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# Effect of Local and Parenteral Immunization on Implantation of *Actinomyces viscosus* T6 in Rats

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**Groups of rats immunized in the vicinity of the major salivary glands or immunized intraperitoneally with *Actinomyces viscosus* T6 and their sham-immunized controls were infected with the homologous bacterium. Significantly higher levels of salivary and serum antibody were induced by intraperitoneal than by salivary gland immunization. There were also significant inverse correlations between the levels of salivary and serum antibody and the levels of implantation of *A. viscosus* T6. The level of implantation of *A. viscosus* T6 was significantly lower in the immunized animals than in the controls. However, antibody had limited capacity to inhibit the establishment of this bacterium.**

The genera *Actinomyces* and *Streptococcus* persist in a wide variety of animals and in humans. *Actinomyces* spp. appear to be organisms whose habitat is limited to the oral cavity, and as such they may be regarded as true indigenes (3, 13). These organisms may account for as much as 86% of the bacteria recovered from human dental plaque, and from 60 approximal samples their isolation frequency was 100% (4). Although *Actinomyces* species have been implicated in both coronal (15, 34) and root-surface caries (2, 19, 29, 32) and in periodontal disease (18, 20–23, 27, 28), there have been few attempts to regulate these bacteria by immunization (7–9, 11, 14) in contrast to the attention afforded *Streptococcus mutans* (reviewed in references 16 and 24).

The purpose of this study was to determine whether immunization with whole cells of *Actinomyces viscosus* T6 was capable of inhibiting colonization of this bacterium. In an attempt to evaluate the relative effectiveness of salivary immunoglobulin A (IgA) and serum-derived IgG antibody, two routes of immunization were chosen: (i) local immunization, injection in the vicinity of the major salivary glands (SG immunization), a route known to effectively stimulate salivary IgA antibodies (10), and (ii) parenteral immunization, intraperitoneal (IP) injection to induce primarily serum IgG and IgM antibodies.

## MATERIALS AND METHODS

**Bacteria.** *A. viscosus* T6, originally isolated from hamster dental plaque (20), was grown for 16 h to late-exponential or early-stationary phase in *Actinomyces* broth (Difco Laboratories, Detroit, Mich.) in an atmosphere of 95% $N_2$ and 5% $CO_2$ at 37°C. Clumps of bacteria were disrupted by exposure to ultrasound for 5 s by using the microprobe of a Sonifier cell disrupter (model W185; Heat Systems Ultrasound, Plainview, N.Y.) at 30 W of power. The bacteria were washed once in 0.01 M phosphate-buffered saline (PBS; pH 7.0), and the number of CFU contained in suspensions of known optical density were determined by plating on blood agar. Portions of the bacterial suspension were also filtered and washed on tared 0.2-μm-pore-size membrane filter disks (Millipore Corp., Bedford, Mass.) and dried to constant weight over phosphorus pentoxide in vacuo, and the dry weight of bacteria (milligrams per milliliter of suspension) was determined.

**Vaccine.** *A. viscosus* T6 was grown to substrate exhaustion in the ultrafiltrate (PM10 membrane; Amicon Corp., Lexington, Mass.) of *Actinomyces* broth maintained at pH 7.0. The bacteria were washed as above and killed by the addition of Formalin to a final concentration of 0.1%. The killed cells were washed free of Formalin and suspended in PBS (pH 7.0).

**Antibody assay.** Antibody activity in saliva and serum was determined by an indirect enzyme-linked immunosorbent assay. Each well of flat-bottomed 96-well enzyme immunoassay microtitration plates (Linbro; Flow Laboratories, Hamden, Conn.) or round-bottomed polyvinyl chloride microtitration plates (1-220-24; Dynatech Laboratories Inc., Alexandria, Va.) was coated with 0.1 ml of a 1-mg/ml suspension of *A. viscosus* T6 in 0.1 M carbonate buffer (pH 9.6) overnight at 4°C. The plates were washed three times with PBS (pH 8.2) containing 0.1% bovine serum albumin. Appropriate dilutions (100 μl) of saliva and serum samples in PBS (pH 8.2) containing 0.05% Tween 20 (PBS-Tween) were added to the wells and incubated overnight at 4°C. The samples were then aspirated, and the wells were washed three times with PBS-Tween. One hundred microliters of an optimal dilution of goat anti-rat γ (lot 16415), anti-rat μ (lot 15508; Cappel Laboratories, Westchester, Pa.), or anti-rat α (lot G365; Miles Laboratories, Elkhart, Ind.) in PBS-Tween was added to each well and incubated for 2 h at ambient temperature. After aspiration of the antisera, the wells were washed three times with PBS-Tween, and 100 μl of an optimal dilution of the IgG fraction of rabbit anti-goat γ conjugated with horseradish peroxidase (lot 16887; Cappel) in PBS-bovine serum albumin was added to each well and incubated for 2 h at ambient temperature in the dark. In addition to isotype-specific antibody activity, total salivary antibody to *A. viscosus* T6 was determined by using horseradish peroxidase-conjugated rabbit anti-rat immunoglobulins (lot 100; Dako, Copenhagen, Denmark). The conjugate was then removed, and the wells were washed as before. Freshly prepared substrate solution consisting of 1 mg of *ortho*-phenylenediamine and 0.5 μl of 30% hydrogen peroxide per ml of citrate buffer (pH 4.5) was added to each well, and color was allowed to develop in the dark. Absorbance was measured at 450 nm in a Titertek Multiskan (Flow Laboratories, McLean, Va.). All antisera were absorbed

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with *A. viscosus* T6 for 48 h at 4°C (10 mg of packed cell per ml of antiserum) before use.

Antibody activity in serum was expressed as the optical density at 450 nm ($OD_{450}$) per min/ml and in saliva as $OD_{450}$ nm per min/mg of total protein.

**Protein determination.** Total protein in rat saliva was determined by the protein dye-binding assay of Bradford (5) adapted for use in microtitration plates (26).

**Quantitation of *A. viscosus*.** *A. viscosus* colonies were counted after cultivation of portions of suspensions obtained from oral swabs and defleshed mandibles on blood agar. In addition, *A. viscosus* was quantitated with fluorescent antibodies (FAs) (33). Smears for FA staining were prepared by placing 5-μl portions of the bacterial suspensions in individual wells on epoxy-coated slides. The density of fluorescent bacteria in the wells was scored from 0 to 4+: 0, no fluorescent cells; 1+, few fluorescent cells (1 to 5%); 2+, moderate numbers of fluorescent cells (5 to 25%); 3+, many fluorescent cells (25 to 75%); 4+, high numbers of fluorescent cells (75 to 100%).

**Determination of the minimum infective dose of *A. viscosus* T6.** Twenty-four weanling Osborne-Mendel rats from the National Institutes of Health (NIH) breeding colony that were free of *Actinomyces* species were divided into six groups of four animals each, placed in steel-bottomed cages, and fed diet NIH 2000 (56% sucrose) and deionized water ad libitum. The rats were inoculated with 50 μl of *A. viscosus* T6 suspended in *Actinomyces* broth (Difco). The first group received ca. $2.4 \times 10^8$ CFU, and each successive group was given 50 μl of a serial 10-fold dilution such that the sixth group received $2.4 \times 10^3$ CFU. Infection was determined by swabbing the mouth with cotton-tipped applicators. The tip of the applicator was placed in 3 ml of sterile PBS (pH 7.0) and subjected to ultrasound at 30 W of power for 60 s at 4°C. Portions of the resulting bacterial suspensions were plated on blood agar. Six days after inoculation, ca. 50% of the animals receiving $2.4 \times 10^5$ CFU were infected, and thus the minimum infective dose was estimated to be ca. $10^5$ CFU.

**Experimental design.** Forty-eight, 19- to 21-day-old Osborne-Mendel rats were divided into four groups of 12. To control for any bias resulting from litter of origin, sex, and weight of the animals, representatives from each litter were assigned to each group. Litter identification was maintained for each animal throughout the study. The animals were maintained on sawdust in plastic shoebox cages and were fed NIH 07 Rat and Mouse Ration (Zeigler Bros., Gardners, Pa.) and deionized water ad libitum. In an effort to compare the relative effectiveness of salivary and serum antibody, two distinct routes of immunization were employed. On day 1 of the experiment, the SG-immunized group was immunized bilaterally in the vicinity of the parotid and submandibular salivary glands with 100 μg (dry weight) of *A. viscosus* T6 in complete Freund adjuvant. Second and third immunizations were performed 14 and 28 days later with 200 and 400 μg of *A. viscosus* T6, respectively, in incomplete Freund adjuvant. The IP-immunized group was immunized by an identical regimen. Groups 3 and 4 served as sham-immunized controls and received PBS instead of antigen. On day 34, the animals were transferred to stainless steel, screen-bottomed cages, fed diet NIH 2000 (56% sucrose) and deionized water ad libitum, and inoculated with $10^5$ CFU of *A. viscosus*. Oral swabs collected 5 days later (day 39) and examined with FA revealed that the bacteria had failed to implant, so the rats were reinoculated with $10^7$ CFU. No implantation was detected with FA by day 43, and the animals were reinoculated with $10^9$ CFU on day 49. Successful implantation was observed on day 53, and the experiment was terminated on day 64. An oral swab was obtained from each animal, and the rats were anesthetized with pentobarbital. Saliva was collected after stimulation with pilocarpine, and blood was obtained by cardiac puncture. Saliva and serum samples were stored at −70°C until assay. The left mandible from each rat was aseptically removed, defleshed, and placed in a vial containing 3 ml of PBS. The plaque was disrupted by exposure to ultrasound (30 W, 60 s), and the number of *A. viscosus* and the total number of recoverable bacteria was determined after cultivation on blood agar.

**Statistical methods.** A two-way analysis of variance model was used to compare the IP-immunized versus IP sham-immunized and SG-immunized versus SG sham-immunized groups separately, retaining the litter effect and possible interactions between litter and treatment group separately in the analysis for each variable. A comparison between the groups immunized via the IP and the SG routes was also made.

To determine whether there was an association between the level of IgA, IgG, or IgM antibody and the level of *A. viscosus* T6, Spearman's rank correlation coefficients were computed for pairs of variables, both in serum and in saliva.

## RESULTS

**Induction of salivary and serum antibody to *A. viscosus* T6 by SG and IP immunization.** Both routes of immunization induced significant levels of antibody to *A. viscosus* T6 whole cells in saliva and serum (Fig. 1). Salivary antibody was expressed in both the IgA and IgG isotypes, and serum antibody was expressed in the IgG and IgM isotypes. Because different antisera were used to determine IgA, IgG, and IgM antibody activity, it was not possible to directly compare the level of antibody activity of one isotype with that of another.

When the two routes of immunization were compared for their effectiveness in stimulating salivary and serum antibody responses, it was clear that the IP route was superior for both antibody responses. There was significantly greater total antibody ($P < 0.004$) and IgA antibody ($P < 0.009$) response in saliva of IP-immunized animals than in SG-immunized animals. Sera of IP-immunized rats contained higher levels of IgG antibody than did sera of the SG-immunized group ($P < 0.066$).

**Effect of *A. viscosus* T6 salivary and serum antibody on colonization of the homologous bacterium.** The level of infection in the IP- and SG-immunized animals and their controls after inoculation with $10^9$ CFU of *A. viscosus* T6 on day 49 is shown in Fig. 2. In an attempt to reduce the large inherent variation associated with enumeration of bacteria on agar media, estimations of the numbers of *A. viscosus* T6 were made with FA in addition to plate counts on blood agar. The level of implantation of *A. viscosus* was determined 4 days after inoculation (day 53) by using FA and at the termination of the experiment (day 64) by using FA and cultivation on blood agar.

Significantly fewer bacteria were recovered from the IP-immunized ($P < 0.01$) and SG-immunized ($P < 0.02$) animals than from their controls 4 days after inoculation. Two weeks later, however, at the termination of the experiment, only the IP-immunized animals exhibited a significant reduction in colonization compared with their sham-immunized controls ($P < 0.02$) as evaluated with FA. When the levels of infection of the groups were compared at the end of the experiment by cultural techniques, the within-group variation was sufficient to preclude any differences reaching

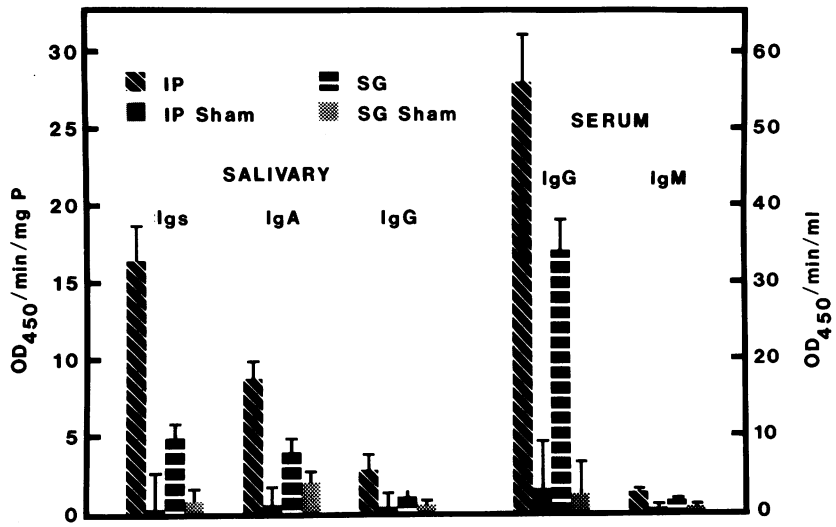


FIG. 1. Levels of anti-*A. viscosus* T6 antibodies in saliva and serum (day 64). Salivary antibody was expressed as $OD_{450}$ per min/mg of protein and serum antibody as $OD_{450}$ per min/ml (mean ± standard error of mean).

statistical significance. At the end of the experiment, *A. viscosus* T6 made up ca. 65% of the total anaerobic bacteria count on blood agar in all groups.

**Relationship between salivary and serum antibody and inhibition of colonization.** Antibody levels in the saliva and sera of the SG- and IP-immunized animals showed statistically significant inverse correlations with the level of *A. viscosus* T6 colonization 4 days after inoculation (Table 1). After a further 2 weeks, however, these correlations were no longer observed (data not shown).

## DISCUSSION

Although preliminary studies indicated that the minimum infective dose of *A. viscosus* T6 for 21-day-old Osborne-Mendel rats was ca. $10^5$ CFU, the present experiment required an increase to $10^9$ CFU before the immunized and control animals could be infected at 70 days of age (experiment day 49). These results differ from a previous report (6) which suggested that rats become more susceptible to infection by *A. viscosus* NY-1 with age. Whether the failure of $10^5$ CFU of *A. viscosus* T6 to infect 70-day-old Osborne-Mendel rats reflects a difference in the strains of *A. viscosus* used (12), the methods of growth, the diet, or the sensitivity of the method of detection remains unclear. It is possible that the immune status of the rats influenced the minimum infective dose because it is clear that the sham-immunized groups exhibited low levels of salivary and serum antibodies reactive with *A. viscosus*.

Significantly fewer *A. viscosus* T6 were detected in the IP- and SG-immunized animals than in their controls 4 days after inoculation with $10^9$ CFU as determined with FA. This

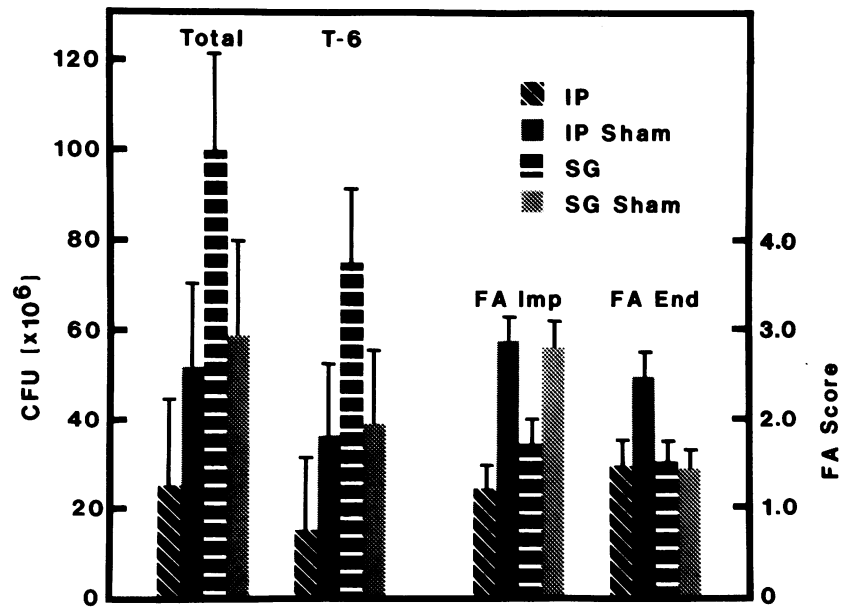


FIG. 2. Level of implantation of *A. viscosus* T6 in immunized and sham-immunized controls. Implantation expressed as CFU × $10^6$ and FA score (FA implantation [FA Imp] on day 53, FA end on day 46; see the text) (mean ± standard error of mean).

TABLE 1. Relationship between antibody activity in saliva and serum and the level of implantation of *A. viscosus* T6 on day 53

| Immunoglobulin | Antibody activity-level of implantation relationship | |
|---|---|---|
| | Correlation coefficient | *P* |
| Saliva | | |
| Total antibody | −0.36 | 0.01 |
| IgA | −0.26 | 0.05 |
| IgG | −0.33 | 0.03 |
| Serum | | |
| IgG | −0.45 | 0.01 |
| IgM | −0.41 | 0.01 |

reduction in implantation was also observed 15 days after inoculation in the IP-immunized group but not in SG-immunized animals. There were statistically significant inverse relationships between the levels of salivary and serum antibody in both immunized groups and the level of implantation of *A. viscosus* T6 4 days after inoculation.

Crawford et al. (11) studied the effects of immunization with Formalin-killed *A. viscosus* T14 in the region of the major salivary glands on colonization of this bacterium and the development of periodontal bone loss in gnotobiotic Sprague-Dawley rats. Salivary gland immunization with *A. viscosus* T14 in incomplete Freund adjuvant induced both salivary and serum agglutinating antibodies but was ineffective in inhibiting colonization. It was interesting that after 60 days of infection the agglutinin levels in the saliva and sera of sham-immunized and infected animals were indistinguishable from those of their immunized counterparts. Salivary antibody was restricted to the IgA isotype. Similarly, Burckhardt and Guggenheim (9) reported that germfree RIC-Sprague-Dawley rats immunized with lyophilized *A. viscosus* NY-1 and monoassociated with the same bacterium accumulated as much plaque as sham-immunized controls. Again, 60 days after infection they found no differences in the serum antibody titers of the immunized and sham-immunized groups. Remarkably, immunization was associated with an increased susceptibility to dental caries.

In the present study, SG and IP immunizations were used in an attempt to compare the relative effectiveness of salivary and serum antibody in inhibition of implantation of *A. viscosus* T6. Salivary gland immunization with particulate and soluble antigens is reported to induce high levels of secretory IgA antibody in saliva (10), whereas parenteral immunization stimulates primarily serum antibodies. It was therefore of particular interest to observe that IP immunization resulted in a significantly greater secretory IgA immune response than did SG immunization. IP immunization could induce a secretory immune response by direct penetration of antigen into Peyer's patches and other parts of the intestinal lymphoid system made permeable to macromolecules by the use of an irritant adjuvant such as complete Freund adjuvant (1).

Alternatively, although the rats did not harbor *A. viscosus* as part of their resident oral or gut microbiota, they may have been primed by exposure to indigenous bacteria sharing common antigens. For example, *Streptococcus sanguis*, *Streptococcus mutans*, and *Lactobacillus salivarius* have been shown to possess antigens cross-reactive with *A. viscosus* (25). Oral priming and parenteral boosting have been demonstrated to be an effective means of inducing a secretory immune response to *Escherichia coli* K-13 polysaccharide in rats (17) and to polio (30) and cholera (31) vaccines in humans. Priming of the secretory immune system to mucosal pathogens by oral or gut commensal microorganisms would, however, be limited to antigens shared by these microorganisms. Priming would not be expected to occur for those antigens unique to the pathogen. The role of naturally occurring secretory antibodies directed against such common antigens in the regulation of colonization is unknown.

Clearly salivary antibodies were capable of impairing the initial implantation of *A. viscosus* T6 but had limited capacity to suppress the establishment of this organism. It is interesting to speculate that the relative inability of salivary antibody to inhibit the establishment of this bacterium may be related to the fact that *A. viscosus* is indigenous to the oral cavity. An important characteristic of autochthonous bacteria may well be their ability to avoid the host immune response.

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