Humoral Immunity to Commensal Oral Bacteria in Human Infants: Salivary Antibodies Reactive with *Actinomyces naeslundii* Genospecies 1 and 2 during Colonization

MICHAEL F. COLE,^{1*} STACEY BRYAN,¹ MISHALL K. EVANS,¹ CHERYL L. PEARCE,¹
MICHAEL J. SHERIDAN,¹ PATRICIA A. SURA,¹ RAOUL WIENZEN,²
AND GEORGE H. W. BOWDEN³

Departments of Microbiology and Immunology¹ and Pediatrics,² Georgetown University
Medical Center, Washington, D.C. 20007, and Department of Oral Biology,
University of Manitoba, Winnipeg, Manitoba R3E 0W2, Canada³

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The secretory immune response in saliva to colonization by *Actinomyces naeslundii* genospecies 1 and 2 was studied in 10 human infants from birth to 2 years of age. *Actinomyces* species were not recovered from the mouths of the infants until approximately 4 months after the eruption of teeth. However, low levels of secretory immunoglobulin A1 (SIgA1) and SIgA2 antibodies reactive with whole cells of *A. naeslundii* genospecies 1 and 2 were detected within the first month after birth. Although there was a fivefold increase in the concentration of SIgA between birth and age 2 years, there were no differences between the concentrations of SIgA1 and SIgA2 antibodies reactive with *A. naeslundii* genospecies 1 and 2 over this period. When the concentrations of SIgA1 and SIgA2 antibodies reactive with whole cells of *A. naeslundii* genospecies 1 and 2 were normalized to the concentrations of SIgA1 and SIgA2 in saliva, the *A. naeslundii* genospecies 1- and 2-reactive SIgA1 and SIgA2 antibodies showed a significant decrease from birth to 2 years of age. The fine specificities of *A. naeslundii* genospecies 1- and 2-reactive SIgA1 and SIgA2 antibodies were examined by Western blotting of envelope proteins. Similarities in the molecular masses of proteins recognized by SIgA1 and SIgA2 antibodies, both within and between subjects over time, were examined by cluster analysis and showed considerable variability. Taken overall, our data suggest that among the mechanisms *Actinomyces* species employ to persist in the oral cavity are the induction of a limited immune response and clonal replacement with strains differing in their antigen profiles.

The genus *Actinomyces* comprises several species of facultatively anaerobic, gram-positive, branching rods that are numerically significant autochthonous bacteria in the oral cavities of humans and other mammals (4, 6). Several species of *Actinomyces* are opportunistic endogenous pathogens that cause actinomycosis and have been implicated in periodontal disease and coronal and root surface caries (3, 5, 6, 25).

The indigenous microbiota of the mouth and other mucosal surfaces exists in a state of homeostasis with the host except when it is perturbed, the mucosal surface is damaged, or the immune system is compromised (6, 20). Adaptive humoral immunity at mucosal surfaces is effected principally by secretory immunoglobulin A (SIgA) (19), which is thought to play a role in the regulation of commensal bacteria (8). However, despite the fact that saliva contains SIgA antibodies reactive with commensal bacteria (29) and commensal bacteria are coated with SIgA (7), these microorganisms colonize and persist on mucosal and tooth surfaces. These findings suggest that, in contrast to exogenous pathogenic bacteria, indigenous oral bacteria are unaffected by, are not subjected to, or are able to avoid immune elimination by mucosal antibodies (reviewed in references 6 and 8). This assertion is supported by the observation that there is no significant difference between the acquisition of the oral and intestinal indigenous microbiotas of transgenic B-cell-deficient mice that lack mucosal and serum immunoglobulins and that of their normal littermates (17).

This observation implies that SIgA does not play a major role in the regulation of the indigenous microbiotas of mice. Furthermore, colonization of mice by commensal enteric bacteria appears to generate a self-limiting mucosal immune response, resulting in a state of chronic hyporesponsiveness (26).

As part of a longitudinal study of the relationships between oral colonization of infants by commensal bacteria and the development of the secretory immune response, we have examined the salivary immune response to *Actinomyces naeslundii* genospecies 1 and 2; these are autochthonous bacteria whose primary habitat is the oral cavity (although strains may be isolated from the tonsils) (5). The results show that colonization by these bacteria is preceded by a SIgA antibody response with changing antigenic specificity in saliva which peaks at 6 months of age but wanes thereafter. The induction of a limited immune response and antigenic variation may be mechanisms by which commensal bacteria avoid immune elimination and persist in the oral cavity and at other mucosal surfaces.

MATERIALS AND METHODS

Study population. Ten healthy, full-term infants were employed in this study. Details of the study population have been published previously (12, 21).

Sample collection and processing. (i) **Oral swabs.** Swab samples were obtained 1 to 3 days, 2 and 4 weeks, and 2, 4, 6, 8, 10, 12, and 24 months postpartum. The mucosal surfaces of the cheeks, buccal sulci, edentulous ridges, tongue, and hard palate were swabbed with the swab from a Vacutainer anaerobic specimen collector (Becton Dickinson Microbiology Systems, Cockeysville, Md.). The swab was then returned to the sealed tube of the collector and transported anaerobically to the laboratory within 1 h of collection. After the swab was placed in 2 ml of reduced transport fluid (31), bacteria were dispersed by ultrasound at 80 W for 10 s with a model 250 sonifier (Branson Ultrasonics Corp., Danbury, Conn.)

* Corresponding author. Mailing address: Rm. S.E. 308A, Med Dent Bldg., 3900 Reservoir Rd., N.W., Washington, DC 20007. Phone: (202) 687-1817. Fax: (202) 687-4973. E-mail: coleman@gunet.georgetown.edu.

equipped with a microprobe. The dispersed sample was serially diluted in reduced transport fluid to 10^{-5} .

(ii) **Whole-mouth saliva.** Whole saliva was collected with sterile 3-ml plastic transfer pipettes. Immediately after collection EDTA was added to a final concentration of 5 mM to prevent formation of heterotypic calcium ion-dependent immunoglobulin-mucin complexes and to inhibit IgA1 protease activity in the sample (12). The saliva samples were held at -85°C until being assayed.

Recovery and identification of *A. naeslundii*. Trypticase soy agar containing 5% sheep blood (TSASB); anaerobic Columbia agar containing 5% sheep blood, cysteine HCl, palladium chloride, dithiothreitol, and hemin (CASB); and CFAT agar (35) (Remel, Lenexa, Kans.) plates were inoculated with a spiral plater (Spiral Systems, Cincinnati, Ohio). The TSASB plates were incubated at 37°C for 48 h in air. The CASB plates were incubated at 37°C for 3 to 5 days in an anaerobic chamber containing an atmosphere of 80% N_2 , 10% CO_2 , and 10% H_2 , and the CFAT plates were incubated at 37°C for 48 h in 5% CO_2 . After incubation, total counts of each colony morphotype were determined from the nonselective and selective media that contained between 30 and 300 CFU. Representatives of each colony morphotype were picked under a dissecting microscope at $\times 20$ magnification and subcultured to purity on TSASB. The purified isolates were stained by Gram's method and tested for the production of catalase. Gram-positive pleomorphic rods were identified as *Actinomyces* species by slide agglutination with a panel of specific rabbit antisera (11, 22). A total of 212 *Actinomyces* isolates were examined, of which 113 were identified as *A. naeslundii* genospecies 1 and 67 as *A. naeslundii* genospecies 2. Thirty-two isolates could not be typed with the panel of antisera.

Production of *A. naeslundii* genospecies 1 and 2 cells for antigen. *A. naeslundii* genospecies 1 (ATCC 12104) and *A. naeslundii* genospecies 2 (W1053) served as the sources of antigen to determine *A. naeslundii* genospecies 1- and 2-reactive SIgA antibodies in the infants' saliva over time by enzyme-linked immunosorbent assay (ELISA) and Western blotting. The bacteria were grown to substrate exhaustion in the ultrafiltrate (10,000 MW cutoff) (Minitan ultrafiltration system; Millipore, Bedford, Mass.) of Todd-Hewitt broth (Difco) supplemented with vitamins, salts, and 1.0% glucose and maintained at a pH of 7.0. The bacteria were harvested and washed by centrifugation at $16,000 \times g$ for 20 min and resuspended first in phosphate-buffered saline (PBS), pH 7.4, and finally in 10 mM HEPES buffer, pH 7.4, containing 5 mM EDTA.

Quantification of SIgA, SIgA1, and SIgA2 in saliva. The concentrations of SIgA, SIgA1, and SIgA2 in saliva were determined by ELISA (12). All antibodies employed in the ELISA were affinity purified, and incubations were performed at room temperature for 1 h unless otherwise stated. The assay volume was 100 μl , and the wells were washed three times with PBS, pH 8.0, containing 0.1% Tween 20 between additions of antibodies, samples, or standards and substrate. Briefly, one-half of the Immulon 2 (Dynatech, Chantilly, Va.) plates were coated overnight with 10 μg of a murine monoclonal antibody (MAb) to human secretory component (SC) (Hybridoma Labs., Baltimore, Md.)/ml. Unbound antibody was washed from the wells, which were then blocked with PBS, pH 8.0, containing 0.1% bovine serum albumin. After the wells were washed, dilutions of saliva or colostrum SIgA, SIgA1, or SIgA2, purified in our laboratory as standards, were added in duplicate and incubated overnight with shaking. A polyclonal goat anti-human α chain antibody (GAH α) (Jackson ImmunoResearch, West Grove, Pa.) at 2.0 $\mu\text{g}/\text{ml}$ and murine MAbs $\alpha 1$ and $\alpha 2$ (Southern Biotechnology Associates, Inc., Birmingham, Ala.) at 0.5 $\mu\text{g}/\text{ml}$ were used to detect bound SIgA, SIgA1, and SIgA2, respectively. Following washing, bound GAH α was amplified with biotinylated rabbit anti-goat γ chain. Bound MAbs $\alpha 1$ and $\alpha 2$ were amplified with biotinylated rabbit anti-mouse γ chain. The amplifying antibodies were obtained from Jackson ImmunoResearch and used at concentrations of 1.0 and 1.5 $\mu\text{g}/\text{ml}$, respectively. The wells were washed again, and streptavidin conjugated with horseradish peroxidase (BioSource International, Camarillo, Calif.) at 0.1 $\mu\text{g}/\text{ml}$ was used to detect the biotinylated antibodies. Following a final wash, *o*-phenylenediamine (1 mg/ml) in citrate-phosphate buffer, pH 4.5, containing 0.012% hydrogen peroxide was added to each well. Absorbances (optical density at 450 nm) were measured with a model EL309 automated microplate reader (Bio-Tek Instruments, Inc., Winooski, Vt.).

Quantification of SIgA, SIgA1, and SIgA2 anti-*A. naeslundii* genospecies 1 and 2 antibodies in saliva. Concentrations of SIgA, SIgA1, and SIgA2 anti-*A. naeslundii* genospecies 1 and 2 antibodies in saliva were determined by ELISA as described above, except that the remaining half of the Immulon 2 (Dynatech) plates were coated overnight with 10 μg (dry weight) of formalin-killed *A. naeslundii* genospecies 1 or 2/ml instead of anti-human SC MAb. A duplicate of each saliva sample was added to both the anti-human SC MAb-coated wells and the *A. naeslundii* genospecies 1- or 2-coated wells on a single 96-well plate. Since the upper-level reagents were the same for both assays, it was possible to interpolate absorbances from the *A. naeslundii* genospecies 1- or 2-coated wells into the

SIgA, SIgA1, and SIgA2 standard curves. The coefficient of variation within and between assays did not exceed 10%.

Quantification of protein in saliva. Total protein in saliva was determined by the bicinchoninic acid assay (Pierce Chemical Co., Rockford, Ill.) according to the manufacturer's protocol.

Fine specificities of SIgA, SIgA1, and SIgA2 anti-*A. naeslundii* genospecies 1 and 2 antibodies in saliva. Cell envelope preparations (CEPs) of *A. naeslundii* genospecies 1 and 2 were prepared as described previously (21). Briefly, a suspension of each genospecies in 10 mM HEPES buffer, pH 7.4, was held on ice and subjected to four 1-min cycles of ultrasound with a GE 50 microultrasonic processor operated at a setting of 6 and equipped with a 3-mm-diameter microprobe (Daigger Corp., Wheeling, Ill.). After sonication, the bacteria were removed by centrifugation, and the supernatant was stored at -80°C .

The fine specificities of *A. naeslundii* genospecies 1- and 2-reactive salivary SIgA, SIgA1, and SIgA2 antibodies were determined by Western blotting. The CEPs together with molecular mass standards (Bio-Rad, Hercules, Calif.) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with the Mini Protean II system (Bio-Rad). The separating gel was 11% acrylamide, and the stacking gel was 5% acrylamide. The separated CEPs were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore) by using a Trans Blot SD system (Bio-Rad). The membranes were blocked for 1 h in 4% bovine serum albumin in Tris-buffered saline, pH 8.3, containing 0.02% NaN_3 . Individual lanes were cut from the membranes, and three lanes of the CEPs were incubated overnight with each saliva sample. The strips were then subjected to three 10-min washes in Tris-buffered saline containing 0.1% Tween and 0.5 M NaCl. Bound SIgA, SIgA1, and SIgA2 antibodies were detected as described above for ELISA, except that 0.5 mg of 3'-diaminobenzidine in 0.01 M PBS, pH 7.4, containing 0.01% H_2O_2 was used as a substrate.

Each Western blot lane, including the molecular mass standards, was scanned with an Ultrosan XL laser densitometer (Pharmacia-LKB, Piscataway, N.J.), and the densitograms were imported into an analytic software program (Gel-Compar 4.0; Applied Maths, Kortrijk, Belgium).

Statistical analysis. The study was designed to examine the following: (i) changes in the concentrations of SIgA1 and SIgA2 and *A. naeslundii* genospecies 1- and 2-reactive SIgA1 and SIgA2 antibodies over time and (ii) interactions between time and SIgA1 and SIgA2 and *A. naeslundii* genospecies 1- and 2-reactive SIgA1 and SIgA2 antibodies. As the differences within individuals over time would be expected to be more highly correlated than differences between individuals over time, a two-factor repeated-measures analysis of variance was used to analyze these data. The within-subjects factor was time (i.e., measurements of samples from the same subject at 1, 6, 12, and 24 months postpartum), and the between-subjects factors were SIgA1 and SIgA2 and *A. naeslundii* genospecies 1- and 2-reactive SIgA1 and SIgA2. The level of statistical significance was set at $\alpha = 0.05$. Cluster analysis was performed within GelCompar 4.0 by using Ward's algorithm (33).

RESULTS

Bacteria began to colonize the infants' oral cavities almost immediately after birth. Between 1 and 3 days postpartum, approximately 10^7 CFU were recovered from swabs of the infants' oral mucosal surfaces that were plated on aerobic (1.1×10^7 CFU) and anaerobic (5.5×10^6 CFU) blood agar. The total viable aerobic and anaerobic counts increased over the first 6 months postpartum to plateau at approximately 10^8 CFU/swab, coincident with the eruption of teeth (Fig. 1A).

Teeth began to erupt at 6 months postpartum in two infants, and all infants had erupted teeth by 12 months (Fig. 1A). Although *Actinomyces* species were recovered from the oral cavities of the mothers at every visit (data not shown), no *Actinomyces* species were recovered from the oral cavities of the infants until after the eruption of teeth (Fig. 1B). There was a lag of approximately 4 months between the eruption of the first tooth and the appearance of *Actinomyces* species in the mouth. Between 10 months and 2 years of age, five oral swab samples were collected from the infants, from which 212 isolates resembling *Actinomyces* species were examined. Of these isolates, 180 were identified as *A. naeslundii* genospecies 1 or 2.

FIG. 1. (A) Total viable aerobic and anaerobic count of oral mucosal surfaces (predentate) and oral mucosa and teeth (postdentate) from birth to 2 years of age (mean CFU per swab $\pm$ standard error of the mean). The vertical bars show the cumulative numbers of infants with erupted teeth. (B) Colonization by *A. naeslundii* genospecies 1 and 2 from birth to 2 years of age (mean CFU per swab $\pm$ standard error of the mean). (C) Concentrations of salivary SIgA1 and SIgA2 from birth to 2 years of age (micrograms per milligram of protein $\pm$ standard error of the mean). (D) Concentrations of SIgA1 antibodies reactive with *A. naeslundii* genospecies 1 and 2 (nanograms per milliliter of saliva and nanograms per milligram of SIgA1 immunoglobulin $\pm$ standard error of the mean). (E) Concentrations of SIgA2 antibodies reactive with *A. naeslundii* genospecies 1 and 2 (nanograms per milliliter of saliva and nanograms per microgram of SIgA2 immunoglobulin $\pm$ standard error of the mean).

FIG. 2. (A) Western blot of *A. naeslundii* genospecies 1 envelope antigens incubated with saliva collected from a single infant at 1, 6, 12, and 24 months postpartum and probed to detect total SIgA, SIgA1, and SIgA2 antibodies. (B) Western blot of *A. naeslundii* genospecies 2 envelope antigens incubated with saliva collected from a single infant at 1, 6, 12, and 24 months postpartum and probed to detect total SIgA, SIgA1, and SIgA2 antibodies.

Thirty-two isolates of gram-positive branching rods from five infants could not be assigned to species by slide agglutination. These isolates have not been examined further. Initially, the infants acquired either *A. naeslundii* genospecies 1 or 2, and there appeared to be an inverse relationship between the counts of these genospecies between approximately 9 and 18 months. However, from 18 to 24 months the counts of both genospecies increased, and by 2 years of age the majority of infants harbored both genospecies.

To control for differences in flow rate, the concentrations of salivary SIgA were normalized to the protein contents of the salivas. SIgA, at a mean concentration of 13.0 $\mu\text{g}/\text{mg}$ of protein, was detected in whole saliva from the neonates within 3 days postpartum (Fig. 1C). The concentrations of SIgA1 and SIgA2 showed significant increases ($P < 0.0001$) from birth to age 2 years. By 2 years of age, the concentration of SIgA in the infants' saliva showed a fivefold increase to a concentration of 64.7 $\mu\text{g}/\text{mg}$ of protein. However, this constituted only 28% of the mean concentration of SIgA (230 $\mu\text{g}/\text{mg}$ of protein) in the mothers' saliva (12). SIgA1 was the dominant subclass in the infants' saliva. SIgA2 represented as little as 12.4% of total SIgA 2 weeks postpartum but reached 25% of total SIgA by 2 years of age, a value that approached the proportion (30.4%) of SIgA2 in the mothers' saliva (12).

Low levels of SIgA1 and SIgA2 antibodies reactive with whole cells of *A. naeslundii* genospecies 1 and 2 were detected within the first month after birth (Fig. 1D and E), at which time they represented $\sim 2\%$ of the total SIgA. Although there was a fivefold increase in the concentration of SIgA between birth and age 2 years, there were no differences between the concentrations of SIgA1 and SIgA2 antibodies reactive with *A. naeslundii* genospecies 1 and 2 when the data were expressed as nanograms of antibody per milliliter of saliva. However, when

the concentrations of SIgA1 and SIgA2 antibodies reactive with whole cells of *A. naeslundii* genospecies 1 and 2 were normalized to the concentrations of SIgA1 and SIgA2 in saliva, the *A. naeslundii* genospecies 1- and 2-reactive SIgA1 and SIgA2 antibodies showed a significant ($P < 0.004$) decrease from birth to 2 years of age. Thus, the proportion of SIgA1 and SIgA2 represented by *A. naeslundii* genospecies 1- and 2-reactive antibodies declined over time.

The fine specificities of *A. naeslundii* genospecies 1- and 2-reactive SIgA1 and SIgA2 antibodies were examined by Western blotting of envelope proteins. Figure 2 shows examples of Western blots of saliva samples collected from a single infant from birth to 2 years of age. The blots show the reactivity of total SIgA, SIgA1, and SIgA2 antibodies with envelope antigens of *A. naeslundii* genospecies 1 (Fig. 2A) and 2 (Fig. 2B). Within 1 month postpartum, SIgA1 and SIgA2 antibodies reactive with envelope proteins between 100 and 20 kDa in size were detected in whole saliva (Fig. 2). Examination of the 40 lanes each of *A. naeslundii* genospecies 1 and 2 CEPs that were reacted with four samples of saliva from the 10 infants and probed to detect SIgA1 and SIgA2 antibodies showed that, initially, salivary SIgA antibodies reactive with envelope antigens were largely confined to the SIgA1 subclass. Over time, however, increasing reactivity was observed in SIgA2, concurrent with the increase in concentration of this subclass in saliva (data not shown). Overall, consistent with the decline in whole-cell reactivity, there was a trend toward a reduction in the number and/or intensities of the envelope antigens recognized over time. Examination of the blot patterns of the infants showed that there were common antigens recognized by the majority of saliva samples. Despite these common responses, it was apparent from cluster analysis of the bands recognized by SIgA1 and SIgA2 antibodies, both within and between subjects over time,

FIG. 3. Individual Western blot strips of *A. naeslundii* genospecies 1 and 2 envelope antigens were incubated with saliva collected from 10 infants at 1, 6, 12, and 24 months postpartum and probed to detect SIgA1 and SIgA2 antibodies. For each IgA subclass, the percentage similarities of the bands recognized by the salivary antibodies both within and among the infants were examined by cluster analysis by Ward's algorithm. The data are displayed as dendrograms. An, *A. naeslundii* genospecies. (A) Anti-*A. naeslundii* genospecies 1 SIgA1 antibodies; (B) anti-*A. naeslundii* genospecies 1 SIgA2 antibodies; (C) anti-*A. naeslundii* genospecies 2 SIgA1 antibodies; (D) anti-*A. naeslundii* genospecies 2 SIgA2 antibodies.

that there was pattern variability. The results of cluster analysis by Ward's method (33) are shown in Fig. 3. Samples from individual infants did not group together in single clusters with high similarities.

DISCUSSION

The genus *Actinomyces* is autochthonous to the oral cavities of humans and other mammals, where it occupies a wide niche (4). In a cross-sectional study, Ellen (9) recovered *A. naeslundii* (*A. naeslundii* genospecies 1) from the saliva of 40% of 15 pre-dentate infants that he examined. However, *Actinomyces viscosus* (*A. naeslundii* genospecies 2) (15) was not detected until after the eruption of teeth, when the organism was isolated from the saliva of 20%, and the dental plaque of 10%, of 21 dentate infants. In contrast, in our longitudinal study, no *Actinomyces* species were recovered from the oral cavities of the infants until approximately 4 months after the eruption of the first tooth. It was interesting to observe that there appeared to be an inverse relationship between counts of *A. naeslundii* genospecies 1 and 2 during the first 9 to 10 months following their colonization. The difference between our findings and those of Ellen (9) could be explained, in part, by the fact that Ellen inoculated undiluted swab samples whereas the lowest dilution of swab sample we plated was 10^{-2} . However, this may be offset to some degree by the fact that we identified *A. naeslundii* genospecies 1 and 2 with specific antisera rather than by the colony morphology employed by Ellen (9).

Although *A. naeslundii* genospecies 1 and 2 did not colonize the mouth until after the eruption of teeth, low levels of SIgA1 and SIgA2 antibodies reactive with these genospecies were detected in saliva shortly after birth by using a sensitive ELISA. It is likely that these antibodies were induced by *Actinomyces* species that were transients in the oral cavity before their establishment after tooth eruption. Consistent with this assertion was the invariable recovery of *Actinomyces* species from parallel oral swabs collected from the mothers of the infants (data not shown). Alternatively, *Actinomyces* species may have colonized the tonsils preferentially (5) soon after birth. However, we did not examine the tonsils of these infants. It could be argued that the *Actinomyces*-reactive antibodies detected in saliva before the establishment of *A. naeslundii* genospecies 1 and 2 were, in fact, cross-reactive antibodies induced by members of the resident oral or intestinal microbiota or bacterial antigens in food (2). While this contention cannot be completely excluded, Kilian (16) failed to observe any cross-reactivity between *A. viscosus* NY1, *A. viscosus* WVU 627 (*A. naeslundii* genospecies 2), and *A. naeslundii* ATCC 12104 (*A. naeslundii* genospecies 1) and *Escherichia* and *Klebsiella* typing sera. On the other hand, it has been reported (23, 24) that *A. viscosus* OMZ104 (*A. naeslundii* genospecies 2), *Streptococcus mutans*, and *Streptococcus sanguis* have common wall-associated and extracellular protein antigens. However, *S. sanguis* was rarely isolated, and *S. mutans* was never isolated, from the mouths of the pre-dentate infants in our study (21). Moreover, neither species was isolated in a study of oral streptococcal colonization of pre-dentate infants conducted by Smith et al. (27). It is also possible that the salivary *Actinomyces*-reactive IgA antibodies were produced without antigenic exposure as the result of anti-idiotypic induction (18).

The low levels of SIgA1 and SIgA2 antibodies reactive with *A. naeslundii* genospecies 1 and 2 were detected in saliva within 1 week postpartum. The subclass distribution of the *A. naeslundii* genospecies 1- and 2-reactive SIgA antibodies paralleled the relative proportions of SIgA1 and SIgA2 concentrations in the neonates' saliva. *A. naeslundii* genospecies 1- and 2-reac-

tive SIgA1 and SIgA2 antibodies (in nanograms per milliliter) reached a plateau at 6 months of age and showed no increase in response to colonization by these *Actinomyces* genospecies. Indeed, when the antibody concentrations were normalized to salivary protein, the *A. naeslundii* genospecies 1 and 2 antibodies showed a statistically significant decline in concentration over time. It is possible that the presence of *A. naeslundii* genospecies 1 and 2 in the mouth served to absorb the SIgA *Actinomyces*-reactive antibodies, rendering them unavailable for assay (since it is known that oral bacteria are coated with SIgA [7]). However, Widerström et al. (34) observed that Western blots of SIgA antibodies reactive with mutans streptococci in parotid and submandibular ductal saliva showed a high degree of similarity to those in whole-mouth saliva. Gleeson and collaborators (13) conducted a longitudinal study of the development of IgA-specific antibodies to *Escherichia coli* O antigen in the saliva of children from birth to 5 years of age. In consonance with the results of our study, the data of Gleeson et al. (13) showed that only low levels of SIgA antibodies reactive with this commensal enteric bacterium were detected during the first 4 years of life, despite the colonization of the large intestines of the neonates by *E. coli* (1). Furthermore, the *E. coli*-reactive SIgA antibodies declined significantly during the period from birth to 1 year of age at a time when the total SIgA level in saliva was essentially constant, a finding that was also observed in our study. In a cross-sectional study, Smith and his colleagues (28–30) examined the induction of salivary IgA antibodies in groups of infants aged between 3 and 27 weeks to the commensal viridans streptococci, *Streptococcus mitis* and *Streptococcus salivarius*, that colonize the mouth almost immediately after birth (21, 27). SIgA antibodies reactive with *S. mitis* cells were detected in the saliva of a single infant by 5 weeks of age and in 78% of the neonates by 12 weeks of age. Forty-one percent of saliva samples from these infants contained SIgA antibodies that reacted in Western blots with culture supernatant antigens of *S. salivarius*, and 92% of saliva samples reacted with culture supernatant antigens of *S. mitis*. In contrast to the findings in our study, reactivity with culture supernatant antigens was observed only after the isolation of the respective streptococcal species from the mouths of the infants.

Although variation in the fine specificities of salivary SIgA antibodies reactive with commensal viridans streptococci has been observed in human infants (28–30), we expected *A. naeslundii* antigens recognized by SIgA antibodies in saliva from the same infant collected over time to have clustered together at high similarity. In fact, this was not the case, although a high proportion of the samples clustered at 80% similarity or above. If the pattern of antigens detected had become much more complex over time, it could have explained differences between samples from the same child. However, the intensities and number of bands declined in later samples. Perhaps a significant factor in explaining the differences in patterns within and between children is the nature of the test antigens. In order to efficiently screen a large number of samples, we used single reference strains from our collection, whole-envelope protein profiles which appeared representative of wild-type *A. naeslundii* genospecies 1 and 2. Thus, each of the saliva samples was tested against an identical range of antigens, representative of these two genospecies. In contrast, the infants were colonized by different strains over time. Initial studies (8a) have shown that infants may be colonized by between four and seven ribotypes of *A. naeslundii* over a period of 24 months. Although we have not shown antigenic variation among the strains from these infants, it is possible that their antigens vary in a way similar to that of *S. mitis* antigens (14). If this is the case, the

antibody responses of each infant could reflect the antigenic profiles of the different strains colonizing the mouth. It is well known that different strains of *A. naeslundii* genospecies 1 and 2 can show considerable variation in their antigenic structures (10, 22, 32). Therefore, the antibody response to newly colonizing organisms would be superimposed on the response to previous strains. It is possible that the demonstration of antigen patterns by using standard strains would not reflect all of the antibodies generated by a variety of strains. One could argue that if the test antigens were standard, the patterns of responses should remain the same over time. Clearly, this was not the case and the patterns varied, with responses becoming less complex over time. It could be suggested that this reduction in pattern complexity, together with a decline in levels of antibody to the standard antigen, reflect a reduction in host response to antigens of the standard strains. It is possible that the host became tolerant to *A. naeslundii* antigens. Although this tolerance should extend to antigens shared by the standard strains and the newly colonizing strains, it might not extend to unique antigens on the new strains. The responses to unique *Actinomyces* antigens could reconcile, in part, the stability of the level of SIgA in saliva with a reduction in antibodies to the two standard strains of *A. naeslundii*.

Taken overall, our data suggest that among the mechanisms *Actinomyces* species employ to persist in the oral cavity are the induction of a very limited immune response and clonal replacement with strains differing in their antigen profiles. Studies are in progress to test this hypothesis. It is clear that such experiments must employ analyses of the infants' salivary SIgA antibody responses to their own *Actinomyces* isolates.

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