13, 20), the 3.8K SA induces only helper activity (20). We suggest that the 3.8K SA may induce a local helper activity which may augment the production of gingival antibodies to SA.

The immune mechanism which prevents colonization of teeth by *S. mutans* may consist of three phases (15). (i) The anti-SA-I/II antibodies pass through the gingival epithelium and adhere to the acquired pellicle on the tooth surface. (ii) Any *S. mutans* making contact with the teeth will bind to the anti-SA-I/II antibodies, since the SA I/II is expressed on the cell wall of *S. mutans* (23, 26, 28, 41). Indeed, local passive immunization with monoclonal anti-SA-I/II antibodies 35 prevents adherence of *S. mutans* (15). The streptococci may fail to proliferate, and they might be further inhibited by enzymes released from the local neutrophils. (iii) Antibodies to SA I/II may also opsonize *S. mutans*, thereby enhancing phagocytosis and killing by neutrophils (19, 31). These mechanisms may prevent colonization of *S. mutans* and the development of caries, in the absence of a significant systemic immune response. It should be also noted that the relatively low incidence of caries in the control animals might be ascribed to dietary fluoride (45 ppm). Hence, local gingival immunization can further prevent caries in animals

which are partially protected by fluoride. Topical immunization might therefore serve as a preventive treatment that is complementary to fluoridation. The principle of local active gingival immunization with low-molecular-weight antigens might prove to be a new approach not only in the prevention of dental caries but also in the prevention of gingival and periodontal disease.

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