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Antibody Response in the Parotid Fluid and Serum of Irus Monkeys (*Macaca fascicularis*) After Local Immunization with *Streptococcus mutans*

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The antibody response of *Macaca fascicularis* in parotid saliva and serum to local immunization by two routes with *Streptococcus mutans* was studied and compared over 1 year. Antibodies were titrated and classified by indirect immunofluorescent staining using specific antiglobulin conjugates. Anti-glucosyltransferase activity was assayed by an enzyme inhibition test. Animals were immunized first by injecting formalin-killed bacterial cells and cell products subcutaneously into the vicinity of the four major salivary glands. The monkeys were next immunized by retrograde instillation of antigen into the parotid duct. Extensive subcutaneous local immunization gave a serum response only. After parotid duct immunization, high titers of immunoglobulin A (IgA) antibody, along with traces of immunoglobulin G (IgG) and immunoglobulin M (IgM) antibody, appeared in the parotid saliva, and in the serum high titers of IgG antibody were present along with lower titers of IgA and IgM. IgA antibodies in parotid fluid were shown by double immunofluorescent staining to be associated with antigenic determinants which cross-reacted with an antiserum directed to human secretory component. Titers in parotid fluids and sera fell sharply when immunization was stopped. This response pattern was reproducible. High concentrations of antibody capable of inhibiting glucosyltransferase prepared from *S. mutans* were found in the sera, but relatively little was detected in the parotid fluids. Extensive immunization via the parotid duct resulted in transient functional impairment of the gland, as evidenced by diminished salivary flow rates. We conclude that parotid ductal immunization can be an effective method for stimulating a salivary secretory IgA antibacterial antibody response.

The establishment of dental caries as an infectious transmissible disease by Keyes (20) and the identification of *Streptococcus mutans* as an etiological agent of caries (10) generated renewed interest in the potential role of immunization in caries prevention. Several studies were designed to examine this role, using bacterial cells or cell product vaccines administered by various routes to different species of rodents used in experimental caries studies. Failure to achieve protection against caries in albino hamsters by active immunization was reported by Fitzgerald and Keyes (J. Dent. Res. **41** [special issue]: abstr. 146) and by passive immunization in rats by Sweeney et al. (27). In contrast, Wagner (Bacteriol. Proc. **67**:99) found that rats immunized with live *Streptococcus faecalis* developed less caries than nonimmunized control animals. Hayashi et al. (19) vaccinated rodents with glucosyltransferases and glycosidic hydrolases and reported protection against dental caries. Guggenheim et al. (18), however, reported that immunization of rats with a glucosyltransferase preparation resulted in no reduction in caries. Tanzer et al. (28) reported variable results in their attempts to immunize rats against *S. mutans*-induced caries.

These inconsistencies were partly the consequence of variations in experimental design. A source of confusion which did not become evident until the pioneering work of Tomasi et al. (32) was the failure to appreciate the independence of the secretory immune system from other immune mechanisms. It was made clear that secretory immunoglobulin A (IgA) was the predominant immunoglobulin class in saliva and other secretions. In humans most salivary antibody is associated with this class. Secretory IgA antibodies are virtually all the end products of local synthesis and externalization via secretions such as saliva. Since dental caries affects tooth surfaces which are in contact with saliva,

it is reasonable to expect that salivary IgA antibodies rather than serum antibodies will be important in ameliorating dental disease. It is clear from the experiments of Genco and Taubman (15, 29) in rabbits and of Ogra and Karzon (26) in humans that local application of antigen may induce IgA antibodies in secretions, and that parenteral administration will generally induce serum antibodies in the absence of secretory antibodies. The difference in rate of appearance and disappearance of secretory antibodies as compared to serum antibodies is well documented (25).

The foregoing considerations underline the importance of monitoring salivary antibody production throughout the course of experiments designed to define the role of immunization in caries prevention. Taubman and Smith (30) have recently reported on the effects of immunization in several groups of pathogen-free and germfree rats with formalized whole-cell vaccine of *S. mutans*. They followed serum and salivary antibodies throughout 4 to 5 months and determined that most of the salivary antibody was of the IgA class. They reported that the immunized animals had lower mean caries scores and fewer lesions than the sham-immunized and infected control groups. This protective effect was almost certainly due to the salivary IgA antibodies found in the immunized animals before and during infection with the challenge organism. This and other careful studies of caries vaccination in rodents have implied the protective potential of salivary antibodies against cariogenic organisms.

Bowen (5) showed that caries can be induced in the irus monkey (*Macaca fascicularis*) by *S. mutans* of human origin, and that the disease in this species resembles caries in man in its clinical, radiographic, and histological characteristics. Bowen (6) also reported that irus monkeys immunized intravenously with a vaccine of live *S. mutans* had fewer carious lesions than control animals. Salivary antibodies were not measured.

The studies reported here were directed to: (i) find a route of immunization that would regularly give rise in irus monkeys to salivary antibacterial IgA antibodies and (ii) study the kinetics and specificity of secretory and serum antibodies in response to such immunization.

The effect of immunization on implantation in the dental plaque of the immunizing strain of *S. mutans* is detailed in a companion manuscript (8).

MATERIALS AND METHODS

Animals. Eight wild-caught female irus monkeys between 3 to 6 years of age were housed separately in metal wire cages. Eight times a day they were fed a high-sucrose, low-fiber diet augmented with Nutri-Cal (Evsco Pharmaceutical Corp., Oceanside, N.J.), a vitamin, mineral, and protein supplement. Sucrose was dissolved in their drinking water at a final concentration of 3% (wt/vol). The water was taken directly from the fluoridated municipal supply. Clinical laboratory tests were carried out when necessary to monitor their health. Their weights remained stable (5 to 7 lb. [ca. 2.3 to 3.2 kg]) throughout the experimental period. All monkeys had 28 teeth when the experiments were begun, though in a few deciduous molars were still present. Mirror and explorer examination and bite-wing radiographs revealed no caries, and periodontal tissues showed mild generalized gingivitis (8). When necessary for the following procedures, monkeys were sedated with a combination of Sernylan (phencyclidine hydrochloride, 4 to 5 mg; Bio-centic Laboratories, St. Joseph, Mo.) and Innovar (0.05 to 0.1 ml; Pitman Moore, Washington Crossing, N.J.).

Preparation of antigens. *S. mutans* 6715 was obtained from Martin Taubman, Forsyth Dental Center, Boston, Mass. This organism was originally isolated from humans by Fitzgerald et al. (11) and was shown to be cariogenic in pathogen-free rodents. *S. mutans* strains GS-5, BHT, and FA-1 were provided by Robert Fitzgerald, Veterans Administration Hospital, Miami, Fla., and Ronald Gibbons, Forsyth Dental Center, Boston, Mass. Organisms were grown for 36 h at 37 C in Trypticase soy broth, and the cells were harvested by centrifugation, washed three times with phosphate-buffered saline (PBS; 0.15 M NaCl, 0.02 M sodium phosphate, pH 7.4), and finally suspended in PBS containing 0.6% formalin. After 24 h at 4 C, the killed cells were washed three more times in PBS, suspended in 0.2% formalin in PBS at a concentration of 10^9 organisms/ml (based on McFarland standards), and stored at 4 C. Such cells were used both for vaccination and for the immunofluroescent antibody assay.

Crude glucosyltransferase preparations (CEP) were derived from culture supernatant of *S. mutans* strains 6715, GS-5, and BHT. Crystalline $(NH_4)_2SO_4$ was dissolved in the supernatant to a final concentration of 2.4 M (35). The precipitate was recovered by centrifugation, dissolved in distilled water, dialyzed against multiple changes of distilled water, lyophilized, and stored at −20 C. The presence of glucosyltransferase in this preparation was evidenced by incorporation of the [^{14}C]glucose moiety of sucrose into ethanol-insoluble polysaccharide (9). This activity remained stable for up to 2 years.

Immunization. The eight monkeys were divided into two comparable groups according to dental age. The schedule for immunization is shown in Fig. 2. Four monkeys (group I) were injected in the vicinity of the four major salivary glands 10 times during a 35-day period. The vaccine was prepared by washing the formalinized *S. mutans* 6715 cells three times with PBS and suspending these at 10^9 cells/ml in a solution of 45 mg of CEP per ml from strain 6715 dissolved in 0.01 M sodium citrate-sodium phosphate buffer, pH 5.0. One-half milliliter of this vaccine was injected through a 27-gauge needle in multiple sites in the

vicinity of each gland, always attempting to deposit the vaccine within the gland. Group II was similarly injected but with the diluent buffer alone.

Intraductal immunization was subsequently carried out on the parotid glands of the animals in group I using the same vaccine. This route was suggested by the work of McDowell and Lascelles (22) in which IgA antibody was found in the whey of sheep after ductal infusion into the mammary gland of *Salmonella* flagellar antigens. Glass micropipettes (Microcaps, Drummond Scientific Co., Broomall, Pa.) of various sizes (10 to 20 μl) were fire polished and fitted to polyethylene tubing 5 cm in length. Retrograde instillation of vaccine into the parotid duct (PD) was carried out using these cannula assemblies. The glass cannula was inserted into the duct, 1 ml of air was withdrawn from the ductal tree, and a stopcock fitted between the cannula assembly and syringe was closed. A syringe containing the vaccine was then fitted to the stopcock. The valve was opened, and 1.0 ml of the vaccine was slowly infused into the gland. The vaccine was delivered to both parotid glands of monkeys in group I, and an equivalent volume of citrate-phosphate buffer was infused into the parotid glands of group II. The entire dead space of the delivery system was less than 0.1 ml.

Prior to immunization a pilot study was performed to examine the mechanics of this technique. One milliliter of radioopaque medium (Hypaque, Winthrop Laboratories, New York, N.Y.) was instilled into the parotid gland of an irus monkey using the cannula and catheter assembly described above. Radiographs (Fig. 1) were taken before, immediately after, 1 min, and 5 min after instillation of the fluid medium. The catheter was left in place in the duct, and during 30 min we witnessed no escape of fluid from the gland either around or via the catheter. The radiographs revealed nearly complete filling of the gland with the 1.0 ml of medium and progressive diffusion of material into the periphery with a concomitant decrease in radioopacity over 5 min. The filling pattern appeared normal, and little resistance was perceived to the instillation of the fluid.

Oral infection with S. mutans. All monkeys were infected with *S. mutans* 6715 on days 241, 248, and 278 using a technique described elsewhere (8). This

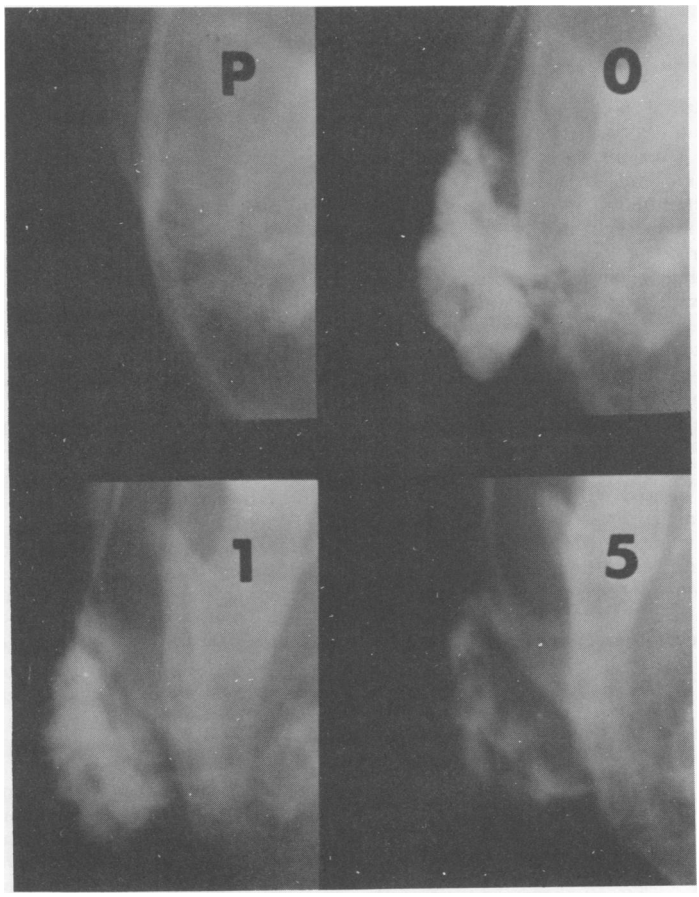

FIG. 1. *Composite radiograph of a monkey parotid gland. P, Prior to instillation into the PD of 1.0 ml of radioopaque medium; 0, immediately after injection of the medium. The stopcock in PD cannula was then closed for 1 min, after which the radiograph labeled "1" was taken; "5" was taken 5 min after the opening of the stopcock.*

organism persisted as part of their oral flora for the remainder of the experiment. All animals were similarly infected with *S. mutans* GS-5 on days 432 and 446.

Saliva and serum collection. Fire-polished micropipettes were fitted to polyethylene catheters and inserted in both PDs in sedated monkeys. Catheters were taped in place, and pilocarpine nitrate (2.0 mg/kg total body weight) was administered subcutaneously (s.c.). The first few drops of parotid fluid were discarded, the catheter was placed in a collecting tube, and the volume of parotid fluid (PF) taken in the first 10 min was measured as an estimate of the flow rate. The remaining "nonparotid" saliva was collected by allowing it to drip into a funnel inserted into a plastic bottle. Parotid saliva (10 to 15 ml) and nonparotid saliva (5 to 10 ml) were usually collected in 90 min. Salivation was terminated by intramuscular injection of 0.5 mg of atropine. PF samples from both glands in a single monkey were pooled, clarified by centrifugation, dialyzed against PBS at 4 C, and frozen at −20 C. Several 0.1-ml samples were frozen at −70 C for antibody assays. Nonparotid saliva was dialyzed at 4 C first against 2% ethylenediaminetetraacetate and then PBS, clarified by centrifugation, and frozen at −20 C.

Blood was collected in glass tubes and allowed to clot overnight at 4 C. Serum was recovered with a pipette and centrifuged (600 × *g*), and the clarified serum was frozen at −20 C. Several 0.1-ml aliquots were frozen at −70 C for antibody assays.

Antibody assays. Two assays for antibody were used: (i) indirect immunofluorescent staining (IFS) and (ii) inhibition of glucosyltransferase activity. For the indirect IFS procedure, preparations of formalinized *S. mutans* described above were used. The cells were washed once with distilled water and suspended at a concentration of 10^8 cells/ml. An acid-cleaned glass microscope slide was placed on a paper template to standardize the position and size of the bacterial smear. A drop of the bacterial suspension was placed on the slide with a platinum loop, smeared to template size, air dried, and fixed by gentle heating. Serum and saliva samples, processed as described above, were serially diluted in PBS. Each dilution was applied to a bacterial smear for 15 min and decanted, and the slide was washed in PBS for 1 h. Three commercial goat antiserum-fluorescein conjugates, each directed to one of the three major human immunoglobulin classes (Meloy Labs., Inc., Springfield, Va.), were used to detect antibody. Molar fluorescein-protein ratios of 3 to 4 were reported for these conjugates. We have confirmed by gel diffusion and immunoelectrophoresis and extensive use in staining experiments that these antisera cross-react strongly and specifically with the analogous monkey immunoglobulins. Extensive cross-reactions of antisera to human immunoglobulins with their subhuman primate analogues have been previously reported (2, 3, 14). Absorption of immune fluids with antisera to a single immunoglobulin class prepared in a manner identical to the conjugates resulted in removal of all antibody activity of that class. Addition of purified monkey immunoglobulin G (IgG) to the antihuman IgG conjugate removed all staining activity. Conjugates were applied for 30 min, the slides were washed for 1 h in PBS, and cover slips were mounted in fresh 10% glycerol in PBS buffered at pH 7.4. Stained smears were evaluated using a Zeiss microscope with the slide oiled to a dark-field condenser and an Osram HBO-200 light source. In the exciting path were a BG-12 filter and a 495-nm interference filter. Barrier filters used were either a no. 50 or a double thickness of Wratten no. 57A gelatin filters. Fluorescence in a pattern with recognizable bacterial morphology was necessary to constitute a positive result. The antibody titer was reported as the reciprocal of the greatest dilution which resulted in staining. The immunoglobulin class of the antibody was determined simultaneously, since staining depended on the class specificity of the conjugate. Controls included preimmunization serum and saliva samples and fluid samples from sham-immunized animals. Smears of *S. mutans* FA-1, selected from serogroup *b*, were included to evaluate the specificity of the antibody. Assays were often repeated on the same samples using independently prepared dilution series, and some results were read several times by different investigators without knowledge of the sample source. The technique proved sensitive enough to use on unconcentrated saliva samples and was reproducible within the range of a single dilution step and easily carried out.

Glucosyltransferase activity was measured by determining incorporation of the radiolabeled moiety of [^{14}C]glucose-labeled sucrose into ethanol-insoluble polysaccharide under conditions in which levansucrase activity was not detectable (9). To test the inhibiting capacity of serum or saliva samples of small volume or low antibody concentration, it was necessary to reduce the volumes previously reported for the glucosyltransferase assay. The modification which we used in these experiments consisted of a 10-fold reduction in final volume to 20 μl which contained about 37 μg of CEP. An increase in the incubation time from 2 to 6 h was included to increase the sensitivity of the assay. These modifications have allowed us to test saliva using 10-μl samples with about a 10-fold increase in sensitivity over that obtained by the reported method (9). Serum and saliva from monkeys immunized with *S. mutans* were tested for their capacity to inhibit glucosyltransferase by incubating various dilutions of the antibody source with the enzyme for 30 min at 30 C prior to the addition of the radioactive substrate and dextran primer. The procedure for determination of glucosyltransferase activity was then followed. The inhibiting capacity of the immune fluids was expressed as the percentage of reduction in radioactivity incorporated compared to control fluids from sham-immunized animals at the same dilution. Enzymes prepared from *S. mutans* GS-5 and BHT were tested to determine the extent of cross-reaction of the inhibiting antibody.

Detection of secretory component. Rabbit antiserum to human secretory component was prepared by immunization with human salivary IgA purified by gel filtration, anion exchange chromatography, and sucrose density gradient ultracentrifugation. The antiserum, which reacted only with IgA, was absorbed with the whole plasma from a patient with an IgA myeloma. The absorbed antiserum precipitated

in gel diffusion and immunoelectrophoresis only with secretory IgA and not with serum immunoglobulins.

In a four-step staining procedure, PF containing IgA antibodies was first applied to a 6715 smear. The rabbit antiserum to human secretory component was then applied and followed by goat anti-rabbit IgG conjugated with rhodamine. In the fourth step, the fluorescein-conjugated antiserum to human IgA was applied. In parotid fluid samples containing only IgA antibody, red staining indicated the presence of secretory component and staining with both fluorochromes showed the association of secretory component with IgA antibody. Preimmune PFs, normal rabbit serum, and a non-cross-reacting strain of *S. mutans* were used as controls.

RESULTS

Immunization by s.c. injection. The ability of irus monkeys to mount a salivary immune response had not been explored. After seven s.c. injections of the vaccine in the vicinity of the salivary glands during 15 days, the mean serum immunoglobulin M (IgM) antibody titer was elevated, and IgG and IgA antibody was also detected (Fig. 2). No antibody could be detected in 20×-concentrated PF from any of the monkeys.

Immunization via the PDs. The same vaccine was then delivered to the gland by instillation into the PD. PF was collected from each animal 7 days after the first PD immunization. Two milliliters was concentrated about 20× using a Minicon apparatus (Amicon Corp., Lexington, Mass.) and assayed for antibody. IgA antibody to *S. mutans* 6715 was found in the concentrated PFs of three of the four animals in group I, whereas no antibody was detectable in concentrated fluids from group II animals. All succeeding antibody assays were performed on unconcentrated samples. Further PD immunization resulted in increases in the mean PF IgA antibody titer to peak at 13.5. On day 225 (Fig. 2) individual titers in the four

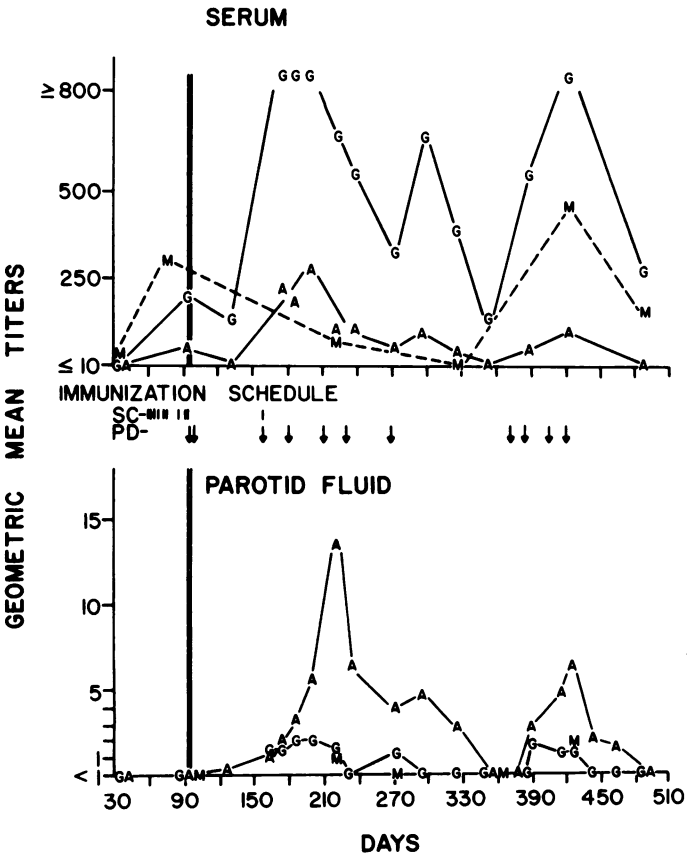

FIG. 2. *Titers of antibody to S. mutans 6715 as measured by indirect IFS. Titers are expressed as the reciprocal of the dilution. Each point represents the mean of samples taken from four animals. Vertical bars indicate subcutaneous (SC) immunizations. Arrows indicate immunizations via the PD. The double vertical lines signify the beginning of PD immunization. The letter at each point denotes the immunoglobulin class of the antibody. All points on abscissa (<1) indicate no detectable antibody.*

immune animals were 8, 16, 16, and 16. IgG antibody was also found in the PF. Its highest mean level occurred on days 186 and 200 and was only found thereafter in PF samples taken within 7 days after immunization. IgM antibody was infrequently found at low levels in PF, and it was always found in concert with IgG antibody. Peaks of mean IgA and IgG antibody titers were found in the serum on day 200. Serum IgG antibody reached titers above 800, the highest dilution tested.

After the first six immunizations via the PD route the monkeys were rested for 5 weeks. Antibody levels in both fluids fell rather sharply during this interval. A single immunization on day 267 resulted in transient elevation of antibody titers in both PF and serum. During the next 9 weeks mean titers declined until antibody was undetectable in the PF. Four subsequent PD immunizations beginning on day 372 and evenly spaced over the next 7 weeks resulted in PF and serum responses with kinetics similar to those seen after the first course of PD immunization. Mean PF IgA antibody titers rose during immunization and afterwards fell sharply to undetectable levels after 9 weeks. Serum IgG, IgM, and IgA antibody titers all increased during immunization and fell when immunization was curtailed. During the entire course of the experiment, the PF IgA antibody titers were higher than IgG titers, whereas in the serum the reverse was true.

One monkey, previously unexposed to the vaccine by any route, was immunized by instillation of the vaccine into the right PD while the left duct was instilled with diluent buffer. The response pattern (Fig. 3) was similar to that seen in the above group of animals. IgA antibody was detected only in the secretions from the immunized gland, except in one sample from the left gland taken on day 265 in which it was detected at a titer of 1. IgG antibody was found in the fluid from the immunized gland during the later series of instillations. As in the

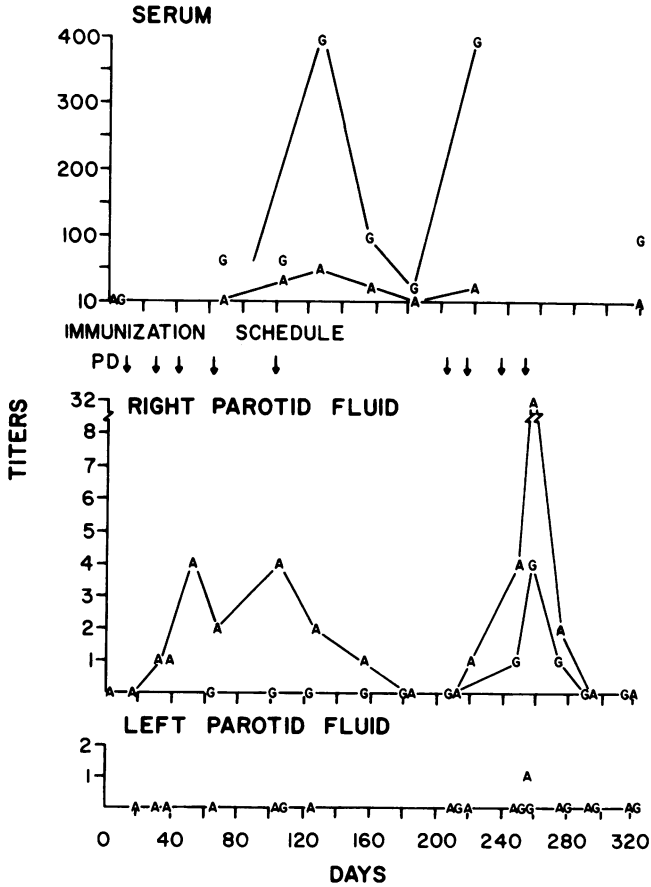

FIG. 3. *Titers of antibody to S. mutans 6715 as measured by indirect IFS. Data were obtained from a single monkey instilled with S. mutans vaccine in the right PD and with a buffer solution in the left. Titers are expressed as the reciprocal of the dilution. Arrows indicate immunizations via the PD. The letter at each point denotes the immunoglobulin class of the antibody.*

initial group of monkeys, titers rose quickly after the beginning of a series of PD instillations and fell soon after immunization was stopped. Again IgA-IgG antibody titer ratios were always greater than unity in PF and less than one in the serum.

The association of secretory component with PF IgA antibody was shown by staining with the anti-human secretory component antiserum alone and by double staining with both the anti-human secretory component and anti-human IgA together. Preimmune PF showed no staining, and substitution of normal rabbit serum for the anti-human secretory component serum resulted in no staining with the goat anti-rabbit IgG. Immune PF applied to the non-cross-reacting strain of *S. mutans* resulted in no staining. Substitution of goat anti-human IgG for anti-human IgA in the final step resulted in red staining only, signifying the presence of secretory component and the lack of significant cross-reaction of the anti-human IgG antiserum with rabbit IgG.

Further confirmation of the association of PF IgA antibody with secretory component was obtained by the absorbtion of all IgA antibody activity from PF with the rabbit antiserum to the human secretory component. Neither normal rabbit serum nor goat anti-human IgG reduced IgA antibody activity in equivalent samples of PF. Anti-human secretory component reduced but did not completely remove IgA antibodies from immune serum, suggesting the presence in serum of some IgA associated with secretory component.

Inhibition of glucosyltransferase activity was measured to further characterize the antibody response to *S. mutans*. Preimmune PF samples were found to have high concentrations of a substance capable of inhibiting glucosyltransferase. This was easily removed by routine dialysis of all PF samples against PBS samples prior to assay. Significant concentrations of inhibiting antibody were not found in the serum (Fig. 4) until 4 months after initial immunization. Over the next 4 months they remained at essentially the same level during repeated immunization via the PD route. Significant inhibition ($\geq$10%) was not detectable in the PF at any time. When the capacity of the serum to inhibit the enzyme was near plateau levels (days 196, 206, 220), low levels of inhibiting activity were found in PF samples in which both IgA and IgG antibody to strain 6715 were found using indirect IFS. When PF samples taken at days 175 and 200 were concentrated 16$\times$ and tested for enzyme inhibition, the mean percentage of inhibitions found were 4 and 42%, respectively. After the last series of PD injections, while the serum and PF responses were at their peak, inhibiting activity was slightly elevated but still at low levels in PF. Sera collected from immune animals at the peak of mean percentage of inhibition (day 195) of 6715 enzyme were found to inhibit enzyme preparations from BHT or GS-5 either poorly or not at all.

PF flow rates were measured as an indicator of gland function during immunization. Flow rates were recorded over a 10-min interval during peak flow beginning 2 to 5 min after administration of pilocarpine. Most often secretions from both glands were collected by two cannulas, but occasionally only one gland could be sampled. All individual flow rates were normalized to milliliters per minute per gland, and group means were calculated for each interval. The sham-immunized monkeys showed little change (Table 1) in flow rate over the experimental period, whereas the flow in the immunized animals temporarily decreased after immunization. Significant differences in flow rates between the groups were not seen until days 425 and 442 after the beginning of the last series of immunizations. Flow rates in the immune animals appeared to be recovering on day 464, when the last samples were collected.

DISCUSSION

Studies in the rabbit (29) and rat (30) showed that antibacterial antibodies could be regularly induced in the saliva by s.c. injection of bacterial components or whole cells in the vicinity of the major salivary glands. Using the same method of immunization and a similar vaccine, we were unable to elicit salivary antibody in irus monkeys; however, serum antibodies were detected. We were able to demonstrate the induction and restimulation of salivary antibacterial antibodies by intraductal instillation of vaccine into the PD. These data provide the first evidence for the induction of a secretory IgA antibacterial antibody response in primate saliva.

Indirect IFS using conjugates specific for a single immunoglobulin class permitted titration and classification of antibodies in a single procedure. Indirect IFS has been compared with indirect hemagglutination in the detection of antibodies to *Plasmodium falciparum* in the sera of Aotus monkeys (4). The sensitivity of indirect IFS was shown to be equal to or greater than that of indirect hemagglutination with titers reported over 2×10^4. Using indirect IFS we have recorded titers of serum antibody to *S. mutans* $> 10^3$ in monkeys and $> 3 \times 10^3$ in rabbits. We have found that staining with conjugates with a fluorescein-protein ratio of

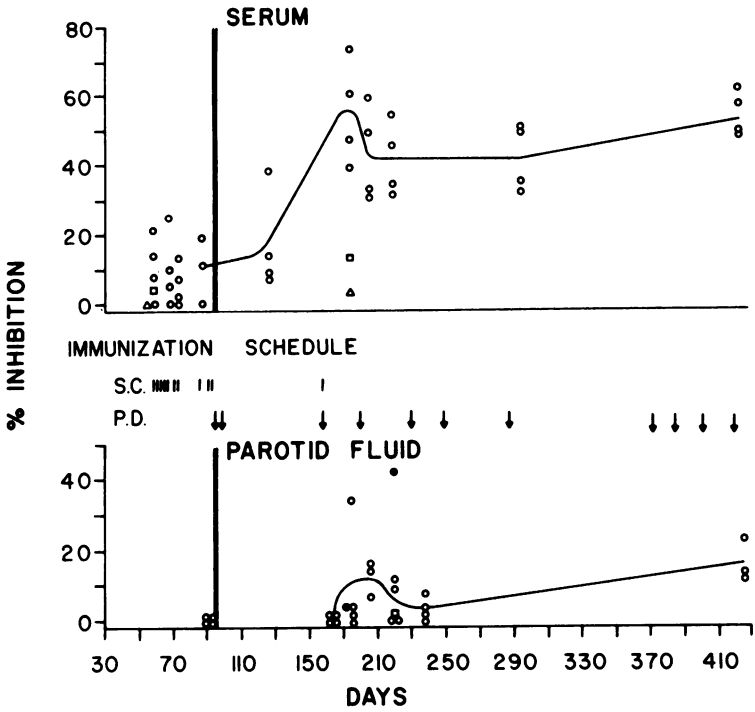

FIG. 4. *Inhibition of glucosyltransferase by monkey serum and PF. Inhibition is expressed as the percentage of reduction in enzyme activity by fluids from immune animals compared to fluids from control animals. Symbols:* O, *percentage of inhibition of enzyme prepared from strain 6715 in a single animal sample;* ●, *percentage of 6715 enzyme by 20×-concentrated PF samples; solid lines, mean percentage of inhibition of 6715 enzyme by four animal samples;* □, *mean percentage inhibition of enzyme prepared from strain BHT;* △, *mean percentage of inhibition of enzyme prepared from strain GS-5. Vertical bars indicate subcutaneous (SC) immunizations. Arrows indicate immunizations via the PD. The double vertical lines signify the beginning of PD immunization.*

about four increases the sensitivity of the assay by two to three double-dilution steps compared to conjugates with fluorescein-protein ratios of 1 to 2. The use of conjugates made from antisera raised to monkey immunoglobulins may also increase the sensitivity of the assay, but these are not yet available. Testing the anti-human immunoglobulin conjugates for specificity to analogous monkey immunoglobulins was usually performed with monkey serum. However, goat antiserum to human IgA, purchased from the same commercial source, precipitated a single sharp band when reacted in double diffusion with concentrated monkey whole saliva. A preparation of irus monkey IgG, purified by diethylaminoethyl chromatography from serum, absorbed all staining activity from the anti-human IgG conjugate. Attempts were made to quantitate immunoglobulins in irus monkey saliva by radial immunodiffusion, but the commercial plates containing antisera to human immunoglobulins were not sensitive enough. Efforts are in progress to prepare antisera to monkey immunoglobulins and to evaluate more sensitive techniques for quantitation of monkey secretory antibodies.

IgA antibodies to strain 6715 were detected in 20×-concentrated PF after only 1 week, during which the monkeys received two PD immunizations. IgA antibody titers were first seen in unconcentrated PF in samples taken from two of the four monkeys 32 days after initial PD instillation similar to the finding in the one PD-immunized monkey. However, samples had not been obtained since day 7 after initial PD instillation. We predicted a significant delay in the early IgA antibody response to intraductal instillation in the monkeys already immunized by s.c. injection. We (7) have shown that the early IgA antibody-forming cell response to local immunization with dinitrophenylated bovine serum albumin in rabbits was inhibited by prior feeding or intraperitoneal injection of the same antigen. The differences in immunization schedules and sampling intervals between the four s.c./PD-immunized monkeys and the single PD-immunized animal make the response patterns difficult to compare. Re-

TABLE 1. *PF flow rates after stimulation with pilocarpine*[a]

Group	Mean PF flow (day)												
	74	93	102	127	161	175	200	273	294	390	417	425[b]	442[b]	464
Immune	0.38 ±0.025[c]	0.31 ±0.102	0.38 ±0.038	0.26 ±0.023	0.21 ±0.042	0.36 ±0.026	0.42 ±0.199	0.17 ±0.016	0.17 ±0.044	0.08 ±0.036	0.10 ±0.031	0.06 ±0.020	0.07 ±0.017	0.17 ±0.068
Control	0.46 ±0.099	0.55 ±0.035	0.32 ±0.049	0.29 ±0.044	0.27 ±0.019	0.45 ±0.094	0.33 ±0.086	0.29 ±0.071	0.21 ±0.095	0.30 ±0.140	0.32 ±0.170	0.38 ±0.045	0.42 ±0.065	0.32 ±0.255

[a] Immunization schedule: s.c. injection in vicinity of glands on days 58, 60, 63, 65, 67, 71, 73, 81, 91, 93, 158, and 180; intraductal instillation on days 95, 99, 158, 210, 228, 267, 372, 384, 405, and 419.

[b] Difference between groups was significant by Student's t test ($P < 0.01$).

[c] Results given in milliliters per minute per gland ± standard error.

cently, however, four young (12 to 15 months) irus monkeys were immunized with the same vaccine via the PD route only. Their response patterns show detectable IgA antibody 20 to 25 days after PD instillation. These later observations support the view suggested by the previous data that prior s.c. immunization resulted in little, if any, delay in the early secretory immune response. Experiments designed specifically to examine the early salivary secretory response to PD instillation must be carried out to obtain a definitive answer in the primate.

PF samples taken during the initial IgA antibody response from the monkeys immunized by both s.c. and PD routes contained IgG antibody, whereas the initial PF response in the unsensitized animal showed no IgG antibody. Since concentrations of serum IgG antibody were much higher in the presensitized animals than in the single unsensitized monkey, it is likely that the IgG antibody in PF reflects transudation from the serum rather than local synthesis. This opinion is supported by the observation that IgM was found in PF in association with IgG only when serum IgM antibody titers were elevated.

Monkeys immunized 1 week after the mean PF IgA titer had reached its first peak on day 221 (Fig. 2) did not respond with elevated antibody titers. A similar observation was made by André et al. (1), who immunized mice by intragastric feeding with sheep erythrocytes and quantitated plaque-forming cells in the spleen. No splenic IgA, IgG, or IgM plaque-forming cell response could be elicited if a second course of immunization followed the first by a week. Their results suggested that full responsiveness had not been restored after 1 month but was present after 3 months. They concluded that the most likely explanation for this refractoriness was either inactivation of the antigen or interference with the absorption of the antigen by antibodies synthesized in response to the preceding course of immunization. Observations by Walker et al. (34) suggest that oral immunization of rats with soluble macromolecular antigens specifically interferes with their intestinal uptake. Our findings showing elevations in antibody titers when PD immunization was carried out during low existing PF titers, and no response to immunization during elevated PF titers can be explained by antibody interference with antigen uptake. In fact, the relatively transient nature of the IgA responses reported both by André et al. (1) and by us probably reflects a continuously changing inverse relationship between antigenic stimulation and antigen washout dependent on antibody concentrations at the absorbing surface.

The net serum antibody response largely paralleled that found in PF with high titers of IgM antibody found early during s.c. immunization. Serum IgM titers steadily decreased until another IgM peak was observed concomitant with the last series of PD injections, which began 1 year later. Investigators (33) have reported that high serum IgG antibody titers will suppress an IgM response, but in our experiments IgG titers were high during the second IgM peak. This difference in behavior may be attributable to the antigen, the route of immunization, or the species.

Several observations lead to the conclusion the IgA antibody found in the PF was synthesized in cells within the salivary glands. In the PF, IgA antibody titers were always higher than IgG. In the serum the reverse was true. These observations are inconsistent with simple transudation of serum antibodies. Immunization into a single parotid gland of an unsensitized monkey (Fig. 3) resulted in high titers of IgA antibodies in the secretions from that gland but only a late, barely detectable IgA response in the fluid from the contralateral control gland. Finally, secretory component was shown to be associated with the IgA antibodies in the PF. Though polymeric IgA and secretory component have been found in the serums of most species, this polypeptide chain is most commonly associated with the secretory IgA dimer, which combines with it after secretion of the dimer from the plasma cell and prior to its addition to the external secretions.

Recent observations suggest the presence of secretory IgA anamnesis (16) and particularly an anamnestic salivary IgA response (30). Our observations agree with those (1, 23, 38) who have concluded that the IgA system shows little or no evidence of immunological memory. Little or no memory would imply the necessity for a relatively continuous supply of antigen to maintain those levels of antibody necessary for effective in vivo function. We were able to elecit secretory antibodies as long as we continued to immunize the parotid glands via the duct but antibody levels dropped quickly when the vaccine was withheld, even though the oral cavity had been infected by the immunizing strain. The length of the duct of the major salivary glands likely precludes retrograde stimulation under normal conditions. However, Mandel (personal communication) has found high levels of secretory antibody to infecting organisms in PFs from glands afflicted with acute parotitis. Minor salivary glands have very short secretory ducts and may well be continuously stimulated by the natural retrograde flow of oral antigens. This constant stimulation could result in sustained elevated titers of antibody in the secretions from these glands. The contribution of the minor glands to total salivary antibody may prove to be a most important oral ecological determinant because of the persistence of the response. Whether or not the minor glands are collectively capable of synthesizing enough antibody to significantly inhibit *S. mutans* implantation depends on both the levels of antibody necessary for inhibition and the concentration of *S. mutans* in plaque necessary to initiate disease. It has recently been shown (J. Dent. Res. **54** [special issue]: abstr. 53) that secretions from human minor salivary glands contain concentrations of IgA four times that found in secretions from the major salivary glands. No IgA antibody was detected in samples of nonparotid saliva from immune monkeys. This finding may have been the result of dilution of antibody by the copious volumes of pilocarpine-stimulated saliva or of removal of antibodies by absorption to oral organisms. Since *S. mutans*, though universally found in human dental plaque, seems to initiate colonization of smooth tooth surfaces with difficulty (17), it may not require high concentrations of salivary antibody to tip the balance away from implantation.

In view of our findings, it is safe to speculate that an immune response can be elicited in minor salivary glands by retrograde instillation. It is suggested that application of high concentrations of antigen to the mucosa overlying these glands might elevate salivary antibodies to the level necessary to inhibit adherence of cariogenic bacteria. Concentrations of these bacteria in dental plaque would be limited by salivary antibody below the level necessary to produce dental caries. The small numbers of organisms which persist in the plaque would provide continuous stimulation, both locally of the glands and of the gut-associated lymphoid tissue. It is suggested that maintenance of this balance between antigenic stimulation and bacterial elemination would result in significant reduction in dental caries.

Levels of serum antibody capable of inhibiting glucosyltransferase were more closely correlated with serum IgG antibody titers, as measured by indirect IFS, than with serum IgA or IgM antibody. It appeared, therefore, that most of the inhibiting activity in the serum was present in the IgG class. Results with enzyme preparations from *S. mutans* strains GS-5 and BHT, belonging to serogroups *c* and *b*, respectively, showed little cross-inhibition. Antigenic differences between enzyme preparations from

representative strains of the various serogroups of *S. mutans* have been reported before (12, 13). Mukasa and Slade (24) have implicated the *a-d* antigenic determinant of group *a* and *d* strains of *S. mutans* as the site on the cell wall participating in binding glucosyltransferase. The *a-d* antigenic site is found on the same polysaccharide molecule as the *a* and *d* serogroup-specific antigenic determinants (21, 23). The affinity of the enzyme for these polysaccharide antigens may result in their association in solution as well as on the surface of the bacterial cell. Enzymes prepared from culture supernatants for use in immunization or in the inhibition assay might be combined with these polysaccharides, permitting inhibition by antibodies directed to the group-specific antigens. This would provide one explanation for the antigenic distinctness of these enzymes exhibited by the inhibition assay. Low levels of inhibiting activity were also found in the PF but always in the presence of both IgG and IgA antibody to 6715. Most IgG antibody was assumed to be transuded from the serum and not locally synthesized.

Significant differences in average mean flow between immune and control groups were only seen during the last series of PD immunizations. The contents of the vaccine were not well defined and some may have been toxic. When the vaccine was introduced via the ducts into an animal with high titers of serum antibody, it was likely that an immunopathological reaction might interfere with secretory function. Experiments are presently being carried out to determine the mechanism of this dysfunction. Vaccines prepared from purified *S. mutans* antigens are being evaluated for their immunogenicity when introduced via the PD route. Immunization with these vaccines may not result in similar functional alterations.

Instillation of antigen via the PD is a relatively simple procedure. A similar technique is used in humans to obtain diagnostic radiographs of the salivary glands and is most often quick and painless. However, the practicality of this method as a mode of mass human immunization is admittedly questionable.

We have demonstrated the feasibility of inducing secretory salivary IgA antibodies in monkeys to a common human oral pathogen. We have examined some of the characteristics of the immune response and some of the properties of the antibody. In a succeeding manuscript we will evaluate the capacity of this antibody to alter the ability of the immunizing organism to colonize the surfaces of teeth. Inhibition of bacterial colonization of smooth surfaces would suggest the use of immunization in the prevention of dental disease.

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