Study of Humoral Immunity to Commensal Oral Bacteria in Human Infants Demonstrates the Presence of Secretory Immunoglobulin A Antibodies Reactive with *Actinomyces naeslundii* Genospecies 1 and 2 Ribotypes

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The mouths of three human infants were examined from birth to age 2 years to detect colonization of *Actinomyces naeslundii* genospecies 1 and 2. These bacteria did not colonize until after tooth eruption. The diversity of posteruption isolates was determined by ribotyping. Using immunoblotting and enzyme-linked immunosorbent assay, we determined the reactivity of secretory immunoglobulin A (SIgA) antibodies in saliva samples collected from each infant before and after colonization against cell wall proteins from their own *A. naeslundii* strains and carbohydrates from standard *A. naeslundii* genospecies 1 and 2 strains. *A. naeslundii* genospecies 1 and 2 carbohydrate-reactive SIgA antibodies were not detected in any saliva sample. However, SIgA antibodies reactive with cell wall proteins were present in saliva before these bacteria colonized the mouth. These antibodies could be almost completely removed by absorption with *A. odontolyticus*, a species known to colonize the human mouth shortly after birth. However, after colonization by *A. naeslundii* genospecies 1 and 2, specific antibodies were induced that could not be removed by absorption with *A. odontolyticus*. Cluster analysis of the patterns of reactivity of postcolonization salivary antibodies from each infant with antigens from their own strains showed that not only could these antibodies discriminate among strains but antibodies in saliva samples collected at different times showed different reactivity patterns. Overall, these data suggest that, although much of the salivary SIgA antibodies reactive with *A. naeslundii* genospecies 1 and 2 are directed against genus-specific or more broadly cross-reactive antigens, species, genospecies, and possibly strain-specific antibodies are induced in response to colonization.

Secretory immunoglobulin A (SIgA) is considered to be the principal mediator of host defense at mucosal surfaces. Consistent with the large surface area of mucosae requiring protection and the fact that mucosal surfaces are open systems, more IgA is synthesized than all of the other immunoglobulin isotypes combined (22). The stimulus for IgA synthesis at mucosal surfaces appears to be colonization of these surfaces by commensal bacteria bearing polyclonal mitogens such as lipo-polysaccharide (23) and perhaps lipoteichoic acid, since germ-free mammals have underdeveloped mucosa-associated lymphoid tissues and lack SIgA in their secretions (9). Although SIgA is thought to act to exclude extrinsic pathogenic microorganisms, it appears to be without effect on commensal bacteria, since these microbes colonize and persist on mucosal and tooth surfaces despite its presence (6). The reasons for this persistence are unknown, although immune tolerance and antigenic variation have been proposed.

The response of the host to bacterial colonization of the mouth may provide a degree of host immune protection if these autochthonous organisms carry antigens that cross-react with antigens of pathogens that are important in virulence. Such antigens could be proteins involved in the adherence of pathogens to tissues (20). Conversely, tolerance of the host to such antigens that contribute to the virulence of pathogens could compromise immune protection. In addition, commensal gram-positive bacteria, in particular lactobacilli and oral streptococci, have been proposed as viable vectors of protective antigens in vaccines (1, 20, 30). In order for such vaccines to be effective, it is important that the organisms colonize the host for a sufficient period of time and stimulate a protective immune response. At present, testing the effectiveness of these vaccine strains has been limited to animals; however, it is proposed that such vaccines could be valuable in providing protection in humans. If this is the case, understanding the development of infants’ salivary SIgA responses to bacteria colonizing the mouth could assist in determining the optimal time for oral immunization to promote both the persistence of vaccine strains and also a significant immune response. The human oral cavity with easy access, convenient surfaces, and saliva containing SIgA provides an ideal habitat to study the nature of mucosal immune responses.

However, in common with other areas supporting a commensal microbiota, the study of the generation and specificity of the immune response in the mouth is not without its difficulties. Among the most significant of these is the variation of
strains of species of commensal bacteria during colonization, introducing the possibility of antigenic variation or drift of the colonizing species over time. We (13) and others (17, 18) have confirmed that strains of commensal bacteria colonizing oral mucosal surfaces demonstrate extensive diversity and, in particular, the streptococci show clonal replacement during colonization (13, 17). In addition, studies in early infancy the volumes of saliva that can be obtained are small, and sensitive methods are necessary to demonstrate the amount and specificity of IgA antibody.

The problem of potential variation of strains during colonization cannot be controlled but genetic typing of the predominant strains can give information on their stability during longitudinal analysis of the immune response. Moreover, storage of these isolates provides strains from individual infants to measure both the magnitude and the specificity of the response of individual infants to strains colonizing their mouths.

In longitudinal studies of human infants with standard strains of bacteria as antigen, we have shown that the salivary IgA immune response to Actinomyces naeslundii genospecies 1 and 2 (7) and to selected species of viridans streptococci (8) is limited. Also, these commensal bacteria induce an IgA antibody response in saliva with changing specificity that peaks at 6 months of age and wanes thereafter. Similarly, in the mouse, colonization by commensal enteric bacteria induces a self-limiting mucosal immune response and a state of chronic hyporesponsiveness (28). Consistent with the high degree of diversity among strains of Actinomyces and streptococci, Western blots of cell wall antigens of these bacteria probed with infants’ saliva have shown extensive variability (7, 8). We have proposed, based on these data, that the induction of a limited immune response, perhaps as a result of antigenic variation, may be among the mechanisms used by commensal bacteria to avoid immune elimination and persist in the oral cavity and at other mucosal surfaces.

In our previous studies (7, 8), we analyzed the IgA antibody response in saliva to selected commensal oral bacteria by enzyme-linked immunosorbent assay (ELISA) and Western immunoblotting with whole cells or cell wall extracts of standard and type strains. In our studies of Actinomyces spp. (7), cluster analysis was used to analyze Western blots of cell wall antigens of two standard strains of A. naeslundii genospecies 1 and 2 reacted with infants’ saliva samples taken during colonization of their mouths. The clusters formed had high internal similarities of 90 to 96% and included saliva samples from different infants at different collection times. This result showed that the IgA antibodies in the saliva from different infants reacted equivalently with the standard antigens, suggesting that all infants produced closely similar responses that did not allow discrimination within or among them.

We considered that one reason for this observation could be that standard laboratory strains of A. naeslundii genospecies 1 and 2 were used as antigen for all of the saliva samples. However, it is possible that cluster analysis of the patterns of salivary IgA antibodies reactive with antigens from strains that colonized each infant, rather than standard strains might provide a more accurate picture of the specificity of an infant antibody response.

Furthermore, consideration of the identities of organisms that initiate the IgA response prior to colonization by A. naeslundii raised the possibility that such responses may be the result of stimulation by antigens common to strains of closely related genera or species. Thus, the response to A. naeslundii could result, in part, from stimulation by strains of A. odontolyticus, because this species has been shown to colonize infants’ mouths as early as 2 months postpartum (27). This suggestion is further strengthened because A. naeslundii and A. odontolyticus have a common peptidoglycan structure, the walls of both include rhamnose and 6-deoxytalose, and there is evidence of serological cross-reaction between strains of the species (4). The removal of IgA antibody activity to selected species of oral streptococci by absorption of saliva with cells of Enterococcus faecalis (8) provided further support for this assertion.

Consequently, in an attempt to determine the specificity of the IgA antibody response in individual infants, we examined IgA antibodies in the saliva of three infants to their own genetically typed strains (ribotypes) of A. naeslundii genospecies 1 and 2. In addition, we measured the effect of absorption of saliva from six other infants with cells of A. odontolyticus on their reactivity with cells of A. naeslundii genospecies 1 and 2 to confirm the presence of cross-reacting antigens.

### MATERIALS AND METHODS

**Study population.** Nine healthy, full-term, breast-fed infants—referred to here as infants 1, 7, 8, 12, 14, 18, 20, 21, and 24—were selected for this study. They comprised five males and four females. Six were white, two were black, and one was Hispanic. The details of the study population have been described previously (7, 8, 13, 14). The experimental procedures used were approved by the Institutional Review Board of Georgetown University Medical Center.

**Sample collection and processing.** (i) Whole-mouth saliva. Whole saliva was collected at 1 to 3 days, at 2 and 4 weeks, and at 2, 4, 6, 8, 10, 12, and 15 months postpartum corresponding to visits 1 to 10. Saliva was collected by using sterile 3-mL plastic transfer pipettes. Immediately, EDTA was added to a final concentration of 5 mM to prevent formation of heterotypic calcium ion-dependent immunoglobulin-mucin complexes and to inhibit the IgA1 protease activity in the saliva (14). The saliva samples were held at −80°C until assay. Saliva collected from infant 18 at 2, 4, and 10 months (visits 4, 5, and 8), from infant 20 at 4, 10, and 15 months (visits 5, 8, and 10), from infant 21 at 2, 4, 8, 10, and 15 months (visits 4, 5, 7, 8, and 10), and from infants 1, 7, 8, 12, 14, and 24 at 4 and 15 months (visits 5 and 10) were used in the current study. The volumes of saliva that could be collected, particularly at the earlier visits, were low, such that only a limited number of assays could be carried out on each sample. Consequently, although saliva samples from infants 18, 20, and 21 were used for Western blotting with their homologous strains, it was necessary to use saliva samples from infants 1, 7, 8, 12, 14, and 24 for absorption. In addition, Western blots of cell wall proteins were limited to the same infant and it was not possible to perform analyses among the babies.

(ii) Oral swabs. In parallel, and after each collection of whole saliva, the mucosal surfaces of the cheeks, buccal sulci, edentulous ridges, tongue and hard palate and teeth, when erupted, were swabbed by using the swab from a Vacutainer anaerobic specimen collector (Becton Dickinson Microbiology Systems, Cockeysville, Md.). The swab was then returned to the labeled tube of the collector and transported under anaerobic conditions to the laboratory within 1 h of collection. After the swab was placed in 2 mL of reduced transport fluid (29), bacteria were released from the swab and dispersed by ultrasound at 80 W for 10 s with a Branson Sonifier 250 (Branson Ultrasound Corp., Danbury, Conn.) equipped with a microprobe. The dispersed samples were serially diluted in reduced transport fluid to 10−5 and plated onto various media.

**Recovery and identification of Actinomyces species.** Trypticase soy agar containing 5% sheep blood (TSASB); Columbia agar containing 5% sheep blood, cysteine HCl, palladium chloride, diithiothreitol, and hemin (CASB); and CFAT agar (32) plates (all from Remel, Lenexa, Kan.) were inoculated by using a spiral plater (Spiral Systems, Cincinnati, Ohio). TSASB plates were incubated at 37°C for 3 to 5 days in an anaerobic chamber containing an atmosphere of 80% N2, 10% CO2, and 10% H2, and CFAT plates were incubated at 37°C for 48 h in 5% CO2 in air. After
incubation, total counts were made of each colony morphotype on plates of the nonselective and selective media that contained between 30 and 300 CFU. Representatives of each morphotype were selected under a dissecting microscope at ×20 magnification and subcultured to purity on TSA/B. The purified isolates were confirmed 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 (5, 11, 24).

Ribotyping of *A. naeslundii* genospecies 1 and 2 isolates. Ribotyping of the *A. naeslundii* genospecies 1 and 2 isolates obtained from the mouths of infants 18, 20, and 21 was performed as described in detail previously (2, 3).

Preparation of wall extract of *A. naeslundii* genospecies 1 and 2 isolates. Cell wall extracts of the *A. naeslundii* genospecies 1 and 2 isolates were prepared as described previously (7). Briefly, cells of each isolate were suspended in ice-cold 10 mM HEPES (pH 7.4) and subjected to four 1-min cycles of ultrasound. The bacteria were then removed by centrifugation and stained by Gram’s method. Microscopic examination confirmed that the bacterial cells remained intact, and there was no evidence of cellular debris. The supernatants were stored at −80°C until use.

Analysis of wall extracts of *A. naeslundii* genospecies 1 and 2 isolates. The wall extracts and molecular weight 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%, and the stacking gel was 4.5% acrylamide. The gels were run at 20 mA. After the gels were stained with Coomassie brilliant blue R-250. The separated wall extracts on the other two sets of gels were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore) by using a Trans-Blot SD system (Bio-Rad). One set of blots was processed for the simultaneous detection of carbohydrate and protein by using the DIG Glycan/Protein double labeling kit (Roche Molecular Biochemicals, Indianapolis, Ind.) according to the manufacturers’ instructions. The second set of blots was blocked for 1 h in 4% bovine serum albumin (BSA) in Tris-buffered saline (TBS; pH 8.3) containing 0.02% NaN₃ in preparation for immunoblotting.

Extraction of *A. naeslundii* genospecies 1 and 2 cell wall carbohydrate. Cell wall carbohydrate from *A. naeslundii* genospecies 1 (ATCC 12104) and 2 (WWU 627) was obtained by extraction from isolated cell walls with trichloroacetic acid, as described previously (5). Briefly, cell walls were produced after cell disruption by a Mickle tissue disintegrator (Mickle Laboratory Engineering Co. Gomshall, Surrey, United Kingdom), washed thrice in distilled water, and lyophilized (model EF 03; Edwards High Vacuum, Crawley, Sussex, United Kingdom). Protein was removed by treatment with protease from *Streptomyces griseus* (Sigma-Aldrich, St. Louis, Mo.). A 50-mg portion of cell wall was incubated for 16 h at 37°C in 30 ml of 0.1 M phosphate buffer (pH 8.0) containing 0.2 mg of protease/ml. Protease-treated walls were washed thrice with distilled water and lyophilized. Lyophilized walls were extracted in 30 ml of 5% aqueous trichloroacetic acid (Fisher Scientific, Pittsburgh, Pa.) for 16 h at 37°C. The wall suspension was centrifuged, and 5 volumes of acetone (Fisher) was added to the pellets. Lyophilized extracts were obtained with an AlphaImager (Alpha Innotech Corp., San Leandro, Calif.). The image files were imported into an analytic software program (GelComp 4.0; Applied Maths, Kortrijk, Belgium) for analysis.

Cluster analysis of Western blot band patterns. Cluster analysis was performed with GelComp 4.0 by the method of Ward (31).

Absorption of saliva. The specificity of *A. naeslundii* genospecies 1- and 2-reactive SLgA antibodies in saliva was tested by absorption. Selected pairs of saliva samples were obtained from three male and three female infants, i.e., infants 1, 7, 8, 12, 14, and 24. Saliva samples selected were those obtained at 4 months of age (visit 5) before *A. naeslundii* genospecies 1 and 2 were isolated from their mouths (termed “early samples”) and at 15 months of age (visit 10) after *A. naeslundii* genospecies 1 and 2 were isolated from their mouths (termed “late samples”). *A. odontolyticus* has been shown to be an early colonizer of the mouth (27), we elected to determine whether *A. naeslundii*-reactive antibodies could have been induced by colonization by *A. odontolyticus*. Briefly, *A. odonto-
yticus* NCTC 9935 (serotype 1), *A. odontolyticus* WWU 482 (serotype 2), and *A. odontolyticus* MCB 120, a serotype 2 isolate obtained from an infant in our study, were grown in Actinomyces Broth (Becton Dickinson Microbiology Systems, Sparks, Md.) at 37°C in 5% CO₂ for 5 days. Aliquots of the saliva samples were serially diluted in PBS-Tween and mixed with an equal volume of packed, washed cells of the *Actinomyces* strains. The suspensions were incubated at 37°C for 2 h and then at 4°C overnight. After absorption, the bacteria were removed by centrifugation, and an ELISA was used to measure the antibacterial antibodies in absorbed and unabsorbed dilutions of the saliva samples as described above. The percentages of residual antibody activity against *A. naeslundii* genospecies 1 and 2 after independent absorption with the *A. odontolyticus* strains compared to the unabsorbed control were determined in microtiter wells coated with *A. naeslundii* genospecies 1 and 2. The titration curves of unabsorbed and absorbed serial dilutions of the saliva were approximately parallel. The effect of absorption with *A. odontolyticus* on *A. naeslundii* reactive SLgA antibodies in the saliva samples was calculated from the linear portion of the titration curve by dividing the optical density at 450 nm of each dilution of the absorbed saliva samples by that of the corresponding dilution of unabsorbed saliva and multiplying by 100. The effect of absorption was expressed as: (i) the number of absorbed samples that retained activity against *A. naeslundii* genospecies 1 and 2 and (ii) the median of the percentages of residual antibody in the absorbed samples. The variances in the data were expressed as the 25th and 75th percentiles.

Detection of SLgA antibodies in saliva reactive with *A. naeslundii* genospecies 1 and 2 cell wall carbohydrate. The ELISA used in the present study has been described in detail elsewhere (7, 8). Briefly, Immunol-2 plates (Dynatech, Chantilly, Va.) were coated overnight with cell wall carbohydrate from *A. naeslundii* genospecies 1 or 2 at a concentration of 5 μg/ml in 0.05 M carbonate buffer (pH 9.6). Unbound carbohydrate was washed off the wells that were then blocked with phosphate-buffered saline (PBS; pH 8.0) containing 0.1% BSA. After the wells were washed, dilutions of saliva samples from each infant were added in duplicate and incubated overnight with shaking. After the diluted saliva was washed out, a biotinylated affinity-purified polyclonal goat anti-human α chain (BGAHs) antibody (Jackson ImmunoResearch, West Grove, Pa.) at 2.0 μg/ml was used to detect bound SLgA antibodies. After being washed, streptavidin conjugated with horseradish peroxidase (SA-HRP; BioSource International, Camarillo, Calif.) at 0.1 μg/ml was added to the wells to detect the biotinylated antibodies. The wells were washed again, and o-phenylenediamine (1 mg/ml) in citrate-phosphate buffer (pH 4.5) containing 0.02% hydrogen peroxide was added to each well. The optical density at 450 nm was measured with a Spectra-Rainbow automated microplate reader (Tecan U.S., Inc., Research Triangle Park, N.C.). As positive controls, rabbit antibodies reactive in precipitin tests with the cell wall carbohydrates and adult human saliva samples (from J. L. Keeling, Emory University) were used. The ELISA Bound rabbit IgG antibodies were revealed by using an affinity-purified goat anti-rabbit IgG antibody conjugated with horse-

RESULTS

Ribotyping. As we have reported previously (7) *A. naeslundii* genospecies 1 and 2 do not colonize the human oral cavity until approximately 4 months after the eruption of teeth. In our study population primary teeth began to erupt at 6 months of age and all infants had erupted teeth by 12 months (7). *A. naeslundii* genospecies 1 and 2 first were detected in infants 18, 20, and 21 at 10 months of age (visit 8). Twenty-two isolates of *A. naeslundii* obtained from the three infants were examined. The 6 isolates from infant 18 and the 3 isolates from infant 21 were identified as *A. naeslundii* genospecies 1. *A. naeslundii* genospecies 1 (3 isolates) and *A. naeslundii* genospecies 2 (10 isolates) were obtained from the third infant (infant 20).

A schematic of the ribotype patterns of isolates of *A. naeslundii* from the infants is shown in Fig. 1. The ribotypes produced by separate digestion of the genomic DNA from *A. naeslundii* genospecies 1 isolates with endonucleases BamHI and BsrRI showed that each was colonized by a single unique
clone. However, although use of these endonucleases allowed differentiation between the *A. naeslundii* clones colonizing the infants the clones shared common patterns. *A. naeslundii* genospecies 1 isolates from infants 18 and 20 shared a common ribotype pattern when genomic DNA was digested with BamHI and isolates from infants 18 and 21 shared a common BbrPI ribotype. Thus, overall, the *A. naeslundii* genospecies 1 isolates from infants 18, 20, and 21 were closely related. In contrast, the two clones of *A. naeslundii* genospecies 2 obtained from infant 20 were distinct. Nine isolates of *A. naeslundii* genospecies 2 grouped into one clone with identical BamHI and BbrPI ribotype patterns, whereas the BamHI and BbrPI ribotype patterns of the remaining isolate were different from those of the other nine isolates.

**Analysis of protein profiles of *A. naeslundii* genospecies 1 and 2.** The cell wall extracts of *A. naeslundii* genospecies 1 and 2 were resolved into approximately 40 bands by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 11% running gels (Fig. 2). Overall, the *A. naeslundii* protein profiles were 57.5% similar and were readily separated into genospecies by using cluster analysis. In the genospecies 1 cluster, the protein profiles of the six isolates obtained from infant 18 (isolates 3486, 3487, 3488, 3489, 3512, and 3513) that comprised a single clone were 92% similar. The protein profiles of the three isolates from infant 21 (isolates 4498, 4499, and 4512) that comprised a third clone were 97% similar.

The genospecies 2 isolates from infant 20 comprised two clones: one with nine isolates and the second with a single isolate. The protein profiles of the 10 genospecies 2 isolates (isolates 3835, 3838, 3839, 3842, 3843, 4645, 4646, 4676, 4677, and 4678) were 90.5% similar, and the protein profiles of the nine isolates comprising the larger of the clones (all of the above accept 4646) were 91.5% similar. These results indicate that the predominant strains of *A. naeslundii* in each infant were relatively stable over time compared to *Streptococcus mitis* and, unlike the streptococci, these strains did not show evidence of clonal replacement (17, 18).

**Effect of absorption of the infant saliva with *A. odontolyticus* cells on *A. naeslundii*-reactive SIgA antibodies.** The results of absorption of the saliva samples are shown in Table 1. The saliva samples were absorbed with cells of the type strain of *A. odontolyticus* (NCTC 9935) (serotype 1), strain WVU (West Virginia University) 482 (serotype 2), and (MCB 120) (serotype 2). Only serotype 2 *A. odontolyticus* strains were recovered from the infants in our study, and MCB 120 was one of these. Absorbed saliva was tested by ELISA with cells of these strains and also *A. naeslundii* ATCC 12104 (genospecies 1) and *A. naeslundii* W1053 (genospecies 2) as antigen. Examination of the data for the early saliva samples shows clearly that absorp-
tion with strains of *A. odontolyticus* reduced the SIgA antibody activity against both genospecies of *A. naeslundii* either to background (0% median residual antibody activity for genospecies 1) or to nearly background levels (0 to 2.6% median residual antibody activity for genospecies 2). However, in contrast to the early saliva samples, cells of the *A. odontolyticus* strains were much less effective at absorbing *A. naeslundii* genospecies 1-reactive SIgA antibodies (14.6 to 30.3% median residual antibody activity) and genospecies 2-reactive SIgA antibodies (9.0 to 21.0% median residual antibody activity) in the late saliva samples.

Reactivity of salivary SIgA with wall carbohydrate from *A. naeslundii* genospecies 1 and 2. After Western transfer of the wall extracts one of each of three replicate strips of each isolate was developed by using the DIG Glycan/Protein double labeling kit (Roche Molecular Biochemicals) to detect glycoconjugates, and several bands staining for both protein and sugars were detected. These bands reacted with the infants salivary SIgA antibody (data not shown). However, when purified wall carbohydrates from the *A. naeslundii* genospecies 1 and 2 reference strains were used to coat the wells of microtiter trays, no carbohydrate-reactive SIgA antibodies were detected in saliva from the infants by using a sensitive ELISA. In contrast, the control rabbit antisera and adult saliva gave strong positive reactions with the purified wall carbohydrate.

Reactivity of salivary SIgA antibodies with wall extracts. Immunoblot patterns were analyzed by using the Ward method (31). An example of the dendrogram from cluster analysis of the immunoblots from infant 18 is shown in Fig. 3. The majority of the *A. naeslundii* genospecies 1 and 2 bands recognized by salivary SIgA antibodies had apparent molecular masses of between 75 and 31 kDa. However, in some lanes salivary SIgA antibodies recognized between one and three bands with molecular masses of >75 kDa and several lower molecular mass bands that ran between the 31- and 14.4-kDa standards. SIgA antibodies in all but one saliva sample recognized two bands with apparent molecular masses of approximately 75 and 53 kDa that were common to both of the *A. naeslundii* genospecies 1 and 2 wall extracts. Computer analysis identified between 7 and 17 bands (mean, 11 bands) of the *A. naeslundii* genospecies 1 wall extracts recognized by salivary SIgA antibodies and between 6 and 17 bands (mean, 12 bands) of *A. naeslundii* genospecies 2 wall extracts recognized by salivary SIgA antibodies. Comparisons were made between the patterns of the blots of the wall extract of each isolate reacted with saliva from each visit within each infant, and details of the strain within clusters and the saliva samples are shown in Table 2. Comparisons of the clusters formed showed that they could be divided into three types termed A, B, and C based on the isolates and the visit at which the saliva samples were obtained (Table 2). Type A clusters were those formed by antibody reactivity patterns of saliva samples from more than one visit reacted with extracts of a single isolate. Type B clusters included antibody reactivity patterns of a saliva sample...
TABLE 1. Residual *A. naeslundii* genospecies 1 and 2 SIgA antibody activity in infant saliva after absorption with *A. odontolyticus* serotypes 1 and 2*

| Test strain (serotype) and parameter | Early saliva samples | Late saliva samples |
|-------------------------------------|----------------------|---------------------|
|                                     | Absorbed with *A. odontolyticus* strain (serotype): | Absorbed with *A. odontolyticus* strain (serotype): |
|                                     | NCTC 9935 (1) | WVU 483 (2) | MCB 120 (2) | NCTC 9935 (1) | WVU 483 (2) | MCB 120 (2) |
| A. odontolyticus                   |                       |                   |
| NCTC 9935 (1)                     |                       |                   |
| No. positive                       | 0                     | 0                 | 8           | 0             | 12          | 11          |
| Median (%)                         | 0                     | 3.9               | 2.1         | 0             | 19.9        | 15.3        |
| 25th percentile                    | 0                     | 0.7               | 0           | 0             | 18.2        | 4.1         |
| 75th percentile                    | 0                     | 21.7              | 7.4         | 0             | 23.3        | 27.8        |
| WVU 483 (2)                        | 3                     | 0                 | 3           | 12            | 0           | 6           |
| No. positive                       | 0                     | 0                 | 0           | 18.1          | 0           | 1.1         |
| Median (%)                         | 0                     | 0                 | 0           | 7.2           | 0           | 0           |
| 25th percentile                    | 0                     | 0                 | 0           | 7.2           | 0           | 0           |
| 75th percentile                    | 0                     | 1.1               | 0.3         | 27.2          | 21.6        |
| MCB 120 (2)                        | 8                     | 9                 | 0           | 12            | 11          | 0           |
| No. positive                       | 2.5                   | 6.8               | 0           | 14.0          | 10.6        |
| Median (%)                         | 0                     | 0                 | 0           | 6.9           | 3.1         |
| 25th percentile                    | 0                     | 0                 | 0           | 6.9           | 3.1         |
| 75th percentile                    | 1.1                   | 13.3              | 3.3         | 38.1          | 37.1        |
| A. naeslundii                      |                       |                   |
| ATCC 12104 (1)                     | 3                     | 5                 | 5           | 11            | 12          |
| No. positive                       | 0                     | 0                 | 0           | 19.9          | 30.3        |
| Median (%)                         | 0                     | 0                 | 0           | 12.7          | 17.4        |
| 25th percentile                    | 0                     | 0                 | 0           | 12.7          | 17.4        |
| 75th percentile                    | 0.1                   | 13.3              | 3.3         | 38.1          | 37.1        |
| W1053 (2)                          | 1                     | 6                 | 7           | 9             | 11          |
| No. positive                       | 0                     | 1.4               | 2.6         | 11.0          | 21.0        |
| Median (%)                         | 0                     | 0                 | 0           | 1.3           | 5.6         |
| 25th percentile                    | 0                     | 0                 | 0           | 1.3           | 5.6         |
| 75th percentile                    | 0                     | 10.9              | 5.9         | 25.0          | 32.2        |

* Whole saliva was collected from six infants before (early samples) and after (late samples), they were colonized with *A. naeslundii* genospecies 1 and 2. These saliva samples were absorbed with *A. odontolyticus* serotypes 1 and 2 whole cells. After absorption, an ELISA was used to measure *A. naeslundii* genospecies 1 and 2 SIgA antibodies in absorbed and unabsorbed dilutions of the saliva samples. The percentages of residual antibody activity against *A. naeslundii* genospecies 1 and 2 after independent absorption with the *A. odontolyticus* strains were determined by dividing the optical density at 450 nm of each absorbed saliva by that of the corresponding unabsorbed saliva at the same dilution and multiplying that value by 100. The results are expressed as the number of samples that retained activity against *A. naeslundii* genospecies 1 and 2 after absorption with *A. odontolyticus*, the medians of the percentages of residual antibody in the absorbed samples and the 25th and 75th percentiles. Duplicate samples of saliva from six infants were assayed on two separate occasions. Serotypes are indicated in parentheses.

from a single visit reacted with several different isolates. However, these divisions were not absolute, and some clusters included antibody reactivity patterns of saliva from more than one visit reacted with several isolates. These were termed type C clusters. In order to simplify the presentation of the data, the points of separation and the internal similarities of clusters for each infant are shown as schematics in Fig. 4 and 5. As an example of the manner in which the simplified data are presented, consider the type A cluster of infant 18 based on strain 3487 (Table 2), which is shown in the dendrogram in Fig. 3 and the schematic in Fig. 4. In the Fig. 4 schematic for infant 18, saliva samples from visit 4 (2 months), visit 5 (4 months), and visit 8 (10 months) form a type A cluster at 88% similarity, and this cluster connects to the B2, C2, B1, and C1 clusters at 64% similarity. Similarly, in the same infant, cluster B2 (based on saliva from visit 5 [4 months] and strains 3486, 3488, 3489, 3512, and 3513) is formed at 94% similarity and joins the other clusters at 83% similarity.

The overall similarity among the band patterns recognized by the SIgA antibodies in the saliva samples collected from the same infant at different visits varied from 74% for genospecies 1 strains from infant 21 (Fig. 4) to 15% for genospecies 2 strains from infant 20 (Fig. 5).

The results of cluster analysis of *A. naeslundii* genospecies 1 isolates are shown in Table 2 and Fig. 4. Despite the overall separation at high similarities, some clusters were defined at lower similarities of between 42 and 83%. For infant 18, cluster A (strain 3487) and cluster B2 (saliva visit 5 [4 months]) separated at 64 and 83%, respectively. For infant 20, cluster B1 (saliva visit 5 [4 months]) separated from clusters B2 and B3 at 42%, making it quite distinct. For infant 21, cluster A (strain 4512) and cluster C4 were separated at 74% from clusters C1, C2, and C3.

In all, eight clusters were defined among the strains of *A. naeslundii* genospecies 2 from infant 20 (Table 2 and Fig. 5). Of particular note, were clusters B4 and B5 (both from saliva collected at visits 5 [4 months] and 8 [10 months]) that were separated from the other clusters at 15% similarity. Also, clusters B2 and B3 based on saliva from visit 10 (15 months) were separated at 60%. The single type A cluster (strain 4676) among the *A. naeslundii* genospecies 2 isolates separated at 75% from clusters B2 to B5.

**DISCUSSION**

The objective of the present study was to examine the specificity of the SIgA antibody responses in individual infants to the strains of *A. naeslundii* that colonized their mouths. The isolates were ribotyped in order to reveal the diversity of strains within an infant and to detect any obvious clonal replacement. Insufficient numbers of strains were isolated from the infants to detect genetic variants that could be present in low numbers. However, we believe that our results reveal the predominant *A. naeslundii* ribotype in these infants and confirm that the ribotypes isolated persisted in their mouths over time, as has been shown to be the case in adults (J. Johnson, M. Kinard, G. Bowden, and C. Schachtele, abstract from the 72nd General Session of the International Association for Dental Research, J. Dent. Res. 73:346, 1994). Each of the three infants (infants 18, 20, and 21) in our study was colonized by a unique ribotype of *A. naeslundii* genospecies 1 from age 10 months (visit 8) when the bacteria were first detected in their mouths until the last sampling at age 15 months (visit 10). Infant 21 was also colonized by *A. naeslundii* genospecies 2, and the isolates were predominantly one ribotype, reflecting the findings for *A. naeslundii* genospecies 1. However, one isolate recovered at age 15 months (visit 10) was a different ribotype, suggesting that infants, like adults (M. Kinard, J. Johnson, G. Bowden, and C. Schachtele, abstract from the 72nd General Session of the International Association for Dental Research, J. Dent.
Res. 73:248, 1994) can be colonized by more than one ribotype of *A. naeslundii* genospecies 2.

The limited clonal diversity and stability of *A. naeslundii* is in marked contrast to *S. mitis* that exhibits extensive clonal diversity and replacement in infants (13, 17, 18). The finding of limited diversity among isolates of *A. naeslundii* colonizing these infants could be considered to be an advantage when comparisons are drawn among antibody responses because it could be argued that, because clonal diversity is minimal, the antigenic stimulus to the infants’ immune system would be relatively stable. Against this it must be recognized that ribotyping may not be sufficiently discriminating to reveal antigenic drift or variation among these strains. Indeed, the persistence of a single ribotype may reflect a balance that has been established between the immune response of the host and that unique ribotype.

### Table 2

The clusters formed from analysis of Western blot patterns of SIgA antibodies in infants’ saliva reacted with cell wall proteins from their autochthonous strains of *A. naeslundii* genospecies 1 and 2

| Infant no. | Genospecies* | Overall pattern similarityb (%) | No. of clusters | Cluster typesc | Strain no. | Visit no. | Infant age(s) (mo) |
|-----------|--------------|---------------------------------|----------------|---------------|------------|-----------|-------------------|
| 18        | 1            | 64                              | 1              | A             | 3487       | 4, 5, 8   | 2, 4, 10          |
|           |              |                                 |                | B1            | 3489, 3512, 3513 | 8          | 10             |
|           |              |                                 |                | B2            | 3486, 3488, 3489, 3512, 3513 | 5          | 4              |
|           |              |                                 |                | C1            | 3486, 3488 | 4, 8       | 2, 10            |
|           |              |                                 |                | C2            | 3486, 3489, 3512, 3513 | 4, 8       | 2, 10            |
| 20        | 1            | 42                              | 3              | B1            | 4651, 4562, 4653 | 5          | 4              |
|           |              |                                 |                | B2            | 4651, 4562, 4653 | 10         | 15             |
|           |              |                                 |                | B3            | 4651, 4562, 4653 | 8          | 10             |
|           |              |                                 |                | B4            | 3835, 3838, 4645, 4646 | 5, 8, 10 | 4, 10, 15 |
|           |              |                                 |                | B5            | 3835, 3839, 3842, 3843, 4646 | 5          | 4              |
|           |              |                                 |                | B6            | 4645, 4677, 4678 | 10         | 15             |
|           |              |                                 |                | B7            | 3839, 3842, 3843 | 8, 10      |                |
|           |              |                                 |                | B8            | 3839, 3842, 3843 | 5, 4       |                |
|           |              |                                 |                | C1            | 4645, 4677, 4678 | 5, 8       | 4, 10            |
|           |              |                                 |                | C2            | 3835, 3836, 4646, 4677 | 5, 8      | 4, 10            |
| 21        | 1            | 74                              | 1              | A             | 4512       | 8, 10     | 10, 15           |
|           |              |                                 |                | C1            | 4499, 4512 | 5, 7      | 4, 8             |
|           |              |                                 |                | C2            | 4498, 4512 | 4, 5      | 2, 4             |
|           |              |                                 |                | C3            | 4498, 4499 | 7, 10     | 8, 15            |
|           |              |                                 |                | C4            | 4498, 4499 | 4, 8, 10  | 2, 10, 15        |

* indicates *A. naeslundii* genospecies 1 or 2.

b The level of similarity that includes all of the patterns.
c Type A clusters are based on a single strain, type B clusters are based on a single saliva sample, and type C clusters contain mixed strains and saliva samples.
Establishing the specificity of the infants' salivary IgA antibody responses to *A. naeslundii* was first approached by absorbing saliva samples from six infants with strains of *A. odontolyticus*, a species known to colonize the mucous membranes of infants prior to tooth eruption (27). Absorption of the early saliva samples (Table 1) with the *A. odontolyticus* strains removed at least 97.4% of *A. naeslundii* genospecies 1- and 2-reactive antibodies. This finding provides support for the suggestion that, in large part, the antibodies were either stimulated by this species or were directed to antigens common among the genus *Actinomyces* (4) or common among other gram-positive bacteria inhabiting the mouth and gastrointestinal tract of these infants. For example, it has been reported that some species of viridans streptococci, lactobacilli, and actinomyces share extracellular and cell wall antigens (27a). Such common antigens of gram-positive bacteria may include glucans and teichoic acid-associated phosphorylcholine (15). Alternatively, it is possible that the binding of IgA antibodies in saliva to the *Actinomyces* species was nonimmune and mediated by interaction of bacterial lectins with O-linked oligosaccharides at the hinge region of the α1 heavy chain (26).

However, the fact that salivary IgA antibodies bound multiple bands on the Western blots of the cell wall proteins argues against a nonimmune interaction. Another possibility is that *A. naeslundii*-reactive IgA antibodies present in saliva prior to colonization of this bacterium are examples of polyreactive antibody (25). Polyreactive IgA antibodies, some with high functional affinity, have been detected in human saliva (25). Although, if this were the case one might have expected reactivity with the *Actinomyces* wall polysaccharide, since polyreactive antibodies commonly bind carbohydrates (25). However, the infants saliva samples were uniformly negative when tested with cell wall carbohydrate.

After the establishment of *A. naeslundii* in the mouth the salivary IgA antibody response appeared to become more specific for this bacterium. The amounts of residual *A. naeslundii*-genospecies reactive antibodies remaining after absorption with the *A. odontolyticus* strains increased. Also, the immunoblot patterns of antibody reactivity of late saliva samples were generally more complex than those of early samples.

In a previous study (7) we examined the fine specificity of the salivary IgA antibody response in infants' saliva by using two standard strains of *A. naeslundii*. The majority of the clusters observed were those that contained saliva from more than one visit that reacted with several isolates (type C clusters), suggesting that there was no individual specificity of the IgA antibodies in the saliva of a given infant. However, we reasoned that this apparent lack of specificity might be attributed to the use of standard strains as common antigens to test the saliva samples. Therefore, we decided to use strains isolated from individual infants as antigens to test the reactivity of their saliva. The results of cluster analysis of the reactivity of IgA antibodies in saliva samples from individual infants and their homologous strains generated some type C clusters. However, unlike our previous results (7), two other types of clusters, types A and B, were observed. The presence of these clusters adds support for the proposal that the IgA response to *A.
naeslundii in infants shows specificity in recognizing strains and also exhibits maturation with increased specificity. Type A clusters were less common than type B clusters, but one example was detected in each infant (Table 2). Type A clusters were based on a single isolate, and these clusters indicate that more than one saliva sample from an infant recognized this strain as unique and separate from other isolates. Therefore, it is most likely that these strains carried unique epitopes recognized by infants’ salivary IgA, supporting the concept that IgA antibodies in saliva can separate and interact with individual strains of A. naeslundii.

Type B clusters were more common than type A clusters ranging from two in infant 18 for A. naeslundii genospecies 1 (Table 2 and Fig. 4) to five among the A. naeslundii genospecies 2 isolates from infant 20 (Table 2 and Fig. 5). However, no type B clusters were formed from the A. naeslundii genospecies 1 strains of infant 21 (Table 2 and Fig. 4). The presence of type B clusters indicates differences among the reactivity of IgA antibodies in saliva collected at different visits. This is particularly evident with cluster B1 of A. naeslundii genospecies 1 in infant 20 that separates at 42% similarity (Fig. 4) and with clusters B4 and B5 of A. naeslundii genospecies 2 in the same infant that separate from the other clusters at 15% (Fig. 5). Also, the extent of differentiation among the clusters could be related to maturation of the immune response if, as the child’s immune system developed, differences could be shown between the reaction of the earliest and the latest saliva samples to antigens from the bacteria. However, such observations require at least two type B clusters for an infant. This is the case in infant 20 for both A. naeslundii genospecies 1 and 2. In infant 20, A. naeslundii genospecies 1 wall proteins reacted with their homologous saliva only gave three clusters, all of type B (Table 2 and Fig. 4). Cluster B1 was based on visit 5 (4 months) and separated from the later visits, 8 and 10 (10 and 15 months), and at 42% similarity compared to B2 and B3 that separated at 88%, suggesting a distinction between the antibody activity in saliva from visit five compared to the later visits. Thus, in infant 20 the reactivity pattern of the earliest saliva sample was relatively distinct from those of later samples. This pattern was even more evident when the infant 20 A. naeslundii genospecies 2 clusters were considered. Clusters B4 (visit 8; 10 months) and B5 (visit 5; 4 months) separated from clusters B1 (visit 5; 4 months) and B2 and B3 (visit 10; 15 months) at 15% similarity. Moreover, clusters B2 and B3 separated from B1 at 60%, clearly distinguishing the later visit 10 (15 months) saliva from those of the earlier visits. The separation of the saliva from the visit 5 (B5) (4 months) and visit 10 (B3) (15 months) clusters could be based on the isolates involved, which were different for these clusters. If so, this would lend support to the concept that the strains of A. naeslundii isolated, despite being very similar genetically, had variations in their antigenic composition that were recognized by the IgA antibodies in saliva.

The results of cross-absorption support the proposal that the IgA antibodies in saliva samples collected after colonization of the mouths of the infants by A. naeslundii show more specificity than those collected prior to colonization by this species. When these data are considered together with those for the type B clusters, the suggestion of maturation and increased specificity of the salivary IgA response is strengthened. Furthermore, the presence of type A clusters and the division of type B clusters such as B1, B2, and B3 from infant 20 (Fig. 4) indicate that the IgA antibody response can recognize antigenic variations among isolates of the same ribotype of A. naeslundii.

In the mouse, the lamina propria of the gut is populated by two types of IgA-secreting cells that have different origins (19). Conventional or B-2 B cells are derived from the bone marrow, predominate in the Peyer’s patches and require cognate help from CD4+ T cells. As a result of affinity maturation (16), IgA antibodies produced by these cells are specific and of high affinity. In contrast, B-1 cells are derived from the peritoneal and pleural cavities early in ontogeny, do not require cognate help from CD4+ T cells, and are self-replenishing (19). As a consequence their receptor repertoire, established during the neonatal period, is limited, but it appears to be selected by colonization of the neonatal mouse by its commensal bacteria. It has been suggested that IgA antibodies produced by B-1 B cells are designed for regulation of indigenous bacteria, whereas IgA antibody produced by conventional B cells is targeted at extrinsic pathogenic bacteria (19). It is intriguing to speculate that the IgA A. naeslundii genospecies 1- and 2-reactive antibodies present in the saliva samples collected from the infants prior to the establishment of this species in their mouths may reflect, at least in part, a B-1 cell-mediated pluralspecific mucosal immune response (25). This B-1 response may then undergo transition into a B-2 response after colonization by these organisms.

Many studies exploring the potential utility of commensal bacteria as vaccine vectors have used the human commensal viridans streptococcus, S. gordonii, in rodent models (12, 21). Although this bacterium is autochthonous in the human oral cavity it is allochthonous in rodents. Thus, the ecological relationship between the bacterium and its host is different in rodents than in humans. Therefore, data obtained from rodent experiments concerning the immune response to the vector organism and the vaccine antigens it expresses and, indeed, the ease with which the organism can be stably introduced into the climax community, may not be readily extrapolated to man. In this context species belonging to the genus Actinomyces that are autochthonous to the oral cavity of humans and many other mammalian species (10) may provide a more suitable vector. Be that as it may, a fuller understanding of the mucosal immune response to commensal bacteria, such as has been begun in the present study, is needed before commensal bacteria can be successfully exploited for vaccine delivery in human beings.

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