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Pioneer Oral Streptococci Produce Immunoglobulin A1 Protease

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As part of a longitudinal study of the relationship between bacterial colonization and the secretory immune response, 367 isolates of pioneer viridans streptococci collected from 40 breast- and bottle-fed neonates within the first month postpartum were tested for the production of immunoglobulin A1 (IgA1) protease and glycosidases. Fifty percent of the streptococci isolated produced IgA1 protease, including all isolates of *Streptococcus oralis* and *S. sanguis*, 60.7% of *S. mitis* biovar 1 isolates, and some isolates that could not be identified. Three cleavage patterns of α1 heavy chains were observed. Six isolates of *S. mitis* biovar 1 that did not produce IgA1 protease attacked the α1 chain. Incubation of IgA1 protease-negative *S. mitis* biovar 1 isolates with IgA1, either prior to or together with *S. sanguis*, rendered the IgA1 paraprotein resistant to cleavage by the IgA1 protease of *S. sanguis*. The ability of some pioneer streptococci in the human oral cavity to produce IgA1 protease and of others to modify the susceptibility of IgA1 to cleavage by IgA1 protease perhaps enhances their ability to survive in this habitat.

Streptococcus species constitute a significant component of the oral flora in humans (2). Frandsen et al. (6) demonstrated the localized distribution of different *Streptococcus* species in oral habitats of adults. For example, *Streptococcus oralis* adhered to pellicle-coated enamel during early plaque formation, *S. mitis* biovar 1 colonized the buccal mucosa, and *S. salivarius* colonized the dorsum of the tongue. It was noted by these authors that the habitats of the immunoglobulin A1 (IgA1) protease-producing species *S. sanguis*, *S. oralis*, and *S. mitis* biovar 1 were largely restricted to tooth surfaces and the buccal mucosa. Moreover, these species were most evident during early colonization of teeth or were present on a surface where cells were lost through shedding, necessitating constant recolonization by the bacteria. These observations were taken by Frandsen and coworkers (6) as evidence that IgA1 protease production could play a role in subverting secretory IgA (S-IgA) antibodies, which normally would prevent or reduce adherence of bacterial cells to oral surfaces (16).

In adults, bacteria colonizing habitats arise from reservoirs within the mouth. This means that organisms adhering to surfaces have done so in an environment containing specific antibodies where the production of IgA1 protease could confer on microorganisms an ecological advantage. A somewhat different situation pertains in colonization of neonates and young infants. In neonates, bacteria colonizing habitats within the oral cavity are transmitted from sources extrinsic to the host. Furthermore, the bacteria colonize in an environment where specific antibodies available to inhibit adherence are absent or are present at low levels (19). This could suggest that IgA1 protease production might not provide an immediate advantage to those bacteria colonizing the oral cavity of neonates. Similarly, the results of a recent study (15) that compared the proportions of oral streptococci recolonizing cleaned tooth surfaces of selectively IgA-deficient and normal subjects suggest that S-IgA antibody may not be a factor controlling the adherence of oral streptococci. Thus, the ability to cleave S-IgA1 antibodies may not provide these bacteria with an ecological advantage in colonization.

During a longitudinal study of the relationships between oral colonization of infants by bacteria and the development of the secretory immune response, 367 isolates of viridans streptococci were collected from 40 healthy full-term neonates at 1 to 3 days and 2 and 4 weeks postpartum. One component of the tests (1, 11) used to identify the isolates was the production of IgA1 protease, and it was evident that this characteristic was common among the isolates. The current paper describes the IgA1 protease-producing species, their diversity and contribution to the oral flora of the neonates, and the characteristics of the IgA1 proteases and glycosidases produced relative to those described by others (9, 13, 17).

MATERIALS AND METHODS

Study population. Forty healthy, full-term infants were enrolled in the study without regard to race or sex. Infant-mother pairs were included after signed, informed consent was obtained and provided that the infant was full term (38 to 42 weeks of gestation), of appropriate weight for gestational age, and without underlying illnesses. Conditions which served to exclude an infant-mother pair from the study were (i) perinatal complications of maternal fever, (ii) prolonged rupture of fetal membranes (greater than 24 h), (iii) antibiotic treatment of mother or infant during the first 30 days of the infant's life, and (iv) serious metabolic or infectious complications during the first 30 days of the infant's life.

Sample collection and processing. Swab samples were obtained 1 to 3 days, 2 weeks, and 4 weeks postpartum. The mucosal surfaces of the cheeks, buccal sulci, edentulous ridges, tongue, and hard palate were swabbed with the swab from a

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Vacutainer Anaerobic Specimen Collector (Becton Dickinson Vacutainer Systems, Rutherford, N.J.). 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 (RTF) (20), bacteria were dispersed by ultrasound at 80 W for 10 s with a Branson Sonifier 250 (Branson Ultrasonics Corp., Danbury, Conn.) equipped with a microprobe and were serially diluted in reduced transport fluid to 10^{-5}.

Recovery of pioneer viridans streptococci. Trypticase soy agar plates containing 5% sheep blood (AO2) and Columbia agar containing 5% sheep blood, cysteine HCl, palladium chloride, dithiothreitol, and hemin (ANO2) (Remel, Lenexa, Kans.) were inoculated by using a spiral plater (Spiral Systems, Cincinnati, Ohio). The AO2 plates were incubated at 37°C for 48 h in air, and the ANO2 plates were incubated at 37°C for 72 h in an anaerobic chamber containing an atmosphere of 80% N_2, 10% CO_2, and 10% H_2. Colonies growing on the AO2 and ANO2 plates were counted and examined under a dissecting microscope at a magnification of ×20. A representative of each distinct colony morphology was subcultured to purity.

Identification of pioneer viridans streptococci. The isolates were examined for hemolysis, Gram stained, catalase tested, and identified by using two taxonomic schemes (1, 11). Briefly, isolates were tested for production of hydrogen peroxide, hydrolysis of arginine and esculin, and fermentation of mannitol, sorbitol, raffinose, melibiose, trehalose, amygdalin, inulin, lactose, arbutin, *N*-acetylglucosamine, and glucose in a basal medium containing thioglycolate broth without glucose or indicator and Purple Broth Base (both from Difco Laboratories, Detroit, Mich.) (1). Production of neuraminidase, α-L-fucosidase, β-D-fucosidase, β-*N*-acetylglucosaminidase, β-*N*-acetylgalactosaminidase, α-glucosidase, β-glucosidase, and α-arabinosidase was determined by using 4-methylumbelliferyl-linked substrates (21). β-Galactosidase activity was also determined for selected isolates. Production of IgA1 protease was determined as described previously (4), using an IgA1(κ) paraprotein (Organon Teknika-Cappel, West Chester, Pa.). Control bacteria were also tested in parallel with the isolates. The panel of determinative tests was repeated for approximately one-fourth of the isolates. IgA1 protease-negative isolates that degraded α1 heavy-chain carbohydrate were reexamined by using an IgA1(λ) paraprotein (Athens Research and Technology Inc., Athens, Ga.).

Effect of glycosidases on IgA1 protease activity. The potential ecological significance of the ability of streptococcal glycosidases to modify the susceptibility of IgA1 to cleavage by IgA1 protease (17) prompted an examination of the isolates of streptococci from the neonates. In separate experiments a reference strain of *S. sanguis* (ATCC 10556) that cleaves but does not deglycosylate the α1 chain was coincubated with two isolates that lack IgA1 protease but deglycosylate the α1 chain and IgA1(κ) substrate. Following incubation for 24 h, cleavage of the IgA1 substrate was determined as described above.

RESULTS

Identity of pioneer streptococci. Of 40 neonates enrolled in the study, swab samples were obtained from 27 subjects at 1 to 3 days, from all 40 subjects at 2 weeks, and from 38 subjects at 4 weeks postpartum.

The streptococci were identified on the basis of the classification suggested by Kilian et al., which enabled the separation of *S. mitis* into two biovars (11). However, both the determinative tests proposed by Kilian et al. and the tests proposed by Beighton et al. (1) were required for the satisfactory identification of the isolates. The species of viridans streptococci comprising the 367 isolates recovered from the neonates at 1 to 3 days, 2 weeks, and 4 weeks postpartum are shown in Table 1. *S. oralis* and *S. mitis* biovar 1 were the dominant species isolated from the neonates, comprising 71.4% of the isolates obtained 1 to 3 days, 52.5% of the isolates obtained 2 weeks, and 49.4% of the isolates obtained 4 weeks postpartum. *S. salivarius* was the next most numerous species, comprising 10.0, 30.0, and 28.4% of the streptococcal isolates recovered 1 to 3 days, 2 weeks, and 4 weeks postpartum, respectively.

TABLE 1. Streptococcal species isolated from neonates

Streptococci	% isolated at the following time postpartum:		
	1–3 days (n = 70)[a]	2 wk (n = 135)[b]	1 mo (n = 162)[c]
S. oralis	41.4	24.4	19.8
S. mitis biovar 1	30.0	28.1	29.6
S. mitis biovar 2	4.3	0.7	1.2
S. salivarius	10.0	30.0	28.4
S. sanguis	4.3	3.0	1.9
S. anginosus	2.9	5.2	4.9
S. gordonii	1.4	1.5	3.7
Unidentified	5.7	7.4	10.5

[a] No bacteria were recovered from 4 of 27 neonates sampled.
[b] No bacteria were recovered from 2 of 40 neonates sampled.
[c] Bacteria were recovered from all 38 neonates sampled.

Prevalence of streptococci producing IgA1 protease. Table 2 shows the percentage of streptococci producing IgA1 protease and the percentage of IgA1 protease-negative streptococcal isolates that degraded the α1 chain as indicated by an increase in its electrophoretic mobility. The IgA1 protease-producing isolates comprised all representatives of the species *S. oralis* and *S. sanguis* and 60.7% of the isolates of *S. mitis* biovar 1.

Patterns of IgA1 breakdown by pioneer oral streptococci. Four patterns of IgA1 breakdown were observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). Patterns 1, 2, and 2a resulted from cleavage of the α chain by IgA1 protease and yielded Fcα fragments of decreasing molecular size. All 10 isolates of *S. sanguis* produced pattern 1 cleavage. The production of pattern 2 and 2a cleavage was almost completely concordant with the production of neuraminidase. Of 94 isolates of *S. oralis* examined, 53 (56.4%) produced pattern 2 cleavage and 39 (41.5%) produced pattern 2a cleavage. Two isolates of *S. oralis* (2.1%), although neuraminidase positive, consistently produced pattern 1 cleavage on repeated examination. Of the 65 isolates of *S. mitis* biovar 1 that produced IgA1 protease, 49 isolates (75.4%) yielded pattern 1 cleavage and 16 isolates (24.6%) yielded pattern 2 cleavage. Eighteen isolates of *S. mitis* biovar 1

TABLE 2. Percentages (n = 367) of streptococci producing IgA1 protease and percentages of isolates lacking IgA1 protease that deglycosylate the α1 chain isolated from neonates

Characteristic of isolates	% isolated at the following time postpartum:		
	1–3 days (n = 70)	2 wk (n = 135)	1 mo (n = 162)
IgA1 protease positive	70.0	45.2	45.1
Deglycosylate α1 chain[a]	1.4	1.5	1.9

[a] Only isolates that increase the mobilities of the α1 heavy chains of both IgA1 paraproteins are included.

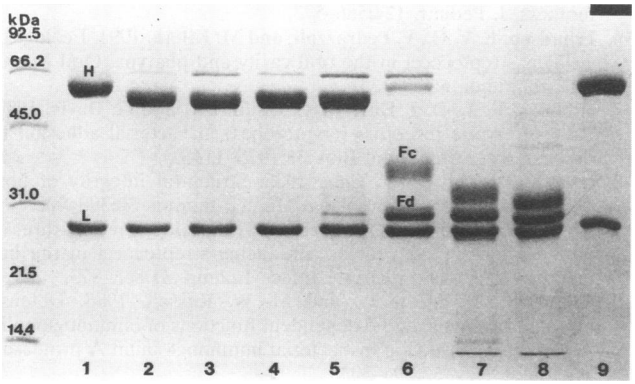

FIG. 1. SDS-PAGE analysis of IgA1(κ) paraprotein incubated with isolates of viridans streptococci from neonates. A comparison of strains producing IgA1 protease (pattern 1, 2, and 2a cleavage) and IgA1 protease-negative isolates that attack the α1 chain (pattern 3 cleavage) is shown. Lanes: 1 and 9, IgA1(κ) control; 2 and 4, isolates of IgA1 protease-negative *S. mitis* biovar 1 (pattern 3); 3 and 5, isolates of IgA1 protease-negative *S. mitis* biovar 1 coincubated with an isolate of *S. sanguis*; 6, isolate of *S. sanguis* (pattern 1); 7, unidentified isolate (pattern 2); 8, isolate of *S. oralis* (pattern 2a). H, heavy chain of IgA1 substrate; L, light chain of IgA1 substrate.

consistently producing pattern 1 cleavage also produced neuraminidase.

Pattern 3 IgA1 breakdown reflected a decrease in the molecular size of the α1 chain compared with that of the negative control. However, pattern 3 isolates lacked IgA1 protease, since no Fcα and Fdα fragments were generated. An increase in mobility of the α1 chain of the IgA1(κ) paraprotein (Organon Teknika-Cappel), indicative of depletion of carbohydrate from the α1 chain (pattern 3), was a characteristic of six IgA1 protease-negative isolates of *S. mitis* biovar 1, six isolates of *S. salivarius*, and a single isolate of *S. anginosus*. However, when these 13 isolates were retested with an IgA1(λ) paraprotein (Athens Research and Technology Inc.), no increase in mobility of the α1 chain was observed following incubation with the *S. salivarius* and *S. anginosus* isolates.

As previously reported (17), coincubation of the pattern 1 *S. sanguis* isolate with the pattern 2 and 2a isolates resulted in a decrease in the molecular size of the Fcα fragment from pattern 1 to those of the patterns 2 and 2a isolates, respectively (data not shown). However, if pattern 3 IgA1 protease-negative *S. mitis* biovar 1 isolates were incubated with the IgA1 paraprotein prior to incubation with *S. sanguis* or if these isolates were coincubated with *S. sanguis*, the immunoglobulin was apparently rendered resistant to cleavage by the *S. sanguis* IgA1 protease (Fig. 1). If, on the other hand, the IgA1 paraprotein was incubated with *S. sanguis* prior to incubation with pattern 3 *S. mitis* biovar 1 isolates, the mobility of the Fcα was converted from pattern 1 to pattern 2a.

DISCUSSION

Despite the secretory immune system and nonimmune host defense factors in saliva, it is clear that commensal bacteria colonize and persist in the mouth. This observation suggests that indigenous oral bacteria are not subjected to, not affected by, or able to avoid host immunity. Little is known about the manner by which oral bacteria evade the host's humoral immune response; however, several mechanisms are possible (2, 14). Many bacteria possess proteases capable of degrading immunoglobulins (8). The IgA1 proteases have been proposed as virulence factors for mucosal pathogens. By cleaving IgA1, the predominant subclass in serum and secretions (5), at the hinge region, the biological activity of the antibody is destroyed (10, 12, 16).

IgA1 protease production has been shown to be a property of three species of oral streptococci, i.e., *S. sanguis* biovars 1 to 4, *S. oralis*, and *S. mitis* biovar 1 (13, 17). The dominant species isolated from the predentate mouths of the neonates up to 1 month after birth in this study and in another study (18) are *S. mitis* biovar 1 and *S. oralis*. These observations are consistent with those of Frandsen et al. (6) that the buccal mucosa, pharynx, and tongue are habitats of *S. mitis* biovar 1, but they conflict with the report by those workers (6) that *S. oralis* was not isolated from mucosal surfaces. The majority of the pioneer *S. mitis* biovar 1 isolates from the neonates produced IgA1 protease. Frandsen and coworkers (6) have also observed that IgA1 protease is produced by two-thirds of the streptococci adhering to the buccal mucosa and by three-fourths of those adhering to cleaned tooth surfaces.

The four patterns of IgA1 degradation observed in the present study are in agreement with the patterns of IgA1 degradation described by Reinholdt et al. (17). Those workers also reported that bacterial glycosidases appear to remove carbohydrate largely from the Fc region of the α1 chain, because the mobilities of the Fdα fragments generated by pattern 1, 2, and 2a isolates appear very similar, although not identical (Fig. 1).

S. oralis, some *S. mitis* biovar 1 and 2 strains, and *S. intermedius* (1) produce neuraminidase. This glycosidase is capable of removing terminal sialic acid from oligosaccharide side chains located at the hinge region and elsewhere along the α1 heavy chain. Once the sialic acid is removed, exoglycosidases, notably β-galactosidase and *N*-acetylglucosaminidase, can also attack the carbohydrate moiety of the α1 heavy chain (17). Removal of carbohydrate has been shown by Reinholdt and coworkers (17) to account for the different cleavage patterns of IgA1 produced by *S. oralis* and *S. mitis* biovar 1 (pattern 2) and by *S. sanguis* (pattern 1). Furthermore, *S. mitis* biovar 2 appeared to be unique in its ability to decrease the molecular size of the α1 heavy chain (pattern 3). Although 8 of 13 strains of *S. mitis* biovar 2 examined by Reinholdt et al. (17) attacked the carbohydrate of the α1 chain, this was not a property of any of the six *S. mitis* biovar 2 isolates obtained from the neonates. In contrast, in the present study six *S. mitis* biovar 1 isolates that did not produce IgA1 protease decreased the molecular size of the α1 chain. These *S. mitis* biovar 1 isolates did not hydrolyze arginine or ferment sorbitol and, therefore, could not be considered to be *S. mitis* biovar 2 (11). Furthermore, when tested with an IgA(κ) paraprotein, six *S. salivarius* isolates and a single isolate of *S. anginosus* also degraded IgA1 carbohydrate. However, this finding could not be confirmed when these isolates were retested with an IgA1(λ) paraprotein, possibly because of differences in the carbohydrate side chains of the α1 heavy chains (17).

Streptococci that represent a numerically significant component of the commensal oral flora and comprise the pioneers of the oral cavity may be aided in colonizing the mouth by the production of IgA1 protease and glycosidases (6, 9, 13, 16). Glycosidases, such as neuraminidase, may increase the susceptibility of S-IgA1 to IgA1 protease (17). Conversely, glycosidases, by extensively deglycosylating the hinge region, also may decrease the susceptibility of S-IgA1 to IgA1 protease (17). The observation made in the present study that coincubation of pattern 3 streptococci with *S. sanguis* rendered the IgA1 paraprotein resistant to cleavage is consistent with this assertion. Furthermore, glycosidases also may serve to expose

cryptic receptors involved in lectin receptor-mediated adherence of oral bacteria (7). Thus, the influence of IgA1 protease and glycosidases on colonization of oral bacteria is potentially quite complex. Glycosidases may increase or decrease the susceptibility of IgA1 antibodies to cleavage by IgA1 protease or may render IgA1 antibodies refractory to IgA1 protease.

The ability to cleave S-IgA1 does not appear to be essential for oral streptococci to adhere to clean tooth surfaces (15). It is unclear whether the production of IgA1 protease by the oral pioneers *S. mitis* biovar 1 and *S. oralis* confers an ecological advantage on these bacteria for their establishment immediately postpartum when the level of S-IgA in saliva is low. However, a study of the ontogeny of salivary S-IgA, recently completed in this laboratory, has shown not only that the levels of S-IgA increase steadily from birth but that salivary IgA immediately postpartum consists almost exclusively of S-IgA of subclass 1 (5a). Furthermore, it is conceivable that IgA1 protease may cleave antibacterial S-IgA1 antibodies in breast milk that may contribute to passive immunity in the oral cavity (3). Finally, the production of IgA1 protease by pioneer bacteria may play a role in autogenic succession by modifying the environment to provide conditions suitable for colonization by oral bacteria that lack this enzyme.

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