Dynamic Changes in the Initial Colonization of *Actinomyces naeslundii* and *Streptococcus gordonii* Using a New Animal Model

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**SUMMARY**: *Actinomyces naeslundii* and *Streptococcus gordonii* are the predominant bacteria and initial colonizers of oral microflora. The binding of *A. naeslundii* and *S. gordonii* and the interaction between them on the salivary pellicle-coated tooth surface play an important role in the biofilm development. Recently, we reported that NOD/SCID.e2f1⁻/⁻ mice are a useful model for studying oral biofilm formation by *Streptococcus mutans* on the tooth surface. In this study, we aimed to determine whether NOD/SCID.e2f1⁻/⁻ mice can be used for studying oral colonization of *A. naeslundii* and *S. gordonii*. Colonization of *A. naeslundii* in mice fed with 1% sucrose water for 24 h before inoculation was higher than that among mice fed with sucrose water for 1 h. *A. naeslundii* colonization using mixed species-inoculation was lower than that using single-species inoculation 30–90 min after inoculation; however, the colonization was higher 120–180 min after inoculation. The mixed inoculation induced better colonization of *S. gordonii* than single-species inoculation 60–180 min after inoculation. Polyclonal and fluorescein isothiocyanate-labeled antibody stained bacteria showed better colonization of *S. gordonii* when a mixed culture is used in vivo. NOD/SCID.e2f1⁻/⁻ mice were useful for studying the initial colonization of *A. naeslundii* and *S. gordonii*. Long-term supply of sucrose water creates a favorable environment for the initial colonization of *A. naeslundii* that, in turn, supports the colonization of *S. gordonii*.

**INTRODUCTION**

Dental plaque is a complex biofilm community of diverse groups of bacteria comprising more than 750 different species (1). Previous culture-based studies suggest that streptococci are prominent during the initial stages of biofilm formation on tooth surfaces (2,3). *Streptococcus gordonii* is an early colonizer of the oral cavity of infants and can comprise a substantial proportion of the biofilm on a healthy dental surface (4–6). Other genera such as *Actinomyces* spp. are also among the earliest colonizers of dental surfaces (2,3,7). These bacteria are present from infancy to adulthood and are involved in the initial stage of infectious disease development (3,8–11). The colonization of tooth or mucosal surfaces by *Actinomyces* spp. provides a substrate for the adherence of other oral bacteria including streptococci (12), resulting in the development of the plaque community. Therefore, these early colonizers play a vital role in biofilm formation in the oral environment. Unfortunately, little attention has been devoted to the dynamic changes in these early colonizers. Previous studies reported the coaggregation of *Actinomyces naeslundii* and *S. gordonii* in vitro (13–16); however, to our knowledge, there are no studies that used an animal model.

Recently, we reported a model for decreased saliva volume in NOD/SCID.e2f1⁻/⁻ mice (17). Further, NOD/SCID.e2f1⁻/⁻ mice lack sIgA and IgG in the saliva and have decreased NK cells. We demonstrated that NOD/SCID.e2f1⁻/⁻ mice are highly susceptible to *Streptococcus mutans* colonization when NOD/SCID.e2f1⁻/⁻ mice are pretreated with human saliva or slgA using a low concentration (1%) sucrose supplement (18). This suggests that there are multiple effects exerted by slgA on *S. mutans* during its colonization; the synergistic effects are evident when a combination of slgA and limited nutrients is used during colonization in NOD/SCID.e2f1⁻/⁻ mice. This further suggests that this mouse can be used as a new animal model to assess prevention methods for dental biofilm-dependent diseases such as dental caries. We also used this mouse for oral infection by *Candida albicans*, to demonstrate the interaction between the indigenous microorganisms and colonization and saliva volume (19). Therefore, the NOD/SCID.e2f1⁻/⁻ mouse may be a useful animal model for the investigation of oral infections by various oral microorganisms.

However, very little is known about the colonization of *A. naeslundii* and *S. gordonii* separately or as mixed infections in the oral cavity using a mouse model. In this
study, we describe the dynamic changes in the colonization of *A. naeslundii* and *S. gordonii* using mixed-species inoculation in the new NOD/SCID.e2f1−/− mouse model. The results of our in vivo study on oral biofilm formation agree with those of in vitro studies.

**MATERIALS AND METHODS**

**Bacteria strains and culture conditions:** *A. naeslundii* X600 and *S. gordonii* ATCC 10558 were cultured in brain-heart infusion (BHI) broth (Difco Laboratory, Detroit, Mich., USA) overnight in an atmosphere of H₂ and CO₂ (GasPack; Becton/Dickinson, Sparks, Md., USA) at 37°C.

**Animals:** Heterozygous NOD/SCID.e2f1+/− mice were bred to produce NOD/SCID.e2f1−/− and NOD/SCID.e2f1+/− (1:2, bone rate). NOD/SCID.e2f1−/− mice genotypes (NOD/SCID.e2f1+/− and NOD/SCID.e2f1−/− mice) were identified using PCR (17). Compared with the NOD/SCID.e2f1− mice, NOD.B10.D2 mice have decreased saliva volume which is higher in level than that in NOD/SCID.e2f1+/− and e2f1−/−/− mice; they also have IgA, IgG, and cellular immunity, and they served as the control group in nonobese diabeticogen (NOD) background mice (20). All mice used in this experiment were female mice aged 4–6 months. The experimental mice were given sterile 1% sucrose drinking water for 1 h or 24 h prior to infection (less than the concentration in juice), and were fed a commercial diet (CMF; water, 8.8%; protein, 27.4%, fat, 8.0%; mineral, 6.1%; fiber, 2.9%; nitrogen, 46.8%; Oriental Yeast Co., Tokyo, Japan); whereas, the control mice in each group were provided with sterile water and CMF. Food and fluids were withheld during the experiment following inoculation. Test animals were maintained in accordance with the guidelines of the National Institute of Infectious Diseases (Tokyo, Japan). Experimental protocols (#210110 and 210111) were approved by the National Institute of Infectious Diseases Animal Resource Committee.

**Measurement of carbohydrates in the oral cavity:** To detect the carbohydrates, a phenol sulfuric acid method was applied to samples from the oral cavity after the feeding of 1% sucrose drinking water for 1 h or 24 h. Samples were collected from the oral cavities by washing with sterilized phosphate-buffered saline (PBS) and by swabbing with a sterilized cotton ball at 1 h or 24 h after finishing the sucrose supply. After sonication at 120 W for 1 min, samples were centrifuged at 10,000 × g for 10 min at 4°C for the supernatant. The samples may have included polysaccharides converted from sucrose by commensal oral bacteria, and sucrose remaining in the oral cavity. A special dose of 2 ml glucose (0.0025 mg/ml, 0.005 mg/ml, 0.01 mg/ml, 0.02 mg/ml, 0.03 mg/ml, 0.04 mg/ml, or 0.05 mg/ml) was prepared to establish the standard curve. After addition of 1-ml 5% phenol solution to the samples, 5 ml of 5% sulfuric acid solution was mixed with each sample and shaken immediately. The samples were placed in a water bath at 25°C for 20 min, and absorbance was read at 490 nm. Carbohydrate concentrations in the samples were calculated under the glucose standard curve.

**Sampling and colony-forming unit (CFU) determination:** Bacterial inoculation, sampling, and CFU counts were performed using procedures and conditions described previously (18,20). *A. naeslundii* and *S. gordonii* were cultured in BHI broth overnight and then washed twice with sterile PBS. Prior to the animal experiment, the ability of disinfect of chlorhexidine (0.2%) was evaluated using the same kind of mice for 180 min. Bacteria were inoculated into the oral cavities of mice at a final concentration of 5 × 10⁷ CFU/ml in 0.25 ml of PBS during 2.5 min. When using the mixed bacteria, the bacteria were concentrated using centrifugation before mixing. Following inoculation, samples were collected from the labial surfaces of the maxillary incisor teeth using a sterile cotton ball every 30 min for 180 min (each time interval using a different mouse from the same cage) and dispersed in 2 ml of PBS. The samples in PBS were sonicated using ultrasonic dispersion (power output, 60 W) for 10 s, and then poured onto BHI, 5% blood, and MS agar plates to determine colony numbers. To avoid confusion with different bacterial colony, colony count number was obtained using microscopy (Olympus TL3-100; Olympus Co., Tokyo, Japan) and compared with the sample colony for each sample.

**S. gordonii observation using mixed-species inoculation:** After sonication, the samples from the animal experiment at 150 min were observed using fluorescent antibody staining. All the samples were washed using PBS once after centrifugation at 1,380 × g and then incubated with anti-IPAe polyclonal antibodies (PAe; surface protein antigen from S. mutans [21]), which cross-react with surface protein antigens from *S. gordonii*, for 1 h at 37°C for the identification of *S. gordonii*. The samples were washed 3 times using PBS and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse (IgG-FITC, Sc-2010; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) for 1 h at 37°C. The cells were washed 3 times using PBS and then observed using laser microscopy (DP70; Epson, Suwa, Japan). To compare studies, the concentrations of bacteria in the animal experiment were the same as that in the in vitro studies for antibody staining. The in vitro samples were cultured for 150 min at 37°C, and then treated with the same steps used in the in vivo samples, as described above. Finally, samples were observed using laser microscopy and photographed.

**Statistics:** CFU data were expressed as mean ± standard deviation. All animal studies were performed independently in triplicates. The independent sample t test was used to compare the colony numbers using SPSS with P values of <0.01 and <0.05 representing statistical significance.

**RESULTS**

*A. naeslundii* colonization on the tooth surface of NOD/SCID.e2f1−/− mice: In comparison to NOD.B10.D2 mice, *A. naeslundii* colonized better on the tooth surfaces of NOD/SCID.e2f1−/− mice at all time points (Fig. 1). All 3 genotypes of NOD/SCID.e2f1 mice were susceptible to *A. naeslundii* colonization. The colony number of NOD/SCID.e2f1−/− mice was almost 30-fold higher than that of NOD.B10.D2 mice using water only at 30 min and colony counts on 5% blood medium agar (Fig. 1A). At 60 min, the colony
number of NOD/SCID.e2f1−/− mice was almost 25-fold higher than that of NOD.B10.D2 mice. At 30 min, the colony number on the tooth surfaces was higher than that at 60 min and 90 min. However, there were no significant differences among NOD.B10.D2, NOD/SCID.wild-type and NOD/SCID.e2f1−/− mice at 90 min. The decrease in the colony number between 30 min and 60 min after inoculation on the tooth surfaces was higher than that of NOD.B10.D2 mice at 30 min. At 60 min after inoculation was much higher than that in NOD/SCID.e2f1−/− mice at 30 min.

**A. naeslundii** colonization in mixed-species inoculation with *A. naeslundii* and *S. gordonii*: Colony numbers of *A. naeslundii* gradually decreased from 30 min to 120 min (Fig. 2). This decrease is dependent on the number of physically remaining and non-adhered bacteria at 30 min and 60 min after inoculation on the tooth surface because a high concentration of *A. naeslundii* was inoculated. After inoculation, bacteria remained physically and non-adhered bacteria decreased immediately by saliva flow. After 120 min, the colony numbers were stable and the bacteria remained on the tooth surface. After 150 min and 180 min after inoculation of the single and mixed species, non-adhered bacteria were almost removed, and adhered and colonized cells remained on the tooth surface. *A. naeslundii* colonization together with mixed inoculation was better than that with single species inoculation with *S. gordonii* 30–90 min after inoculation (Fig. 2). However, from 120 min after inoculation, the colony number of *A. naeslundii* with mix-
Fig. 2. *A. naeslundii* mixed with *S. gordonii* compared to single species initial attachment on tooth surfaces. NOD/SCID.e2f1+/− mice were used; for *A. naeslundii* colonization, samples were poured on 5% blood agar plates every 30 min after inoculation and this was continued till 180 min. Data are expressed as the mean ± SD of 3 independent assays. One mouse was used each time, and a total of 6 mice were used at 6 time points in 1 experiment carried out for each strain. The animal experiment was independently repeated 3 times. The colony numbers in the culture samples from the oral cavities were counted. One asterisk represents $P < 0.05$ (*A. naeslundii* mixed with *S. gordonii* compared with single species at 150 min and 180 min).

ed-species inoculation (250.0 ± 80.2 and 220.0 ± 83.2 at 150 min and 180 min, respectively) was significantly higher than those with single-species inoculation (86.7 ± 23.1 and 73.3 ± 83.2), and the difference was observed at 150 min and 180 min.

*S. gordonii* colonization in mixed-species inoculation with *A. naeslundii* and *S. gordonii*: The decrease in *S. gordonii* colony numbers with mixed-species inoculation was smaller than that with single-species inoculation. *S. gordonii* colonized better in single culture than in mixed inoculation with *A. naeslundii* at 30 min after inoculation (Fig. 3). From 90 min, the colony number of *S. gordonii* with mixed-species inoculation was higher than that with single-species inoculation. This was also observed at 150 min, and the colony number was significantly higher at 180 min.

Antibody staining of the samples in animal experiments: The polyclonal anti-rPAc mouse antibodies, which cross-reacted with the surface protein antigens of *S. gordonii*, but not of *A. naeslundii*, was used to differentiate *S. gordonii* from *A. naeslundii* where the *S. gordonii* fluoresced green using laser light (Figs. 4D and F); the *A. naeslundii* cells were dark (Fig. 4B). Photographs without the laser light showed single bacterial species and aggregation between *S. gordonii* and *A. naeslundii* (Figs. 4A, C, and E). Photographs of the in vivo samples reveal that *S. gordonii* numbers were fewer in the single-species samples than that in the mixed-species samples at the same time intervals (Figs. 5B and D). In the mixed-species in vivo samples, the number of *S. gordonii* was higher than that of single species, and many of them were aggregated with *A. naeslundii* (Figs. 5C and D).

**DISCUSSION**

The data reveal that the colony number of *A. naeslundii* at 90 min after inoculation is much higher in NOD/SCID.e2f1−/− mice than that in NOD.B10.D2 and NOD/SCID.e2f1+/+ mice that were fed 1% sucrose.
water before inoculation. NOD.B10.D2 mice have normal immunity and decreased saliva volume, which is higher than that in the NOD/SCID.e2f1-+/- and e2f1--mice. We demonstrated that the NOD/SCID.e2f1--mice that have a decreased salivary volume, lack IgA and IgG, and have decreased NK cells (17) are useful for studying A. naeslundii and S. gordonii colonization, which are the initial colonizers on human tooth surfaces (2–4,6,7). Further, saliva flow and immunity may play a central role in the initial colonization by these bacteria.

Long-term (24 h) feeding with 1% sucrose enhances the colonization of A. naeslundii in NOD/SCID.e2f1-+/-mice, compared with short-term (1 h) feeding. Polysaccharides converted from sucrose by the original oral bacteria, such as streptococci in the mouse oral cavity, and the remaining sucrose were present in the oral cavity after feeding with 1% sucrose water for 24 h because the carbohydrate concentrations in mice fed 24 h was higher than that in mice fed for 1 h. Further, A. naeslundii degrades sucrose and polysaccharides such as fructan, giving free fructose and glucose (22,23); the presence of sucrose in the diet greatly increases the ability of A. naeslundii to produce fermentable products from sucrose through β-fructofuranosidase activity (22). The end products of sucrose degradation by A. naeslundii are volatile acids, especially acetic acid and lactic acid, and intracellular and extracellular polysaccharides (22), which are required for A. naeslundii to remain in the oral cavity and for other bacteria such as streptococci. Therefore, long-term 1% sucrose supply provides a more favorable condition for A. naeslundii and S. gordonii colonization on the tooth surface.

Dental plaque is a complex microbial ecosystem in which A. naeslundii and S. gordonii are early colonizers. The dynamic changes in the colonization of these bacteria are shown in an in vitro model, an artificial oral cavity environment that shows biofilm formation. However, the oral cavity is a complicated, multi-bacterial environment where saliva flow, tongue movement, and mastication are fundamental. In this study, we demonstrate the relationship of these bacteria in vivo and the dynamic changes show that colonization by both species is better from 150 min to 180 min when inoculated together than when inoculated separately (Figs. 2 and 3).

Direct observation of the 2 species was performed using photographs of the mixed-species inoculation and antibody staining of S. gordonii. Using in vitro samples, S. gordonii was identified using laser light and compared to photographs obtained using normal light (Figs. 4C and D). A. naeslundii did not fluoresce using laser light when compared to photographs with normal light (Figs. 4A and B). This revealed the fluorescent antibody staining was efficient in differentiating S. gordonii and A. naeslundii. The aggregation of A. naeslundii and S. gordonii can be clearly observed in Fig. 4F. Comparing the photographs of single species to 2 species, the number of S. gordonii observed was greater when using the mixed-species inoculation in the animal model (Figs. 5B and D). The photographs show that A. naeslundii and S. gordonii aggregated with each other at 150 min after inoculation (Fig. 5D). This aggregation is possibly because the colonization of the 2 species was higher than...
that of a single species. Several factors may enhance the coexistence of these species. *A. naeslundii* has sialidase activity (24,25), which is not present in *S. gordonii* (24,26); this activity could potentially supply nutrients for *S. gordonii*. Further, *S. gordonii* produces several glycolytic and proteolytic enzymes lacking in *A. naeslundii* (24).

Furthermore, the ability of *A. naeslundii* to bind surface receptors on *S. gordonii* contributes to its retention in biofilms under flowing saliva (26). Additionally, H$_2$O$_2$ production results in *S. gordonii* benefiting from *A. naeslundii*, as it is protected against oxidative stress (15). The aggregated cells in the mix induce physical space that is more numbers of ligands to salivary pellicle receptors on the tooth surface than the non-aggregated cells in single inoculation. Therefore, the aggregation may be important for supporting initial colonization on the tooth surface.

In summary, the NOD/SCID.e2f1 mouse model is useful for studying the initial attachment of *A. naeslundii* and *S. gordonii*. Long-term sucrose supply enhances *A. naeslundii* colonization in NOD/SCID.e2f1$^-$ mice. To our knowledge, this study is the first to observe the dynamic changes in the initial attachment of 2 species using an in vivo model. The NOD/SCID.e2f1 mouse model can be used in the future to elucidate the interaction between multiple species.

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**Conflict of interest**  None to declare.

**REFERENCES**


