OMVs of *P. gingivalis* retain the immunodominant determinants

We next examined whether OMVs function as a reservoir of immunoreactive antigens. We performed whole-cell ELISA to test the reactivity of preimmune serum and three mouse antisera (raised against whole *P. gingivalis* wild type by conventional immunization) to the wild type and *galE* mutant. All three antisera showed a significantly higher reaction to unwashed wild type than to unwashed *galE* mutant (asterisk-1 in Fig. 2), while background reactivity of preimmune serum to the wild type and *galE* mutant were comparable, irrespective of washing. All antisera showed significantly lower reactivities to both washed wild type and the washed *galE* mutant, compared to when the corresponding bacteria without washing served as ELISA antigen (asterisk-2 and -3 in Fig. 2). Washing resulted in a larger decrease in antisera reactivity for the wild type (asterisk-2 in Fig. 2) than for the *galE* mutant (asterisk-3 in Fig. 2), indicating that cell washing drastically reduced surface antigenicity due to loss of OMVs and probably other bacterial appendages as well. When washed cells were used as antigen, two of the three antisera, antiserum-1 and -3, had significantly stronger reactivity to the *galE* mutant than to the wild type (asterisk-4 in Fig. 2). It is possible that the *galE* mutant is more antigenic than the wild type, because antigenic determinants on the outer membrane of the *galE* mutant may be more readily exposed to the environment by deglycosylation of LPS [23] and/ or outer membrane glycoproteins [27].

In Figure 2, we showed that OMVs associated with bacteria enhanced antigenicity. However, LPS may also play a key role in eliciting antibody production and therefore may affect the antigenicity of Gram-negative bacteria. To determine whether LPS and/or OMVs are involved in antigenicity, we examined the reactivity of mouse serum IgG to LPS and OMVs using preimmune serum and one of three antisera against *P. gingivalis* that showed reactivity to the whole cells, antiserum-2 (Fig. 2). The reactivity of preimmune serum against OMV or LPS was low (Fig. 3A). Antiserum-2 reacted strongly to OMV, but not to LPS (Fig. 3A).

To provide further confirmation, we performed an absorption assay where specific antibodies were absorbed from the sera before ELISA. To remove LPS- or OMV- specific antibodies, the sera were absorbed by pre-incubation with LPS or OMV. The reactivity of antiserum-2 to whole *P. gingivalis* cells decreased significantly after pre-incubation with OMV in comparison to pre-incubation with PBS (Fig. 3B). However, pre-incubation with LPS did not influence the reactivity of the antiserum-2 against *P. gingivalis* (Fig. 3B). We also obtained similar results obtained by absorption assay using the other antisera (antiserum-1 and -3).

OMVs of *P. gingivalis* elicit *P. gingvalis*-specific humoral immune responses

To investigate whether OMVs have the potential to induce not only antibodies in blood, but also mucosal antibodies that recognize *P. gingivalis* in mice, we designed an intranasal immunization protocol using OMVs and a mucosal adjuvant (Fig. 4A). Double-stranded RNA has been shown to be an effective adjuvant for mucosal vaccination against influenza virus [28,29]. Therefore, we chose Poly (I:C), a double-stranded RNA adjuvant, as our vaccine adjuvant. Briefly, after intranasal immunization of OMVs or whole-cell *P. gingivalis* twice on day 0 and day 21, mice were sacrificed at week five, and Ig titers were determined by whole cell ELISA. Intranasal immunization with *P. gingivalis* whole cells did not effectively induce *P. gingivalis*-specific antibodies (Fig. 4B–E). In contrast, immunization with OMVs strongly induced *P. gingivalis*-antibodies in mice (Fig. 4B, C, and E). Notably, OMVs also strongly induced nasal wash IgA, as well as serum IgG and IgA. As with the nasal wash, we also observed strong induction of saliva IgA in mice immunized with OMVs (Fig. 4F), but not in either sham-immunized (PBS) (Fig. 4F) or pre-immune mice (data not shown). *P. gingivalis*-specific serum IgM was not found due to similar reactivity among all mouse groups (Fig. 4D).

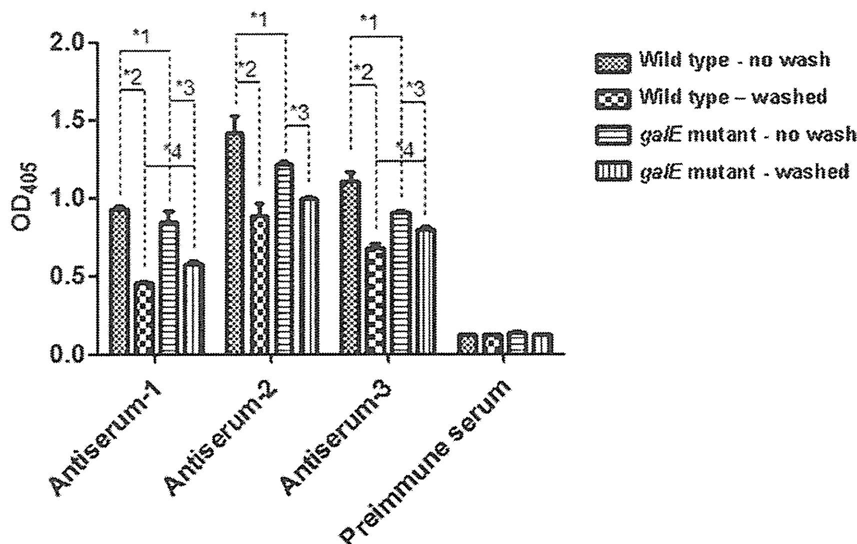


Figure 2. Analysis of antigenicity of whole cells from wild type and the *galE* mutant using *P. gingivalis* antisera. ELISA plates were coated with freeze-fried *P. gingivalis* wild type or the *galE* mutant. Bacteria were either washed twice with PBS or left unwashed before coating. *P. gingivalis* antisera from three different mice and a pre-immune serum were used at dilutions of 1:1,000. Sera reactivity was determined as the absorbance at 405 nm (mean ± SD) for triplicate assays after a 30-min incubation with alkaline phosphatase substrate. Asterisks-1, -2, -3, and -4 denote statistically significant differences ($p<0.05$).
doi:10.1371/journal.pone.0026163.g002

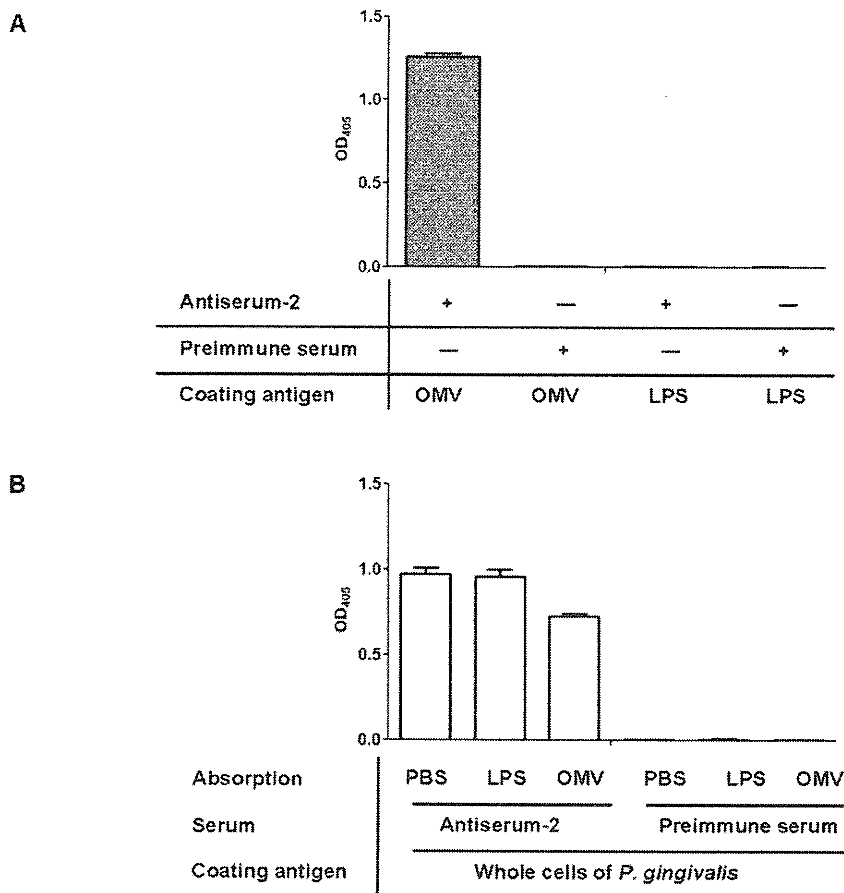


Figure 3. *P. gingivalis* antiserum cross-reacts strongly with OMVs, but not with LPS. (A) ELISA plates were coated with LPS and OMVs of *P. gingivalis*. *P. gingivalis*, antiserum-2 and pre-immune serum were used at dilutions of 1:1,000. Sera reactivity was determined as the absorbance at 405 nm (mean ± SD) for triplicate assays after a 30-min incubation with alkaline phosphatase substrate. (B) To test whether antiserum-2 cross-reacts with OMVs or LPS, we absorbed the serum against LPS and OMVs and removed bound antibodies. ELISA results are expressed as absorbance at 405 nm (mean ± SD) after a 30-min incubation with alkaline phosphatase substrate.
doi:10.1371/journal.pone.0026163.g003

Discussion

In Fig. 1A and B, we demonstrated that OMVs were not detectable in the *galE* mutant. Growth of the *galE* mutant was similar to that of the wild type (Fig. 1D), however, the limulus activity of the respective supernatants was quite different. The limulus activity of the wild type strain supernatant increased steadily through culture day 3, while that of the *galE* mutant remained similar to baseline (Fig. 1C). These data suggest that OMV probably plays an important role in dissemination of LPS to the external environment during growth. However, since *galE* mutation causes pleiotropic effects [23,27], it is also possible that changes in OMV formation and LPS release are two unrelated events in this mutant.

When washed bacterial cells were used as antigen for ELISA, all *P. gingivalis* antisera exhibited drastically decreased reactivity to the wild type (asterisk-2 in Fig. 2), but only mildly decreased reactivity to the *galE* mutant (asterisk-3 in Fig. 2). *P. gingivalis* antiserum recognized OMV, but not LPS (Fig. 3A). Absorption assays revealed that *P. gingivalis* antiserum reactivity to whole bacteria decreased after pre-incubation with OMVs (Fig. 3B). Our data suggest that OMVs play a pivotal role in the antigenicity of *P. gingivalis* and other appendages loosely tethered to the outer membrane, such as fimbriae may also affect the antigenicity of *P. gingivalis*.

The presence of OMVs on *P. gingivalis* (Fig. 1A) may confer increased antigenicity simply because the vesicles effectively expand the bacterial surface area. On the other hand, surprisingly, *P. gingivalis*-specific antibody was not detectable when mice were immunized with whole *P. gingivalis* cells, while OMV immunization strongly elicited specific antibodies (Fig. 4). Therefore, an alternative reason that both antigenicity and immunogenicity were enhanced by the presence of OMVs might be that immunodominant determinants are more concentrated on OMVs than on the bacterial surface itself. Many reports have shown that virulence factors are associated with OMVs in Gram-negative bacteria (reviewed by [30]), including *P. gingivalis* [14,15,16,17,18]. Thus, we suggest that our strategy of OMV vaccination via the nasal cavity might be applicable to *P. gingivalis* bacterial infections whose virulence factors are enriched in the OMV.

Double-stranded RNA, such as the Poly (I:C) and Ampligen®, is a Toll-like receptor 3 (TLR3) agonist. Promising results have been obtained using Poly(I:C) or Ampligen® as an adjuvant in flu vaccine delivered intranasally to mice [28,29]. The safety of Ampligen® also has been established in clinical trials for patients with chronic fatigue syndrome in the U.S. [31]. On the other hand, in many animal studies cholera toxin (CT) B subunit or the mutant CTB [32] has been used as a strong adjuvant to induce protective immunity. However, use of heat-labile

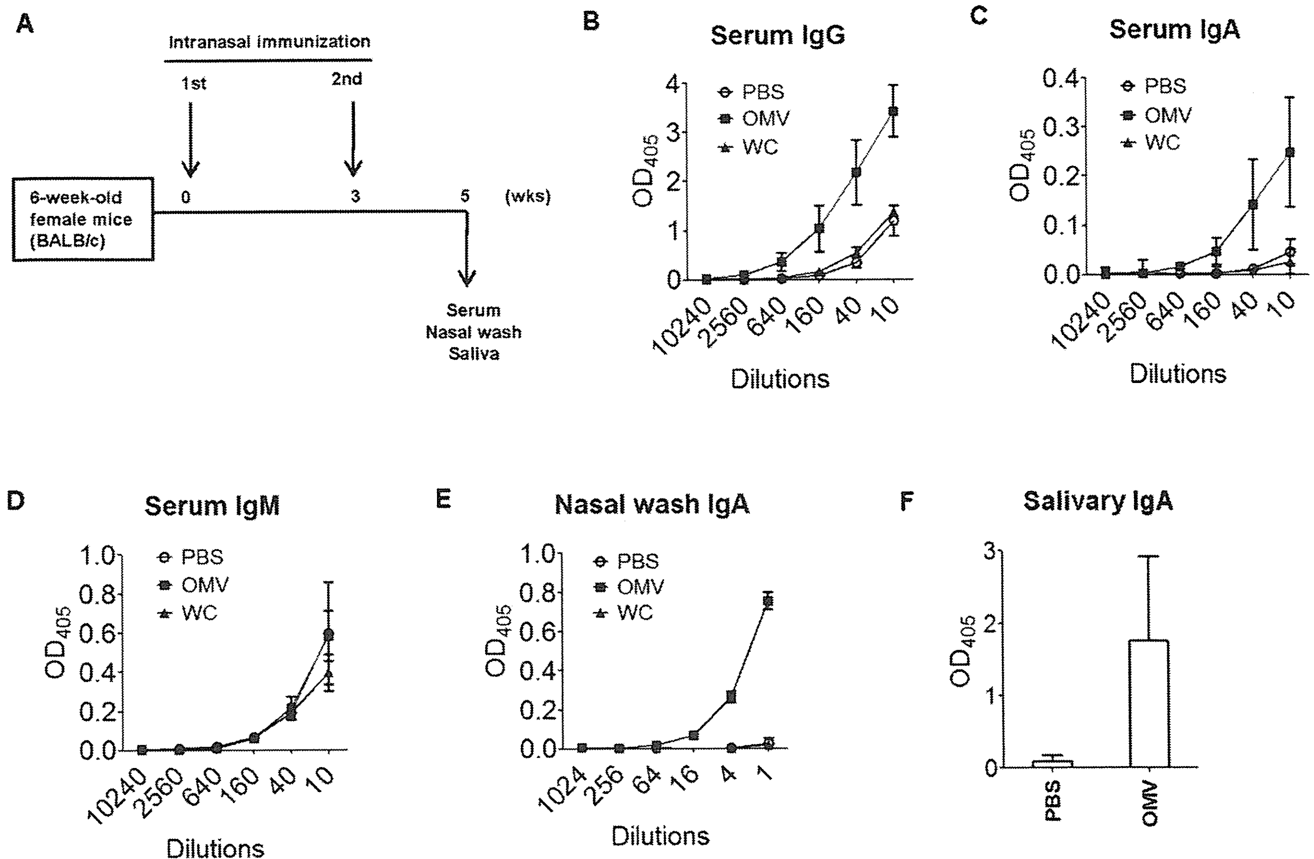


Figure 4. Immunogenicity of OMVs and whole cells of *P. gingivalis* after intranasal immunization. (A) The timeline of immunization is shown. (B–E) ELISA plates were coated with *P. gingivalis* whole cells. Samples of serum, nasal washes and saliva taken from mice immunized with *P. gingivalis* OMV, *P. gingivalis* whole cells (WC), and sham-immunized (PBS) mice. *P. gingivalis*-specific serum IgG (B), serum IgA (C), serum IgM (D), and nasal wash IgA (E) were examined by ELISA. For the salivary IgA (F), un-diluted saliva samples from OMV-immunized mice were compared with those from sham-immunized (PBS) mice. The results of triplicate assays are expressed as absorbance (mean ± SD) at 405 nm after a 30-min incubation with alkaline phosphatase substrate. In (A) to (E), the serum and nasal wash samples were from four mice per OMV-immunized group, four mice per *P. gingivalis* whole cells-immunized group (WC) and two mice per PBS control group. In (F), the saliva samples were from two PBS-immunized mice and three OMV-immunized mice.
doi:10.1371/journal.pone.0026163.g004

enterotoxin (LT), which is structurally and functionally similar to CT, has been linked to severe complications, such as several cases of Bell's palsy (facial paralysis) [33]. Therefore, at present, an adjuvant derived from a toxin is impractical for use in a human vaccine, especially for periodontal disease vaccine, because the benefit of the vaccine must far outweigh the risk of serious side effects.

In this study, we applied Poly (I:C) as an adjuvant for OMV intranasal immunization of mice. Without using a toxin-derived adjuvant, we successfully elicited an s-IgA response in saliva as well as a serum IgG response. In periodontal pockets, periodontopathic bacteria float as planktonic cells or form biofilms in the fluid composed of gingival cervicular fluid (GCF) and saliva. While the GCF contains abundant immunoglobulins (mostly IgG) exuding from blood vessels, the saliva contains abundant s-IgA. Therefore, both the systemic and mucosal immune responses contribute to humoral immunity in the oral cavity and are important in the context of a vaccine strategy against periodontal diseases. In particular, s-IgA is regarded as a main player in immunological defense at the mucosal surface because pathogen-specific s-IgA can inactivate the pathogen before it invades the host. In addition, s-IgA is generally more cross-reactive against pathogen variants than IgG and other classes of immunoglobulins.

It has been reported that intraperitoneal administration of OMVs derived from *Salmonella typhimurium* activated *Salmonella*-specific T and B cell responses and elicits protective immunity against challenge with live bacteria in mice [34]. A recent report showed that intranasal administration of OMVs derived from *V. cholerae* successfully induced protective immunity in mice [21], although it remains unknown whether undesirable molecules such as CT are present as containants in the OMV preparation and whether clinical use is safe. As OMV is a cell-free antigen, its use as a vaccine is safer than the conventional live-attenuated vaccine. In addition, an OMV vaccine is superior to other formulations, such as a purified protein vaccine, for economical reasons and in terms of its stability at ambient temperature. In the present study, we characterized the immunological properties of *P. gingivalis* OMV. In conclusion, we suggest that *P. gingivalis* OMV might have application as a periodontal disease vaccine. To our knowledge, this is the first study using a combination of bacterial OMV and Poly (I:C) for strong induction of bacterial-specific s-IgA in saliva and nasal washes as well as IgG and IgA in serum. Further studies will be required to examine whether this strategy can protect against bacterial challenge and to elucidate the mechanism of humoral immune responses to intranasal administration of OMV.

Acknowledgments

We thank Ms. Noriko Saito for technical assistance. We thank Dr. Emiko Kudo for kindly providing the human sera.

Author Contributions

Conceived and designed the experiments: RN HH KO ST AA MO HW HS. Performed the experiments: RN HH HS. Analyzed the data: RN. Contributed reagents/materials/analysis tools: RN HH KO ST AA HW HS. Wrote the paper: RN HH MO HW HS.

References

1. Lamont RJ, Jenkinson HF (1998) Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. Microbiology and molecular biology reviews : MMBR 62: 1244–1263.
2. Desvarieux M, Demmer RT, Rundek T, Boden-Albala B, Jacobs DR, Jr., et al. (2005) Periodontal microbiota and carotid intima-media thickness: the Oral Infections and Vascular Disease Epidemiology Study (INVEST). Circulation 111: 576–582.
3. Ishihara K, Nabuchi A, Ito R, Miyachi K, Kuramitsu HK, et al. (2004) Correlation between detection rates of periodontopathic bacterial DNA in coronary stenotic artery plaque [corrected] and in dental plaque samples. Journal of clinical microbiology 42: 1313–1315.
4. Ojima M, Takeda M, Yoshioka H, Nomura M, Tanaka N, et al. (2005) Relationship of periodontal bacterium genotypic variations with periodontitis in type 2 diabetic patients. Diabetes care 28: 433–434.
5. Dorn BR, Dunn WA, Jr., Progulske-Fox A (1999) Invasion of human coronary artery cells by periodontal pathogens. Infection and immunity 67: 5792–5798.
6. Lalla E, Lamster IB, Feit M, Huang L, Spessot A, et al. (2000) Blockade of RAGE suppresses periodontitis-associated bone loss in diabetic mice. The Journal of clinical investigation 105: 1117–1124.
7. Li L, Messas E, Batista EL, Jr., Levine RA, Amar S (2002) *Porphyromonas gingivalis* infection accelerates the progression of atherosclerosis in a heterozygous apolipoprotein E-deficient murine model. Circulation 105: 861–867.
8. Nikaido H (2005) Restoring permeability barrier function to outer membrane. Chemistry & biology 12: 507–509.
9. Beveridge TJ (1999) Structures of gram-negative cell walls and their derived membrane vesicles. Journal of bacteriology 181: 4725–4733.
10. Balsalobre C, Silvan JM, Berglund S, Mizunoe Y, Uhlin BE, et al. (2006) Release of the type I secreted alpha-haemolysin via outer membrane vesicles from *Escherichia coli*. Molecular microbiology 59: 99–112.
11. Wai SN, Lindmark B, Soderblom T, Takade A, Westermark M, et al. (2003) Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin. Cell 115: 25–35.
12. Kouokam JC, Wai SN, Fallman M, Dobrindt U, Hacker J, et al. (2006) Active cytotoxic necrotizing factor 1 associated with outer membrane vesicles from uropathogenic *Escherichia coli*. Infection and immunity 74: 2022–2030.
13. Kesty NC, Mason KM, Reedy M, Miller SE, Kuehn MJ (2004) Enterotoxigenic *Escherichia coli* vesicles target toxin delivery into mammalian cells. The EMBO journal 23: 4538–4549.
14. Deslauriers M, ni Eidhin D, Lamonde L, Mouton C (1990) SDS-PAGE analysis of protein and lipopolysaccharide of extracellular vesicules and Sarkosyl-insoluble membranes from *Bacteroides gingivalis*. Oral microbiology and immunology 5: 1–7.
15. Grenier D, Mayrand D (1987) Functional characterization of extracellular vesicles produced by *Bacteroides gingivalis*. Infection and immunity 55: 111–117.
16. Imamura T, Potempa J, Pike RN, Moore JN, Barton MH, et al. (1995) Effect of free and vesicle-bound cysteine proteinases of *Porphyromonas gingivalis* on plasma clot formation: implications for bleeding tendency at periodontitis sites. Infection and immunity 63: 4877–4882.
17. Smalley JW, Birss AJ (1991) Extracellular vesicle-associated and soluble trypsin-like enzyme fractions of *Porphyromonas gingivalis* W50. Oral microbiology and immunology 6: 202–208.
18. Haurat MF, Aduse-Opoku J, Rangarajan M, Dorobantu L, Gray MR, et al. (2011) Selective sorting of cargo proteins into bacterial membrane vesicles. The Journal of biological chemistry 286: 1269–1276.
19. Furuta N, Tsuda K, Omori H, Yoshimori T, Yoshimura F, et al. (2009) *Porphyromonas gingivalis* outer membrane vesicles enter human epithelial cells via an endocytic pathway and are sorted to lysosomal compartments. Infection and immunity 77: 4187–4196.
20. Holst J, Martin D, Arnold R, Huergo CC, Oster P, et al. (2009) Properties and clinical performance of vaccines containing outer membrane vesicles from *Neisseria meningitidis*. Vaccine 27 Suppl 2: B3–12.
21. Schild S, Nelson EJ, Camilli A (2008) Immunization with *Vibrio cholerae* outer membrane vesicles induces protective immunity in mice. Infection and immunity 76: 4554–4563.
22. Kesavalu L, Ebersole JL, Machen RL, Holt SC (1992) *Porphyromonas gingivalis* virulence in mice: induction of immunity to bacterial components. Infection and immunity 60: 1455–1464.
23. Nakao R, Senpuku H, Watanabe H (2006) *Porphyromonas gingivalis galE* is involved in lipopolysaccharide O-antigen synthesis and biofilm formation. Infection and immunity 74: 6145–6153.
24. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry 72: 248–254.
25. Matsumoto N, Salam MA, Watanabe H, Amagasa T, Senpuku H (2004) Role of gene E2f1 in susceptibility to bacterial adherence of oral streptococci to tooth surfaces in mice. Oral microbiology and immunology 19: 270–276.
26. Tsuha Y, Hanada N, Asano T, Abei T, Yamaguchi S, et al. (2004) Role of peptide antigen for induction of inhibitory antibodies to *Streptococcus mutans* in human oral cavity. Clinical and experimental immunology 137: 393–401.
27. Nakao R, Tashiro Y, Nomura N, Kosono S, Ochiai K, et al. (2008) Glycosylation of the OMP85 homolog of *Porphyromonas gingivalis* and its involvement in biofilm formation. Biochemical and biophysical research communications 365: 784–789.
28. Ichinohe T, Watanabe I, Ito S, Fujii H, Moriyama M, et al. (2005) Synthetic double-stranded RNA poly(I:C) combined with mucosal vaccine protects against influenza virus infection. Journal of virology 79: 2910–2919.
29. Ichinohe T, Ainai A, Tashiro M, Sata T, Hasegawa H (2009) PolyI:polyC12U adjuvant-combined intranasal vaccine protects mice against highly pathogenic H5N1 influenza virus variants. Vaccine 27: 6276–6279.
30. Kuehn MJ, Kesty NC (2005) Bacterial outer membrane vesicles and the host-pathogen interaction. Genes & development 19: 2645–2655.
31. Clinical Trials.gov. A service of the U.S. National Institutes of Health Website. Available: http://clinicaltrials.gov/ct2/show/NCT00215800. Accessed 2011 Sep 27.
32. Yamamoto S, Takeda Y, Yamamoto M, Kurazono H, Imaoka K, et al. (1997) Mutants in the ADP-ribosyltransferase cleft of cholera toxin lack diarrheagenicity but retain adjuvanticity. The Journal of experimental medicine 185: 1203–1210.
33. Couch RB (2004) Nasal vaccination, Escherichia coli enterotoxin, and Bell's palsy. The New England journal of medicine 350: 860–861.
34. Alaniz RC, Deatherage BL, Lara JC, Cookson BT (2007) Membrane vesicles are immunogenic facsimiles of Salmonella typhimurium that potently activate dendritic cells, prime B and T cell responses, and stimulate protective immunity in vivo. Journal of immunology 179: 7692–7701.

Roles of Salivary Components in *Streptococcus mutans* Colonization in a New Animal Model Using NOD/SCID.*e2f1*$^{-/-}$ Mice

Tatsuro Ito[1,2], **Takahide Maeda**[1], **Hidenobu Senpuku**[2]*

1 Department of Pediatric Dentistry, Nihon University Graduate School of Dentistry at Matsudo, Chiba, Japan, 2 Department of Bacteriology, National Institute of Infectious Diseases, Tokyo, Japan

Abstract

Streptococcus mutans plays an important role in biofilm formation on the tooth surface and is the primary causative agent of dental caries. The binding of *S. mutans* to the salivary pellicle is of considerable etiologic significance and is important in biofilm development. Recently, we produced NOD/SCID.*e2f1*$^{-/-}$ mice that show hyposalivation, lower salivary antibody, and an extended life span compared to the parent strain: NOD.*e2f1*$^{-/-}$. In this study we used NOD/SCID.*e2f1*$^{-/-}$ 4 or 6 mice to determine the roles of several salivary components in *S. mutans* colonization *in vivo*. *S. mutans* colonization in NOD/SCID.*e2f1*$^{-/-}$ mice was significantly increased when mice were pre-treated with human saliva or commercial salivary components. Interestingly, pre-treatment with secretory IgA (sIgA) at physiological concentrations promoted significant colonization of *S. mutans* compared with sIgA at higher concentrations, or with human saliva or other components. Our data suggest the principal effects of specific sIgA on *S. mutans* occur during *S. mutans* colonization, where the appropriate concentration of specific sIgA may serve as an anti-microbial agent, agglutinin, or an adherence receptor to surface antigens. Further, specific sIgA supported biofilm formation when the mice were supplied 1% sucrose water and a non-sucrose diet. The data suggests that there are multiple effects exerted by sIgA in *S. mutans* colonization, with synergistic effects evident under the condition of sIgA and limited nutrients on colonization in NOD/SCID.*e2f1*$^{-/-}$ mice. This is a new animal model that can be used to assess prevention methods for dental biofilm-dependent diseases such as dental caries.

Citation: Ito T, Maeda T, Senpuku H (2012) Roles of Salivary Components in *Streptococcus mutans* Colonization in a New Animal Model Using NOD/SCID.*e2f1*$^{-/-}$ Mice. PLoS ONE 7(2): e32063. doi:10.1371/journal.pone.0032063

Editor: Bernard Beall, Centers for Disease Control & Prevention, United States of America

Received September 29, 2011; **Accepted** January 20, 2012; **Published** February 21, 2012

Copyright: © 2012 Ito et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was supported in part by Grants-in-Aid from the Scientific Research of the Ministry of Education, Culture, Sports, Science, and Technology of Japan (19791360, 22791822, and 21390506); the Ministry of Health, Labor and Welfare (H19 Iryo-Ippan-007). This work was also supported in part by a Strategic Research Foundation Grant-aided Project for Private Universities from Ministry of Education, Culture, Sports, Science, and Technology in Japan (MEXT), 2008–2012 (S0801032). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: hsenpuku@nih.go.jp

Introduction

Oral streptococci are present in large numbers in dental plaque, which co-interact with the enamel salivary pellicle to form a biofilm on tooth surfaces [1,2]. Streptococcal cell wall components mediate adherence to various salivary receptors [3–6]. The ability of oral streptococci to bind to the salivary pellicle is of considerable etiologic significance in oral disease [4,7]; and is important for biofilm development [8,9]. The glucans synthesized by streptococcal glucosyltransferases convert sucrose into glucan; and provide binding sites through interaction with bacterial cell-associated glucan-binding proteins that promote the accumulation of microorganisms on the tooth surface, and help establish pathogenic biofilms [10,11]. *Streptococcus mutans* plays an important role in biofilm formation on the tooth surface and is a primary causative agent for dental caries [2]. *S. mutans* produces two extracellular glucosyltransferase (Gtfs) that convert sucrose into insoluble glucans [10], where GTF I and GTF SI (water-insoluble glucan) are encoded by *gtfB* and *gtfC*. Animal experiments [12] suggest that the expression of these two *S. mutans gtf* genes is required for maximal virulence in causing dental caries.

It is difficult to extrapolate *in vitro* experimental results to predict the impact of a specific salivary factor in biofilm development. However, the problem facing *in vivo* oral biofilm research is the lack of a natural, reproducible, longitudinal monitoring system permitting the assessment of oral bacterial infection in the same animal throughout the duration of a study. Studies using *S. mutans* infection in animal oral cavities have been performed by feeding the animals powdered Diet 2000 containing unnatural amounts of sucrose (56%). Even when experiments employed feeding a low sucrose content (1 or 5%), longitudinal (more than 2 weeks) feeding with frequent inoculation was performed [13–17]. When these methods were used, *S. mutans* was found to produce a larger amount of insoluble glucan in the oral cavities of mice fed foods containing excess amounts of sucrose. These experiments although interesting do not represent human diet styles.

The mechanical forces of salivary flow and tongue movement tend to dislodge and expel bacteria from tooth surfaces and the oral cavity [18,19]. This controls microbial colonization in the oral cavity as shown with insulin-dependent diabetes mellitus (IDDM), Sjögren's syndrome (SS), and drymouth where these patients suffer from a rapid overgrowth of biofilm and caries that make them highly susceptible to oral infections [20,21]. E2F-1 is a member of

the transcriptional factor controlling the initiation of DNA synthesis [22–24] and subsequent transition of cells from the G0/G1 to S phase in the cell cycle [25,26]. Several recent studies have demonstrated that a mutation of the *e2f1* gene in mice causes enhanced T-lymphocyte proliferation, leading to testicular atrophy, splenomegaly, salivary gland dysplasia, and other types of systemic and organ-specific autoimmunity [27–30]. C57BL/6.*e2f1*$^{-/-}$ mice show high susceptibility to oral streptococci because they do not produce sufficient volumes of saliva and salivary proteins [31]. Further, the combination of E2F-1 deficiency and the NOD gene background induced a rapid progressive development of IDDM and SS compared to NOD mice. This is caused by enhanced auto-reactive Th1-type T cells. NOD.*e2f1*$^{-/-}$ mice do not survive long; therefore they are not suitable for long-term bacterial infection experiments [32]. A recent study using NOD/SCID background E2F-1 deficient mice (NOD/SCID.*e2f1*$^{-/-}$) (T and B cells do not develop to observe E2F-1 function in the NOD background mice without an auto-reactive response) showed E2F-1 may be associated with the differentiation of exocrine cells in the salivary duct [33].

The NOD/SCID.*e2f1*$^{-/-}$ mouse has a decreased saliva volume, lacks sIgA and IgG in the saliva, and has decreased NK cells. This may be a useful mouse for studying oral bacterial infection, colonization, and biofilm formation. These mice have long survival because they do not develop IDDM and SS. Therefore, they may be useful as a model animal for oral bacterial colonization under humanized conditions. Establishment of a humanized experimental system could lead to better understanding of the pathogenic conditions associated with oral bacterial infections and the development of more effective agents for control of bacterial infection associated with oral diseases.

Materials and Methods

Bacterial strains and culture conditions

Streptococcus mutans UA159 was used for colonization study and ELISA. *Actinomyces naeslundii* X600 was used for ELISA as control oral bacteria. All bacteria were grown in an atmosphere of H_2 and CO_2 (GasPack, Becton/Dickinson, Sparks, MD) in Brain Heart Infusion broth (BHI, Difco Laboratory, Detroit, MI) at 37°C.

Animals

NOD/LtJ mice naturally develop IDDM, SS, and dry mouth; and were the parent strain to develop NOD/SCID.*e2f1*$^{-/-}$ mice. They were used as the control to compare *S. mutans* susceptibility to NOD back ground E2F-1$^{-/-}$ mice (NOD.*e2f1*$^{-/-}$) and NOD/SCID back ground E2F-1 heterogeneous (NOD/SCID.*e2f1*$^{+/-}$) and homogeneous deficient NOD/SCID mice (NOD/SCID.*e2f1*$^{-/-}$) [33]. NOD/SCID mice were the parental lines to produce NOD/SCID.*e2f1*$^{-/-}$ mice [33] and were used as control mice in bacterial inoculation experiments. Heterozygous NOD/SCID.*e2f1*$^{+/-}$ mice were bred to generate NOD/SCID.*e2f1*$^{-/-}$ mice. Three types (+/+, +/− and −/− of *e2f1*) of NOD/SCID mice were screened using PCR [33]. All strains were female, 4 months of age and were maintained in accordance with the guidelines for the Care and Use of Laboratory Animals from the National Institute of Infectious Diseases. Experimental protocols (#209125, 210110, and 21124) were approved by the National Institute of Infectious Diseases Animal Resource Committee.

Human saliva collection

Saliva samples were collected from volunteers with good oral health, after stimulation by chewing paraffin gum. The volunteers refrained from eating, drinking, and brushing for at least 2 h prior to collection. The saliva was placed into ice-chilled sterile bottles for 5 min; then centrifuged at 10,000 g for 10 min to remove cellular debris. For the inoculation assay and the enzyme-linked immunosorbent assay (ELISA), the clarified saliva was used after filter sterilization through a 0.22 µm Acrodisc filter (Pall Corporation, Ann Arbor, MI). After filtration, they were pooled and stored at −20°C until used.

Preparation of immunoglobulin, amylase, and mucin

Lyophilized secretory Immunoglobulin A (sIgA) from human colostrum, α-amylase from human saliva, and mucin from bovine submaxillary glands (Sigma-Aldrich, St. Louis, MO) were mixed in PBS and adjusted to similar physiological concentrations as in human-saliva: 0.25, 0.4 and 2.7 mg/ml, respectively. These reagents were stored at −20°C until used.

Bacterial sampling and colony-forming unit (CFU) estimate

Bacterial inoculation, sampling and CFU estimates were performed using procedures and conditions described previously [31,34,35]. All oral streptococci were cultured in BHI broth overnight and then washed twice with sterile phosphate-buffered saline (PBS). Our previous study demonstrated that colony counts of *S. mutans* were significantly higher than that of other streptococci (i.e. *S. sanguis*, *S. sobrinus*, and *S. salivarius*) in mice that ingested 1% sucrose in water one day before inoculation [31]. Thus, mice were given drinking water containing 1% sucrose (less than the usual concentration in juice) one day prior to *S. mutans* inoculation to reproduce the early adherence of *S. mutans* in conditions resembling a natural state. Chlorhexidine (0.2%) soaked sterile cotton swabs were used to disinfect the oral cavities of the mice including the maxillary incisor teeth. The cavity was immediately washed with sterile PBS. Four or 6 mice were treated with 100 µl of human saliva or salivary components for 2.5 min with the aid of micropipette. Casein was used as a control as a non-salivary component for the treatment. Five min after treatment, mice were washed with 100 µl of PBS. *S. mutans* solutions were introduced to the oral cavities of all females at 4 months of age at a final concentration of 7×10^9 CFU in 250 µl of PBS during 2.5 min. Mice were separated into four groups based on the feeding conditions 24 h after inoculation. During the 24 h, one group was fed food with distilled water compared to another fed food with 1% sucrose-water; and the other set was food-deprived with 1% sucrose water or distilled water. Following inoculation, samples were collected from the labial surfaces of the maxillary incisor teeth with a sterile cotton ball and then dipped in 2 ml of PBS. To evaluate NOD/SCID.*e2f1*$^{-/-}$ mice as compared with previous results and to obtain stable data, samples collected from incisors were tested as parameters used in previous studies [31,35]. The samples in sterile PBS were sonicated using ultrasonic dispersion (power output, 60 W) for 10 s, and then poured onto Mitis-Salivarius agar plates containing 0.02 M bacitracin (MSB). CFUs were determined by counting rough-surface colonies on MSB plates after 48 h using anaerobic incubation at 37°C.

ELISA

To determine if sIgA reacts with *S. mutans* in vitro and if sIgA is absorbed on the tooth surface after treatment with human saliva, ELISA was performed with some modifications as described previously [33]. 96-well microtiter H-plates (Sumitomo Bakelite, Tokyo, Japan) were coated overnight at 4°C with a culture of *S. mutans* or *A. naeslundii* (1 µl/ml) in Na_2CO_3 coating buffer at pH 9.6 and incubated at 4°C overnight. In the sandwich assay to

detect absorbed sIgA, we used 1/1,000 mouse anti-human immunoglobulin A antibody (Sigma-Aldrich, St. Louis, MO). The bacteria and antibody were diluted in Na_2CO_3 coating buffer at pH 9.6 and incubated at 4°C overnight. The plates were washed with PBS containing 0.1% (v/v) Tween 20 (PBST); and blocked with 1% (wt/vol) skim milk in PBST for 1 h at 37°C. Excess skim milk was removed by washing three times with PBST. To determine the presence of sIgA on the tooth surface, the tooth surface was swabbed using a sterile cotton ball after treatment with human saliva, and the swabbed ball was soaked in 2 ml coating buffer and shaken for 1 min. A 100 µl aliquot of 0.25 mg/ml sIgA, human saliva, or the soaked sample was added to the wells and the plates were incubated for 1 h at 37°C. The wells were washed three times with PBST; and further incubated for 1 h at 37°C with 100 µl 1/1,000 alkaline phosphatase conjugated goat anti-human immunoglobulin A antibodies (Zymed Laboratories, South San Francisco, CA). After three washings with PBST, the bound antibodies were detected after the addition of 50 µl of 3 mg/ml para-nitrophenyl phosphate as a substrate and incubated for 30 min at 37°C. Absorbance at 405 nm (A_{405}) was measured using a microplate reader (Multiskan Bichromatic; Laboratory Japan, Tokyo, Japan). The mean value for each sample was used to calculate the ELISA value: $Abs_{405} \times 100/t$ (t: time of reaction). Triplicate measurements were performed and means calculated with standard error.

Removal of *S. mutans*-specific sIgA

To determine if specific sIgA is employed for *S. mutans* colonization on the tooth surface, an absorption procedure was performed to remove specific antibody against *S. mutans*. Solutions of 1 mg/ml sIgA in PBS were absorbed with 0.5 mg (dry weight)/ml whole cells of lyophilized *S. mutans* UA159 at 37°C for 1 h and then overnight at 4°C. The mixture was centrifuged at 8,000 rpm for 10 min to remove *S. mutans*-IgA complex. Protein concentrations in the sIgA sample were measured using the Bio-Rad Protein Assay kit (Bio-Rad Laboratory, Hercules, CA) based on the method of Bradford and measured at 595 nm. The concentration of sIgA was adjusted to 0.25 mg/ml after the absorption procedure.

Inhibiting effects of FruA in biofilm formation with *S. mutans*

To determine if the animal model could be used for the analysis of inhibitors for colonization and biofilm formation of *S. mutans* on the tooth surface, fructanase (FruA), a candidate inhibitor for biofilm formation of *S. mutans* [36], was used in the *in vivo* assay. The inhibiting activity of FruA at 1.25 units/ml was assayed in 96 well microtiter plates coated with human saliva [36]. FruA at 1.25 units/ml was also added within a 1% sucrose solution in drinking water (DW). FruA does not digest sucrose at 20~25°C in 1% sucrose drinking water and does at 37°C in the oral cavity after mice drink the water [36]. After pre-treatment of sIgA following bacterial inoculation, all NOD/SCID.*e2f1*$^{-/-}$ mice were fed and supplied 1% sucrose water containing or not containing FruA. After 24 h inoculation, samples were collected and the CFU was counted as described above.

Statistical analyses

The CFU and ELISA data were expressed as means ± standard deviations. GraphPad Prism version 5.0 d for Mac OS X (GraphPad Software, San Diego, CA) was used to perform tests of significance. The statistical significance of differences between two groups was determined using the unpaired *t*-test. For comparison between multiple groups, one-way analysis of variance (ANOVA) and Tukey-Kramer tests were used. P-values less than 0.001, 0.01 or 0.05 were considered statistically significant using two-tailed comparisons. All experiments were repeated and analyzed independently.

Results

Colonization of *S. mutans* in mice treated with human saliva

Human saliva is thought to play a significant role in the attachment of *S. mutans* to the tooth surface. We evaluated human saliva in bacterial colonization of NOD/SCID wild type, NOD/SCID.*e2f1*$^{+/-}$ mice, and NOD/SCID.*e2f1*$^{-/-}$ mice. *S. mutans* colonization in each mouse was significantly increased at all time points after the inoculation when they were treated with human saliva (Fig. 1 A, B and C). Bacterial numbers on the tooth surfaces were significantly higher in NOD/SCID.*e2f1*$^{-/-}$ mice than those in NOD/SCID wild type or NOD/SCID.*e2f1*$^{+/-}$ mice after 90 and 120–180 min post inoculation (Fig. 1 D). Colony numbers of *S. mutans* gradually decreased from 30 min to 90 min; however, after the colonization phase, the CFU gradually increased from 90 to 180 min in human saliva-treated NOD/SCID.*e2f1*$^{-/-}$ mice; whereas the other mice did not show a difference comparing time points.

Effects of human saliva and salivary components in *S. mutans* colonization

To determine if salivary components induce colonization of *S. mutans* on the tooth surface using the *in vivo* model, α-amylase, mucin and sIgA, receptors for *S. mutans* adhesins, were used to treat teeth before bacterial inoculation. CFUs were lower within non-treated mice compared to NOD/SCID.*e2f1*$^{+/-}$ and $^{-/-}$ 18 mice treated with all components other than casein treatment (control; non-salivary component) in NOD/SCID.*e2f1*$^{+/+}$ mice (data not shown). NOD/SCID.*e2f1*$^{-/-}$ mice had a higher colonization than NOD/SCID.*e2f1*$^{+/-}$ and NOD/SCID.*e2f1*$^{+/+}$ in each pre-treatment using the salivary components (Fig. 2 A, B and C). Bacterial colonization on teeth treated with 0.25 mg/ml sIgA at physiological concentrations was increased significantly in NOD/SCID.*e2f1*$^{-/-}$ mice (13,992±6,423); however, there was no significant difference in treating with saliva compared to sIgA (Fig. 2 C). In NOD/SCID.*e2f1*$^{+/+}$ and NOD/SCID.*e2f1*$^{+/-}$ mice, treatment with 0.25 mg/ml sIgA did not show greater colonization (Fig. 2 A, B). Further, higher concentrations of sIgA (0.4 mg/ml) did not result in higher colonization by *S. mutans* in comparison with BSA and casein in NOD/SCID.*e2f1*$^{+/-}$ and NOD/SCID.*e2f1*$^{-/-}$ mice (Fig. 2 B and C). Treatment in NOD/SCID.*e2f1*$^{-/-}$ mice with mucin (at 0.4 and 2.7 mg/ml) or with BSA did not result in increased levels of *S. mutans* colonization; these pre-treatments yielded significantly lower CFU counts compared to treatment with 0.25 mg/ml sIgA and considerably higher counts compared to treatment with 0.4 mg/ml amylase. Treatment with amylase at 0.1 mg/ml showed significantly higher colonization than at 0.4 mg/ml in NOD/SCID.*e2f1*$^{+/-}$; whereas there was no significant difference using NOD/SCID.*e2f1*$^{-/-}$ mice.

SIgA was taken from human colostrum, and therefore may include various antibodies to pathogens. To confirm whether sIgA reacts with *S. mutans*, ELISA was performed using *S. mutans*-coated 96 well microtiter plates. *A. naeslunidii* was also used for coating as another oral bacterium. 0.25 mg/ml sIgA reacted strongly with *S. mutans* but not *A. naeslundii* (Fig. 3 A). The specificity of sIgA was observed by absorption of specific antibody to *S. mutans* in pre-incubation using *S. mutans* whole cells within sIgA. The absorbed sIgA was used for the ELISA assay and showed no significant

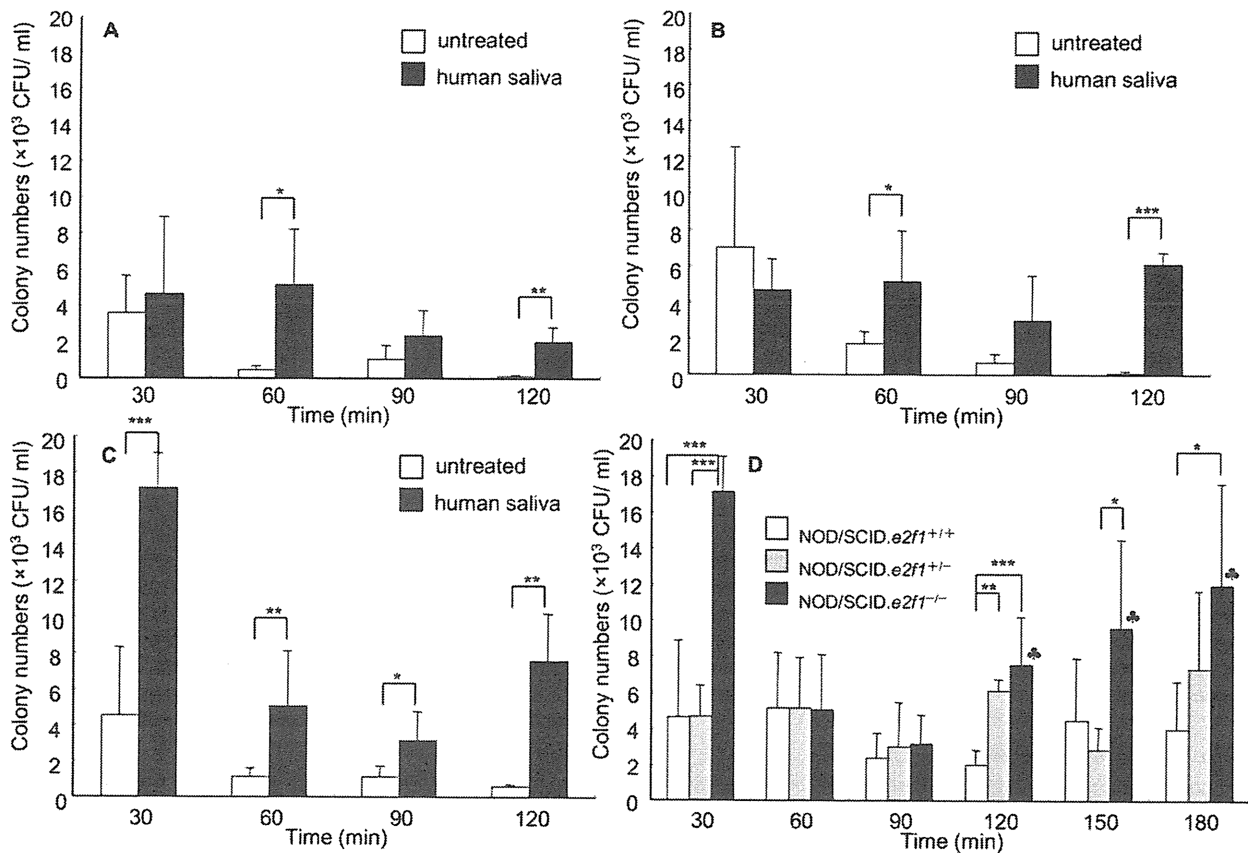


Figure 1. Colonization of *S. mutans* in human-saliva treated mice. Colony numbers of *S. mutans* in (A) NOD/SCID wild type, (B) NOD/SCID.*e2f1*$^{+/-}$, (C) NOD/SCID.*e2f1*$^{-/-}$ female mice, and 4 months of age pre-treated with and without human saliva prior to bacterial inoculation. Asterisks show significant differences (vs. untreated group, *$P<0.05$, ** $P<0.01$, *** $P<0.001$). (D) Time-course analysis of *S. mutans* colonization for each mouse strain pre-treated with human saliva prior to bacterial inoculation. Data were obtained from three independent experiments with 4 mice from each strain, and values are expressed as the means ± standard daviations (SDs) of the data (*$P<0.05$, ** $P<0.01$, *** $P<0.001$, represents significant differences vs. 90 min, $P<0.05$).
doi:10.1371/journal.pone.0032063.g001

reactivity to *S. mutans* (Fig. 3 A). Using human saliva, specific antibody to *S. mutans* was also observed using the ELISA assay (Fig. 3 A). The 0.25 mg/ml absorbed sIgA was used for the colonization assay in NOD/SCID wild type, NOD/SCID.*e2f1*$^{+/-}$ and NOD/SCID.*e2f1*$^{-/-}$ mice, and the effect of absorbed sIgA was compared with 0.25 mg/ml non-absorbed sIgA in all mice. The absorbed sIgA did not increase colonization of *S. mutans* in comparison with non-absorbed sIgA at 120 min after inoculation of *S. mutans* (Fig. 3 B). Therefore, increased colonization of *S. mutans* was dependent on specific antibody to *S. mutans* in sIgA and human saliva using this animal model. To determine whether sIgA remained on the tooth surface after treatment with human saliva, the surface was swabbed using a sterilized cotton ball at 120 min after the treatment in mice; and sIgA in the swabbed sample was measured using ELISA. The level of human-IgA that remained on the teeth for 120 min was significantly higher in NOD/SCID.*e2f1*$^{-/-}$ mice as compared to the other two strains (Fig. 3 C). This shows that specific sIgA antibody to *S. mutans* remains on the tooth surface after treatments with sIgA and human saliva in mice having decreased saliva and lack of IgA and IgG, the NOD/SCID.*e2f1*$^{-/-}$ mice. To determine whether a lack of IgA, by inserting the SCID type in NOD.*e2f1*$^{-/-}$ mice, promoted the colonization of *S. mutans*, the parent strain (NOD.*e2f1*$^{-/-}$ mice) and previous the parent strain (NOD mice) to NOD.*e2f1*$^{-/-}$ mice were used for the colonization assay after pre-treatment with 0.25 mg/ml sIgA and compared with NOD/SCID.*e2f1*$^{-/-}$ mice. We found that the colonization at 120 min after inoculation was significantly lower in NOD and NOD.*e2f1*$^{-/-}$ mice than NOD/SCID.*e2f1*$^{-/-}$ mice (Fig. 3 D). Therefore, lack of IgA and decreased saliva allowed specific IgA to remain on the tooth surface and to promote colonization of *S. mutans* in NOD/SCID.*e2f1*$^{-/-}$ mice.

Synergistic effects of sucrose water and diet, and human saliva on *S. mutans* long-term colonization

Long-term colonization is necessary in a mouse model to study several agents for the prevention to oral diseases. We observed that after inoculation, the colonization of *S. mutans* was slight at 24 hours in NOD/SCID, NOD/SCID.*e2f1* $^{+/-}$ and NOD/SCID.*e2f1*$^{-/-}$ mice pre-treated with human saliva (Fig. 4 A). Drinking water and diet including sucrose helped biofilm formation in other studies [14,31]. A low concentration of 1% sucrose water was selected and supplied as drinking water with the usual animal diet for mice to establish an animal model that avoided high sucrose concentration-dependent colonization. The significant colonization was not observed in only the 1% sucrose water group as compared to that in non-sucrose water and non-diet group (Fig. 4 A, B). However, the group supplied with the combination of 1% sucrose-water and diet showed the most CFU/ml of *S. mutans*; colony numbers in NOD/SCID.*e2f1*$^{-/-}$

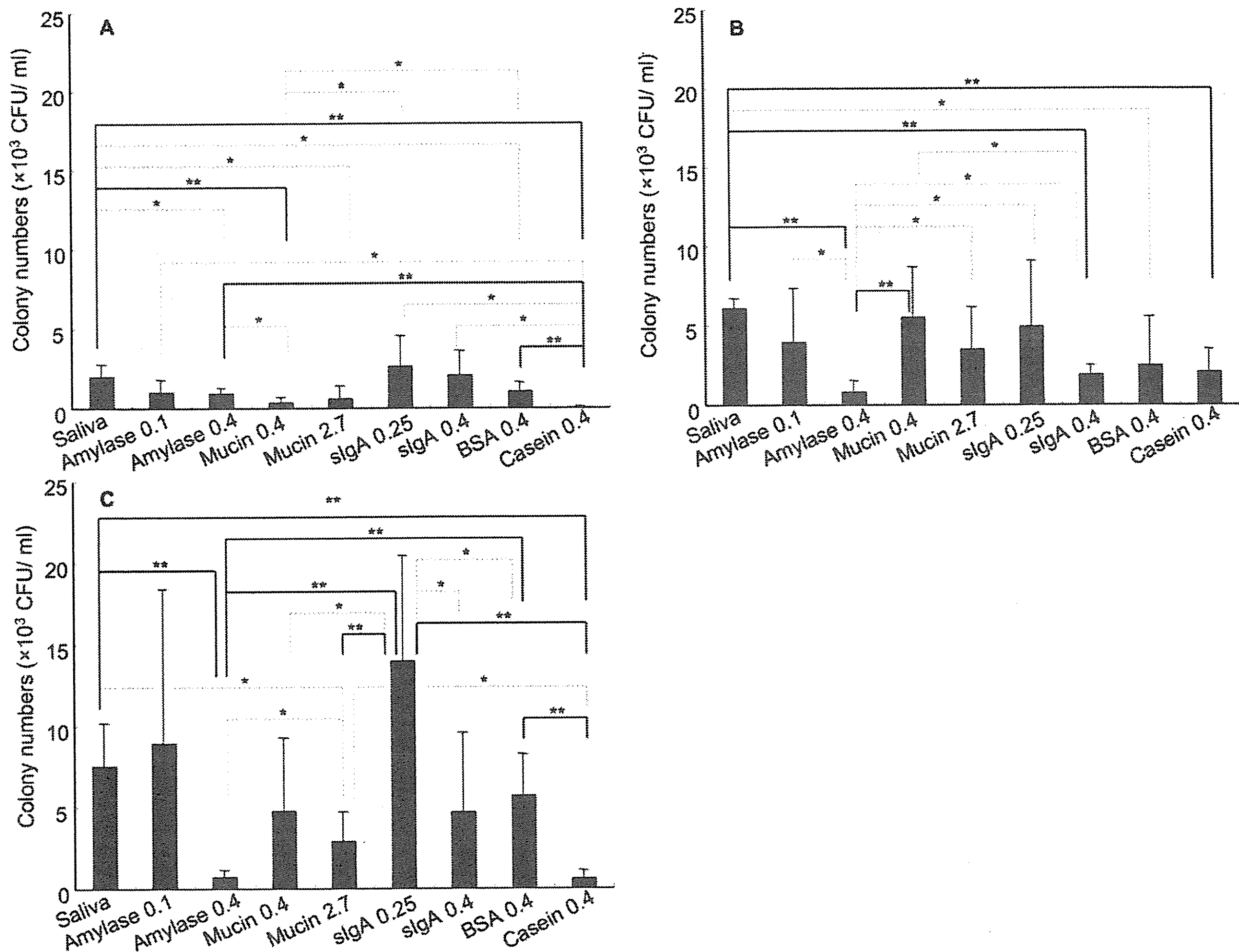


Figure 2. Effects of human saliva and salivary components in *S. mutans* colonization. Colony numbers of *S. mutans* in (A) NOD/SCID wild type, (B) NOD/SCID.*e2f1*$^{+/-}$, (C) NOD/SCID.*e2f1*$^{-/-}$ female mice, 4 months of age, at 120 min after inoculation. All mice were pre-treated with human saliva or salivary components prior to bacterial inoculation. Data are expressed as the means ± SDs of the results for 6 mice per strain (*$P<0.05$, ** $P<0.01$).
doi:10.1371/journal.pone.0032063.g002

(693±500 CFU/ml) and in NOD/SCID.*e2f1*$^{+/-}$ (193±190) were significantly higher than those in wild type mice (17±32) (Fig. 4 D). The colonization was significantly higher in 1% sucrose-water and diet than non-sucrose water and diet in NOD/SCID.*e2f1*$^{-/-}$ mice (Fig. 4 C, D).

Inhibition effects by FruA on colonization of *S. mutans* in vivo

In our previous report, purified and commercial fructanase (FruA) from *Aspergillus niger* completely inhibited *S. mutans* GS-5 biofilm formation on saliva-coated polystyrene and hydroxyapatite surfaces [36]. Therefore, we examined inhibition using FruA in *S. mutans* colonization in the established mouse model system. The bacterial load in NOD/SCID.*e2f1*$^{-/-}$ mice pre-treated with sIgA and supplied sucrose-water containing FruA (13±20 CFU/ml) decreased as compared to that without FruA (104±159); however, there was no significant difference ($P=0.088$).

Discussion

In this study we demonstrated homozygous E2F-1-deficient NOD/SCID (NOD/SCID.*e2f1*$^{-/-}$) mice are highly susceptible to *S. mutans* colonization when NOD/SCID.*e2f1*$^{-/-}$ mice are pre-treated with human saliva or sIgA using a low concentration (1%) sucrose supplement (Fig. 1 D, Fig. 4 D). The colonization levels were remarkably higher in NOD/SCID.*e2f1*$^{-/-}$ mice than other mouse strains including commercial strains: C57BL/6, B10.D2 and NOD mice [31,37]. The high *S. mutans* susceptibility in NOD/SCID.*e2f1*$^{-/-}$ mice may be explained because of impaired salivary clearance. The systemic dysfunction of the salivary gland (e.g., enlarged nuclear size, increased numbers of ducts) caused by the E2F-1 deficiency is the principal reason for the decrease of saliva volume in the mice [33]. Previously we showed that the percent inhibition of saliva production volume (µl/100 g BW) in NOD/SCID.*e2f1*$^{-/-}$ mice was higher than that in other NOD-background mice [33,35,37].

Salivary component molecules that agglutinate bacteria include sIgA, mucins, parotid agglutinin, lysozyme, β_2-microglobulin, and Ca^{2+} ions [38]. Some reports suggest that salivary components may promote colonization of certain strains of bacteria [8,39]. Here we show the positive and negative effects of exogenous human salivary components in *S. mutans* colonization on the tooth surface. In particular, 0.25 mg/ml sIgA promoted colonization of *S. mutans* as compared to mucin, α-amylase, and others. SIgA is the

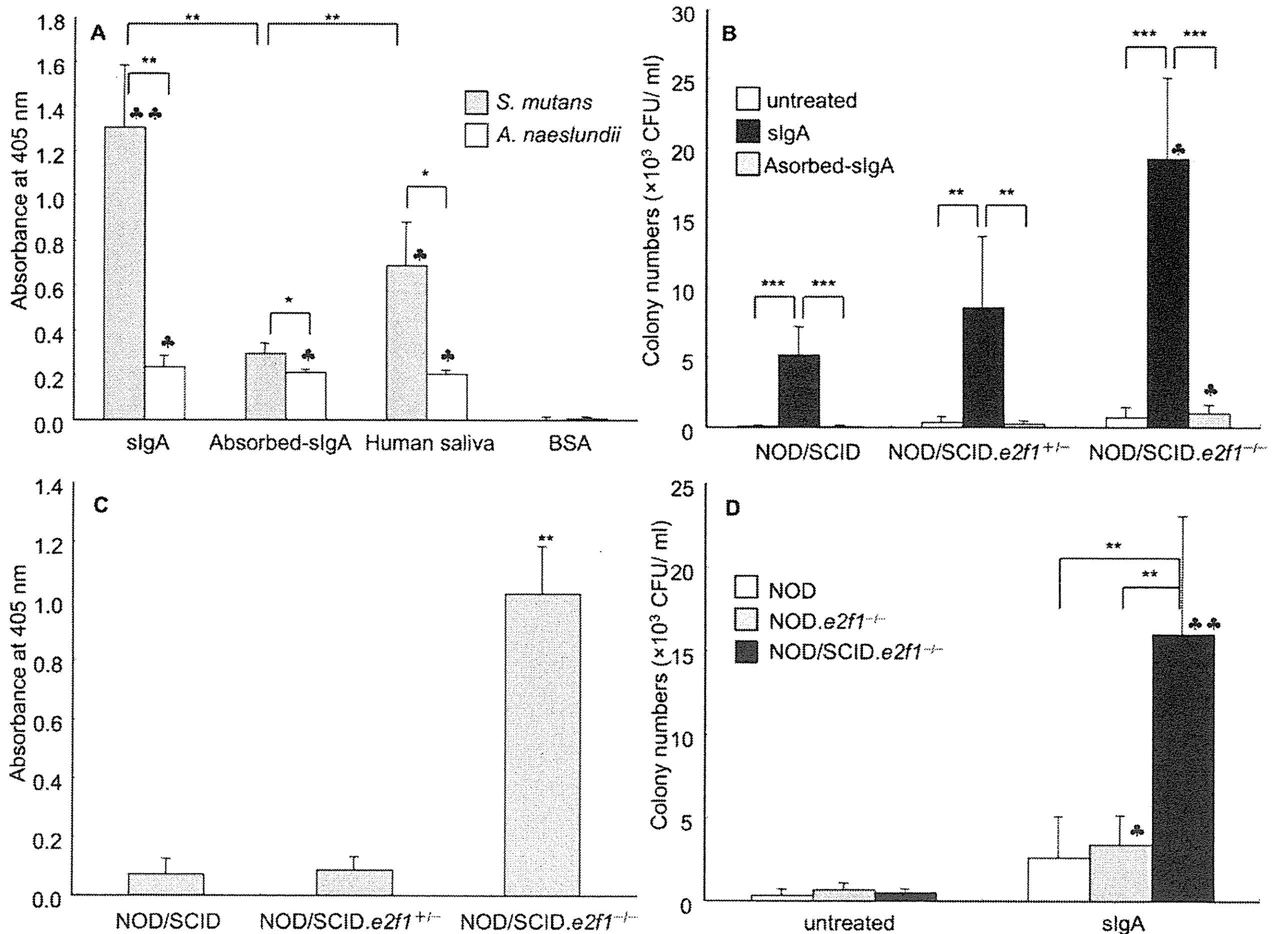


Figure 3. Effect of sIgA in *S. mutans* colonization. (A) Measurement of sIgA, absorbed sIgA and human saliva with *S. mutans*. BSA was the control. The ELISA results are expressed as the mean ± SD of absorbance obtained in three independent experiments. (Significant differences vs BSA, $P<0.01$, $P<0.001$). (B) Colonization assay on the tooth surface from NOD/SCID.*e2f1*$^{-/-}$ mice pre-treated with sIgA and absorbed sIgA at 120 min after inoculation. Untreated mice were controls. The results are expressed as the mean ± SD of absorbance obtained in six mice independent experiments. (Significant differences vs NOD/SCID, $P<0.05$). (C) Confirmation of residual sIgA on the tooth surface using ELISA, at 120 min after treatment. The ELISA results are expressed as the mean ± SD of absorbance obtained in three independent experiments. (Significant differences vs NOD/SCID and NOD/SCID.*e2f1*$^{+/-}$, $P<0.05$). (D) Evaluation of antibody deficiency and decreased saliva in the colonization assay, at 120 min after inoculation. The pre-treatment of sIgA was compared with the untreated group. Data are expressed as the means ± SDs of the results for 6 mice per strain (Significant differences vs untreated group, $P<0.01$, $P<0.001$).
doi:10.1371/journal.pone.0032063.g003

predominant immunoglobulin found in all mucosal secretions including saliva. In general, sIgA is thought to participate in the local disposal of environmental antigens in the oral cavity [38]. Indeed, the inhibitory effects of sIgA against bacterial biofilm formation are well demonstrated [40,41]. However, conversely in this study, sIgA played a role in aiding the colonization of *S. mutans* onto the tooth surface.

Physiological concentrations of amylase, mucin, and sIgA in human saliva are 0.4, 2.7 and 0.25 mg/ml respectively. Amylase at 0.4 mg/ml and 2.7 mg/ml mucin showed significantly lower colonization by *S. mutans* than 0.25 mg/ml sIgA, which showed higher colonization than human saliva treatment in NOD/SCID.*e2f1*$^{-/-}$ mice (Fig. 2). We considered that sIgA supported the attachment because specific sIgA against *S. mutans* was associated with the colonization. The activities of human saliva for colonization show dependency on specific sIgA (Fig. 3). In contrast, higher concentrations (0.4 mg/ml) of sIgA than physiological concentrations showed inhibiting activities as compared to physiological concentrations. The negative effects are also indicated by the effects of specific sIgA antibody on attachment. Therefore, multiple effects of specific sIgA may be dependent on sIgA concentration. The antibody titer to surface protein antigen from *S. mutans* was negatively correlated with the numbers of *S. mutans* in saliva from humans and mice [40,42,43]. The concentration of absorbed sIgA may be an important step for the colonization of *S. mutans* on the tooth surface and regulates the microbial flora in the oral cavity. Hapfelmeier *et al.* recently report reversible microbial colonization in germ-free mice during a dynamic IgA immune response [44]. They indicated the intestinal IgA system lacks classical immune memory characteristics; the intestinal IgA repertoire is characterized by constant attrition and thus represents the dominant species currently present in the intestine. In the oral cavity, a similar function of IgA production to intestinal IgA may cause and control commensal microbial flora. Our findings also show the dynamics of sIgA immune response, and sIgA may function to equalize the bacterial numbers in the oral cavity for the continuous presence of commensal oral bacteria.

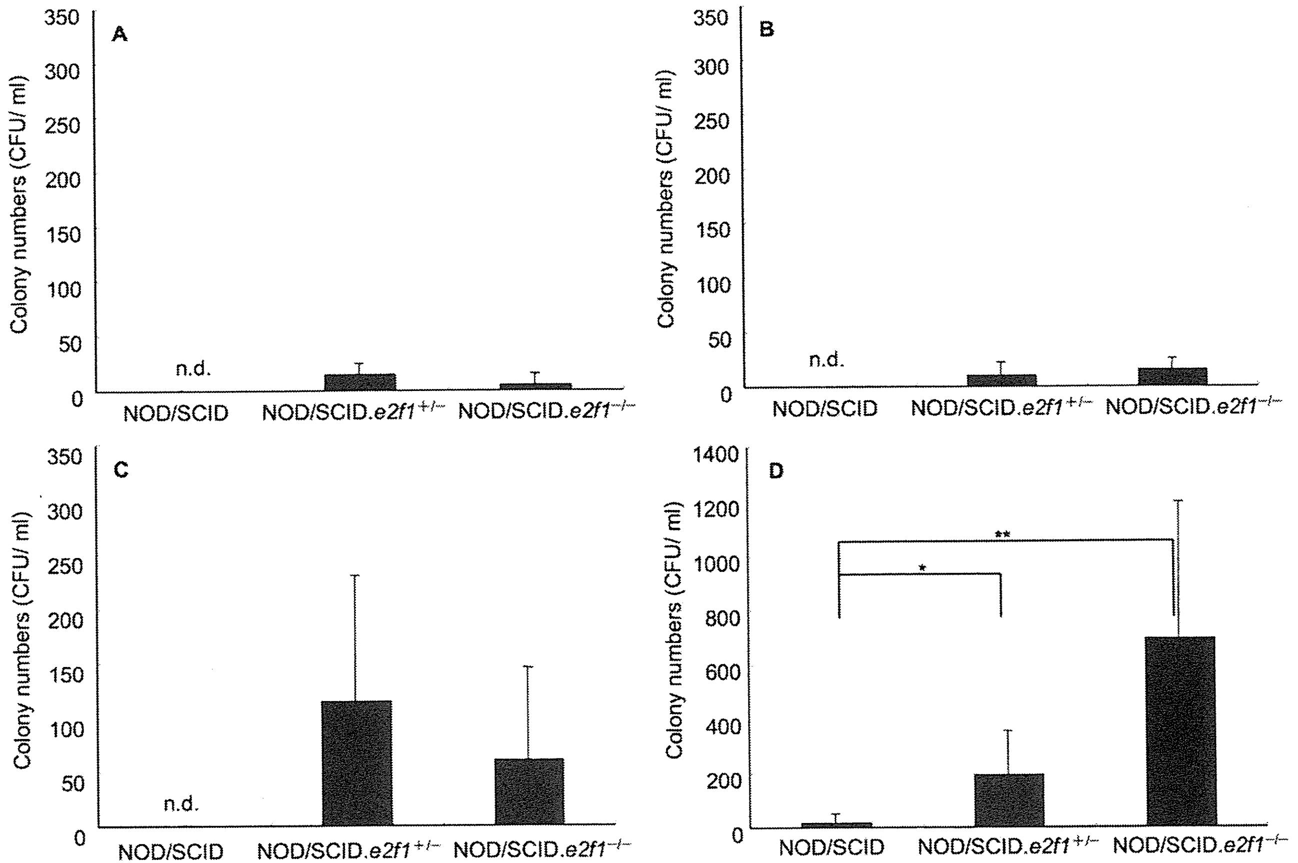


Figure 4. Comparison of different diet groups in *S. mutans* colonization. (A) group was not supplied with water or diet; (B) group was supplied with only 1% sucrose water; (C) group was supplied with both water and diet; (D) group was supplied with both 1% sucrose-containing water and diet. Samples were collected at 24 h after inoculation in NOD/SCID, NOD/SCID.*e2f1*$^{+/-}$ and NOD/SCID.*e2f1*$^{-/-}$ mice pre-treated with human saliva. Data are expressed as the means ± SDs of the results for 6 mice per strain (*$P<0.05$, ** $P<0.01$).
doi:10.1371/journal.pone.0032063.g004

Our *in vivo* colonization mouse system has a number of advantages to study specific sIgA effects because sIgA was absorbed on the tooth surface after exposure of sIgA to NOD/SCID.*e2f1*$^{-/-}$ mice. In our previous report, the production of protein per minute in 1 µl of saliva was significantly lower in NOD/SCID.*e2f1*$^{-/-}$ mice as compared to NOD/SCID mice [33]. NOD/SCID.*e2f1*$^{-/-}$ mice lack mature immunoglobulins due to severe combined immunodeficiency in NOD.*e2f1*$^{-/-}$ mice and a decreased volume of saliva as compared to both parent strains; NOD and NOD/SCID mice [33]. Therefore, sIgA was easily absorbed without competition with mouse IgA, and by decreasing the supply of proteins and poor salivary clearance on the tooth surface in NOD/SCID.*e2f1*$^{-/-}$ mice as compared to NOD.*e2f1*$^{-/-}$ mice [32] and NOD/SCID mice (Fig. 3 C). Further, absorbed specific sIgA against *S. mutans* was responsible for the colonization of *S. mutans* (Fig. 3 D). We show sIgA from human colostrum included sIgA against various microorganisms including *S. mutans*. Therefore, exposure of specific sIgA in the oral cavity may induce the first colonization and initial attachment of bacteria.

The effect of specific sIgA did not persist with the colonization over a long-term and as a result showed small numbers of *S. mutans* at 24 hours after inoculation, enough time to construct the biofilm on the tooth surface. SIgA supports attachment of *S. mutans*, but its effect was limited in the natural condition exposed with commensal bacteria and saliva in the oral cavity. Therefore, the sucrose water and diet were given as nutrients for *S. mutans* biofilm formation. Using 1% sucrose water and the usual mouse diet after inoculation supported long-term colonization in NOD/SCID.*e2f1*$^{-/-}$ and NOD/SCID.*e2f1*$^{+/-}$ mice in comparison to NOD/SCID mice (Fig. 4 D). We demonstrated that a concentration of 1% sucrose in drinking water with non-sucrose diet could induce significant colonization at 24 hours after inoculation. This shows the solid diet without sucrose enhanced colonization in combination with 1% sucrose drinking water (Fig. 4 B and D). The diet contains a few other carbohydrates, and carbohydrates in food debris or sucrose involved in debris absorbed with sucrose water after eating the diet and drinking for 24 hours may be employed in the production of the biofilm matrix. This was not observed previously using animal models for *S. mutans* infections. Possibly this biofilm formation closely resembles the natural environment of the oral cavity when humans consume various foods. In previous reports, conditions were dependent on excessive insoluble glucan formation in high sucrose water [45–48]. Their data showed rapid insoluble glucan formation and they likely generated these extreme effects under the high-sucrose experimental conditions favorable for production of biofilm. Humans eat a variety of foods, but they consciously control the oral condition to maintain oral health and view control of the intake of sucrose as very important. Therefore, we propose that the mouse model system observed here is more representative of the normal human oral environment; and better than previous model systems utilized for demineralization studies.

If this animal model system is used for assessment of various preventive dental caries agents, new preventative materials may be developed. Recently we reported fructanase (FruA) from *Streptococcus salivarius* and *Aspergillus niger* as a preventative. FruA can digest sucrose and prevent colonization [36,49]. In this animal model, experiments using FruA in the mice supplied with 1% sucrose drinking water and diet at 24 hours after the inoculation, FruA inhibited the colonization by *S. mutans*; however, there were no significant differences ($p = 0.088$). It was considered that the animal model system may be useful in assessment of inhibiting agents recognized *in vitro*. However, the present system requires modifications to develop models for various oral infectious diseases as well as for dental caries. Our future studies will use this animal model to find inhibitory agents for infection by biofilm bacteria using the interaction of saliva, nutrients, and bacteria.

Acknowledgments

We thank Naoki Narisawa, Norihiko Kanaguchi, Xi Zhang, Yousuke Kinoshita, and Ryoma Nakao for their technical support, helpful discussions, and advice.

Author Contributions

Conceived and designed the experiments: HS TI TM. Performed the experiments: TI HS. Analyzed the data: TI TM. Contributed reagents/materials/analysis tools: HS TI. Wrote the paper: TI HS.

References

1. Hamada S, Slade HD (1980) Biology, immunology and cariogenicity of *Streptococcus mutans*. Microbiol Rev 44: 331–384.
2. Loesche WJ (1986) Role of *Streptococcus mutans* in human dental decay. Microbiol Rev 50: 353–380.
3. Demuth DR, Golub EE, Malamud D (1990) Streptococcal-host interactions. Structural and functional analysis of a *Streptococcus sanguis* receptor for a human salivary glycoprotein. J Biol Chem 265: 7120–7126.
4. Rudney JD, Ji Z, Larson CJ, Liljemark WF, Hickey KL (1995) Saliva protein binding to layers of oral streptococci in vitro and in vivo. J Dent Res 74: 1280–1288.
5. Rudney JD, Hickey KL, Ji Z (1999) Cumulative correlations of lysozyme, lactoferrin, peroxidase, S-IgA, amylase, and total protein concentrations with adherence of oral viridans streptococci to microplates coated with human saliva. J Dent Res 78: 759–768.
6. Russell MW, Mansson-Rahemtulla B (1989) Interaction between surface protein antigens of *Streptococcus mutans* and human salivary components. Oral Microbiol Immunol 4: 106–111.
7. Rudney JD, Ji Z, Larson CJ (1996) Saliva protein binding to streptococcal layers placed at different oral sites in 48 persons. J Dent Res 75: 1789–1797.
8. Lamont RJ, Demuth DR, Davis CA, Malamud D, Rosan B (1991) Salivary-agglutinin-mediated adherence of *Streptococcus mutans* to early plaque bacteria. Infect Immun 59: 3446–3450.
9. Liljemark WF, Gibbons RJ (1972) Proportional distribution and relative adherence of *Streptococcus miteor (mitis)* on various surfaces in the human oral cavity. Infect Immun 6: 852–859.
10. Kuramitsu HK (1993) Virulence factors of mutans streptococci: role of molecular genetics. Crit Rev Oral Biol Med 4: 159–176.
11. Kopec LK, Vacca-Smith AM, Wunder D, Ng-Evans L, Bowen WH (2002) Influence of antibody on the structure of glucans. Caries Res 36: 108–115.
12. Yamashita Y, Bowen WH, Burne RA, Kuramitsu HK (1993) Role of the *streptococcus mutans gtf* genes in caries induction in the specific-pathogen-free rat model. Infect Immun 61: 3811–3817.
13. Jespersgaard C, Hajishengallis G, Huang Y, Russell MW, Smith DJ, et al. (1999) Protective immunity against *Streptococcus mutans* infection in mice after intranasal immunization with the glucanbinding region of *S. mutans* glucosyltransferase. Infect Immun 67: 6543–6549.
14. Suzuki N, Kurihara Y, Kurihara Y (1998) Dental caries susceptibility in mice is closely linked to the H-2 region on chromosome 17. Caries Res 32: 262–265.
15. Zhang P, Jespersgaard C, Lamberty-Mallory L, Katz J, Huang Y, et al. (2002) Enhanced immunogenicity of a genetic chimeric protein consisting of two virulence antigens of *Streptococcus mutans* and protection against infection. Infect Immun 70: 6779–6787.
16. Keyes PH, Jordan HV (1964) Periodontal lesions in the Syrian hamster. III. Findings related to an infectious and transmissible component. Arch Oral Biol 9: 377–400.
17. Michalek SM, McGhee JR, Shiota T, Devenyns D (1977) Virulence of *Streptococcus mutans*: Cariogenicity of *S. mutans* in adult gnotobiotic rats. Infect Immun 15: 466–471.
18. Bergdahl M, Bergdahl J (2000) Low unstimulated salivary flow and subjective oral dryness. association with medication, anxiety, depression, and stress. J Dent Res 79: 1652–1658.
19. Dawes C (2008) Salivary flow patterns and the health of hard and soft oral tissues. J Am Dent Assoc 139: 18S–24S.
20. Almstahl A, Kroneld U, Tarkowski A, Wikstrom M (1999) Oral microbial flora in Sjögren's syndrome. J Rheumatol 26: 10–4.
21. Almstahl A, Wikstrom M (1999) Oral microflora in subjects with reduced salivary secretion. J Dent Res 78: 1410–1416.
22. Helin K, Lees JA, Vidal M, Dyson N, Harlow E, et al. (1992) A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. Cell 70: 337–350.
23. Kaelin WG, Jr., Krek W, Sellers WR, DeCaprio JA, Ajchenbaum F, et al. (1992) Expression cloning of a cDNA encoding a retinoblstoma-binding protein with E2F-like properties. Cell 70: 351–364.
24. Shan B, Lee WH (1994) Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. Mol Cel Biol 14: 8166–8173.
25. Nevins JR (1992) E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science 258: 424–429.
26. Sørensen TS, Girling R, Lee CW, Gannon J, Bandara LR, et al. (1996) Functional interaction between DP-1 and p53. Mol Cel Biol 16: 5888–5895.
27. Field SJ, Tsai FY, Kuo F, Zubiaga AM, Kaelin WG, Jr., et al. (1996) E2F-1 functions in mice to promote apoptosis and suppress proliferation. Cell 85: 549–561.
28. Lillibridge CD, O'Connell BC (1997) In human salivary gland cells, over expression of E2F1 overcomes an interferon-γ-and tumor necrosis factor-α-induced growth arrest but does not result in complete mitosis. J Cell Physiol 172: 343–350.
29. Murga M, Fernandez-Capetillo O, Field SJ, Moreno B, Borlado LR, et al. (2001) Mutation of E2F2 in mice causes enhanced T lymphocyte proliferation, leading to the development of autoimmunity. Immunity 15: 959–970.
30. Rounbehler RJ, Rogers PM, Conti CJ, Johnson DG (2002) Inactivation of E2f1 enhances tumorigenesis in a Myc transgenic model. Cancer Res 11: 3276–3281.
31. Matsumoto N, Salam MA, Watanabe H, Amagasa T, Senpuku H (2004) Role of gene *E2f1* in susceptibility to bacterial adherence of oral streptococci to tooth surfaces in mice. Oral Microbiol Immunol 19: 270–276.
32. Salam MA, Matin K, Matsumoto N, Tsuha Y, Hanada N, et al. (2004) *E2f1* mutation induces early onset of diabetes and Sjögren's syndrome in nonobese diabetic mice. J Immunol 173: 4908–4918, 2004.
33. Matsui-Inohara H, Uematsu H, Narita T, Satoh K, Yonezawa H, et al. (2009) E2F-1-deficient NOD/SCID mice developed showing decreased saliva production. Exp Biol Med 234: 1519–1524.
34. Senpuku H, Matin K, Salam MA, Kurauchi I, Sakurai S, et al. (2001) Inhibitory effects of MoAbs against a surface protein antigen in real-time adherence in vitro and recolonization in vivo of *Streptococcus mutans*. Scand J Immunol 54: 109–116.
35. Salam MA, Matsumoto N, Matin K, Tsuha Y, Nakao R, et al. (2004) Establishment of animal model for initial adhesion of oral streptococci using recombinant NOD.*B10.D2* mice. Clin Diagn Lab Immunol 11: 379–386.
36. Ogawa A, Furukawa S, Fujita S, Mitobe J, Kawarai T, et al. (2011) Inhibition of *Streptococcus mutans* Biofilm Formation by *Streptococcus salivarius* FruA. Appl Environ Microbiol 77: 1572–1580.
37. Senpuku H (2010) Model mouse designed for oral biofilm formation studies. Int J Oral-Med Sci 8: 125–131.
38. Scannapieco FA (1994) Saliva-bacterium interactions in oral microbial ecology. Crit Rev Oral Biol Med 5: 203–248.
39. Brack CM, Reynolds EC (1988) Colonization of rat molar teeth by mutans streptococci with different salivary agglutination characteristics. Arch Oral Biol 33: 695–699.
40. Russell MW, Hajishengallis G, Childers NK, Michalek SM (1999) Secretory immunity in defense against cariogenic mutans streptococci. Caries Res 33: 4–15.
41. Murthy AK, Chaganty BK, Troutman T, Guentzel MN, Yu JJ, et al. (2011) Mannose-containing oligosaccharides of non-specific human secretory immunoglobulin A mediate inhibition of *Vibrio cholerae* biofilm formation. PLoS ONE 6: e16847.
42. Tsuha Y, Hanada N, Asano T, Abei T, Yamaguchi S, et al. (2004) Role of peptide antigen for induction of inhibitory antibodies to *Streptococcus mutans* in the human oral cavity. Clin Exp Immunol 137: 393–401.
43. Takahashi I, Okahashi N, Matsushita K, Tokuda M, Kanamoto T, et al. (1991) Immunogenicity and protective effect against oral colonization by *Streptococcus mutans* of synthetic peptides of a streptococcal surface protein antigen. J Immunol 146: 332–336.
44. Hapfelmeier S, Lawson MAE, Slack E, Kirundi JK, Stoel M, et al. (2010) Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. Science 328: 1705–1709.
45. Culp DJ, Quivey RG, Bowen WH, Fallon MA, Pearson SK (2005) A mouse caries model and evaluation of Agp5-1-knockout mice. Cries Res 39: 448–454.

46. Ma T, Song Y, Gillespie A, Carlson EJ, Epstein CJ, et al. (1999) Defective secretion of saliva in transgenic mice lacking aquaporin-5 water channels. J Biol Chem 274: 20071–20074, 1999.
47. Krane CM, Melvin JE, Nguyen HV, Richardson L, Towne JE, et al. (2011) Salivary aciner cells from aquaporin 5-deficient mice have decreased membrane water permeability and altered cell volume regulation. J Biol Chem 276: 23413–23420.
48. Catalán MA, Scott-Anne K, Klein MI, Koo H, Bowen WH, et al. (2011) Elevated incidence of dental caries in a mouse model of cystic fibrosis. PLoS ONE 6: e16549.
49. Meyers C (2011) A bacterium that acts like a toothbrush. Science Now 1 April: http://news.sciencemag.org/sciencenow/2011/04/a-bacterium-that-acts-like-a-toothbrush.html?ref=hp.

解説

「歯科領域と口腔感染症について」

泉福英信

口腔バイオフィルム：口腔は、700種類以上の微生物が存在し、5％CO_2環境下においてBrain Heart Infusion寒天培地上で唾液を培養すると1 ml中1 x 10^8以上の微生物が検出される。実際には、培養できない菌も存在しており、莫大な菌数の存在する環境である。食事をして菌数が減り、食後口腔清掃してさらに大幅に菌数が減るが、2～3時間もするとまた元に戻る。よって、口腔には常に一定の菌数が維持されている。それらの菌の中で多くの割合を占めるのが連鎖球菌であり、*Streptpococcus mitis, Streptococcus sanguinis, Streptococcus oralis, Streptococcus gordonii*などである。その他に、Actinomyces, Naisseria, *Veillonella*など多くの菌が存在している[1)]。これらの菌は、歯表面において初期付着菌と呼ばれている。これらの菌が歯表面に生息する理由は、主に唾液タンパク質と相互作用する菌群だからである（図１）。

歯表面を構成しているハイドロキシアパタイトはタンパク質吸着性が強く、唾液に常に曝されている歯表面では唾液タンパク質が吸着している。それを、獲得ペリクルと呼んでいる。この獲得ペリクルに結合できる菌群を初期付着菌と呼び、それらが初めに菌叢を形成する。一度全体に菌叢が出来てしまうと、たとえ新しい菌が口腔に入ってきても定着しにくくなる。初期付着菌は、一定の他の菌とも相互作用することができ、その結果、菌と菌の凝集が起こってくる（図２）。

口腔粘膜上でも、唾液成分や口腔粘膜細胞と相互作用しながら細菌叢が形成されて、それが歯表面細菌叢とともに常在菌叢を形成している。このような常在菌叢を近年では、バイオフィルムと呼ぶようになってきている。これは、菌体が表面に付着し生存している様を表している。歯表面では、このバイオフィルムを昔から歯垢と呼んでいる。これは、一般的な呼称として広く浸透している。このような常在細菌叢は、外環境から侵入した微生物を口腔に定着させないように働いている。その一方、糖の含まれた食事を摂取した際に糖を栄養源として取り込み、代謝する。その結果、口腔ではたちまちのうちに酸が微生物から産生されて、口腔内pHが低下してくる。しかし、唾液による緩衝作用が働き、pHを

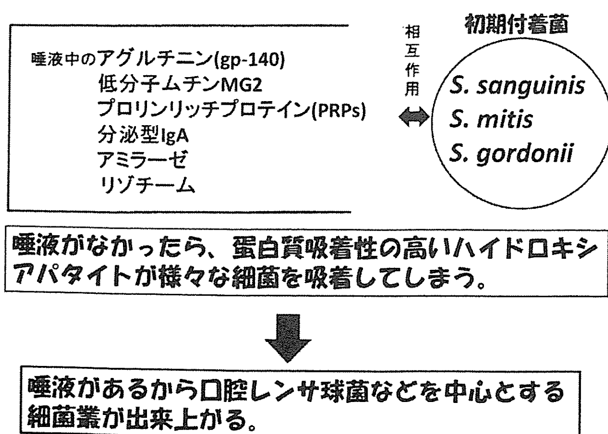


図１　バイオフィルム形成における歯表面唾液の意義

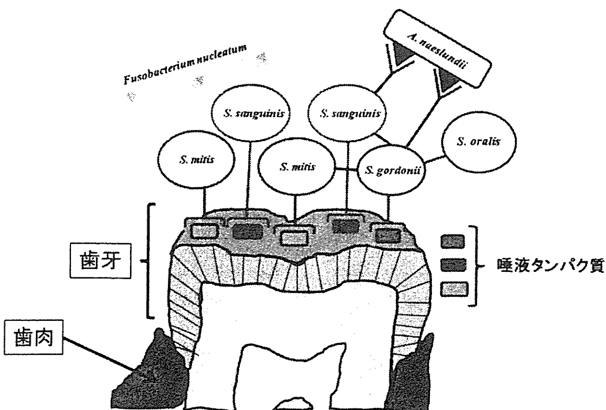


図２　初期の口腔バイオフィルム形成　唾液タンパク質と口腔連鎖球菌、口腔連鎖球菌と他の菌の相互作用（カラーページｉに再掲）

すぐに元に戻す。このような唾液の作用によって、口腔内 pH は中性に維持され、酸性環境下では脱灰してしまう歯を守っている。よって、唾液は、外来微生物を感染させないように菌に対して抗菌的に働きかつ酸産生菌を引き寄せると同時に歯にとって有害な酸を緩衝能力により中和し何も問題なく口腔の健康を維持している。このように口腔に存在する物質は、それぞれが意味をなして存在している。

う蝕：砂糖を頻回に摂取すると、主に歯表面の連鎖球菌群では過少な存在である *Streptococcus mutans* は、分泌した酵素作用により砂糖を基質として水に溶けにくい非水溶性グルカンを合成する[2)]。この非水溶性グルカンが存在すると、粘着性物質であるため自分以外の様々な菌を取り込んで分厚く密なバイオフィルムが形成されてしまう。さらに、唾液に含まれる重炭酸イオンなどがバイオフィルム内に浸透しにくい状況となる。その結果、糖成分の代謝によるバイオフィルム内 pH の低い状態が、唾液の緩衝作用により元に戻らず低い pH が維持され、Ca と P でできているハイドロキシアパタイトは、Ca と P がイオン化し遊離するようになる。これが、続くと歯表面の一部が欠損するほどの脱灰が起こり、これがう蝕発症である。よって、う蝕は *S. mutans* の感染のみで発症するのではなく、食物摂取における砂糖の過剰摂取や口腔清掃習慣の乱れなど生活習慣のバランスが崩れたことが関わって発症するものである。微生物の感染により起こることに間違いないが、他の条件が揃って発症するものであり、一般的な全身感染症とは異なっている。

このように口腔疾患は、感染症であるものの、様々な因子のバランスが加わって発症するものである。具体的な因子には、年齢、歯の修復物、唾液分泌量、義歯、食物の摂取、全身疾患などがある。このような因子のバランスが崩れてくると、う蝕に加えて歯周病、口腔粘膜疾患、誤嚥性肺炎なども発症してくる。これらのバランスの崩れに対応することが、口腔疾患を予防および治療することにつながってくる。バランスの崩れに敏感に反応するのが、バイオフィルム形成である（図3）。

米国モンタナ州モンタナ州立大学の Costerton らによって、川の中の石表面や船底に存在するぬるぬるした菌の固まりと歯牙表面にできる歯垢は似た構造物でありそれらを総称としてバイオフィルムと呼ばれるようになった[3)]。すでにバイオフィルムは歯科の世界でも一般的に使われるようになり、口腔の病原性を発揮する主な原因物質と考えられている。う蝕と共に口腔に2大疾患である歯周病もバイオフィルムが形成されることで導かれる。

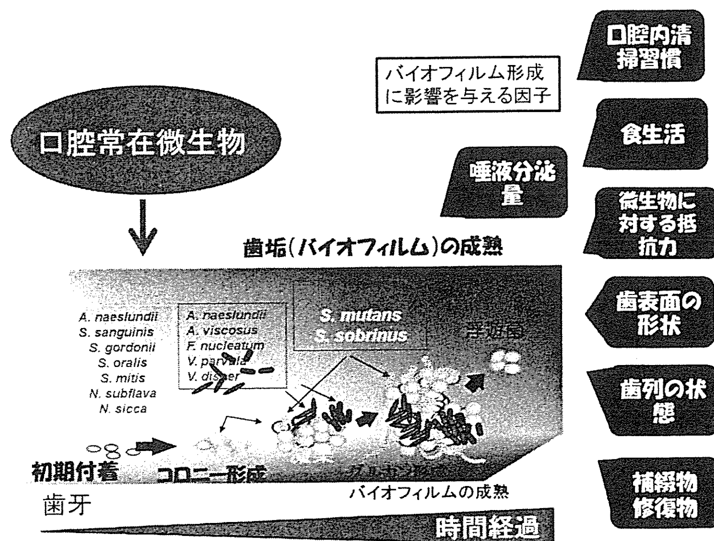


図3　様々な口腔バイオフィルム形成に影響を与える印紙（カラーページ i に掲載）

歯周病：このS. mutans依存した病原性バイオフィルムは、摂取された砂糖を介した非水溶性グルカン合成が関与しているが、歯周病発症に関わるバイオフィルムは必ずしも砂糖摂取に依存しているわけではない。歯石形成によるポケット内の嫌気性環境下に増える嫌気性菌がバイオフィルムを形成し残存する。それらがバイオフィルムから遊離し歯周組織へ大量に侵入、炎症を引き起こし、歯槽骨の吸収の結果、骨量が減少し歯を支えられなくなることが歯周病の原因と考えられる（図4）。

歯周病発症の最初のスイッチに関わる歯石形成は、そのメカニズムについて明確にされていないが、バイオフィルムが形成されそれが石灰化した結果と考えられる。歯垢は、その中身を調べてみるとかなりの割合で死菌が存在している。バイオフィルム内での死菌が形成される研究が近年行われ、そのメカニズムも明らかになってきた。ある一定レベル以上の菌が蓄積し、分厚いバイオフィルムが形成されると、Quorum Sensingが起こり、自菌や他菌を破壊する酵素を分泌するようになり、バイオフィルム内細菌が破壊される[4]。その結果、死菌が蓄積してくる。これら死菌が多くなることが、歯石形成に関わっている可能性が考えられる。歯周病は、近年、糖尿病発症、低体重児出産、誤嚥性肺炎発症、バージャー病発症、HIVウイルスの活性化に関わることが報告された[5、6]。しかし、賛否両論があり定説になるまでには至っていない。今後の検証が必要とされているが、いずれにしても歯周病が発症しないように予防しようという概念は変わらない。

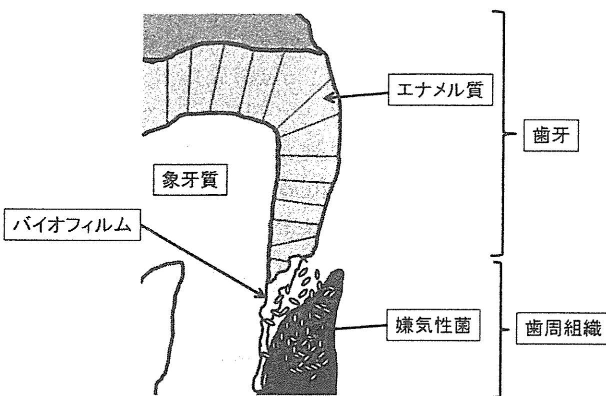
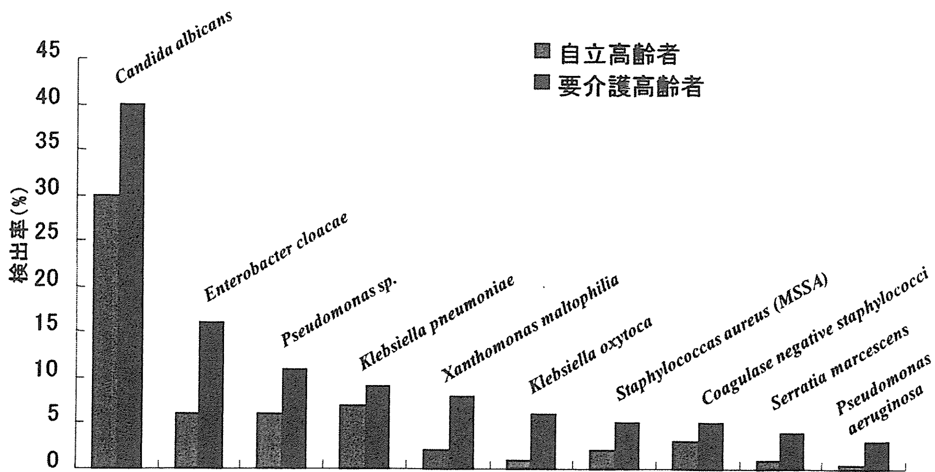


図4 バイオフィルムから嫌気性菌の歯周組織への侵入（カラーページⅰに再掲）

誤嚥性肺炎：現在の日本は少子高齢化が進んでいる。以前の日本では、歯が無くなる前に死亡するケースが多かったが現在では長寿のため歯が無くなることが問題となってきている。高齢者になっても美味しく自分の歯で食べられた方が楽しく生きられるというQuality of Lifeに歯は関わっている。一方、歯を多く残した状態で要介護施設へ入居すると、介護士による口腔ケアは無歯顎の高齢者より



Senpuku et al., Systemic diseases in association with microbial species in oral biofilm from elderly requiring care. Gerontology 2003; 49: 301-309.より改変

図5 歯垢における日和見菌検出率（自立高齢者と要介護高齢者の比較）

も大変になる。よって、全身状態も関わるが要介護施設入居高齢者の口腔は、常在菌以外に日和見菌が多種類検出されるケースが増えている[7]（図５）。

脳梗塞を起こした高齢者では、嚥下障害が重なり、口腔の日和見菌を含めた微生物が肺に入り、誤嚥性肺炎を起こして亡くなるケースが増えてきている。よって、要介護施設高齢者の口腔ケアは重要な位置づけとなっている。歯科衛生士や歯科医師などによる専門的な口腔ケアにより、歯石や口腔バイオフィルムを除去し、日和見菌を限りなく減少させ、口腔常在菌のみに満たされるような口腔環境に整えることが重要と考えられている。

その他：HIV 感染者、白血病、骨髄移植患者など口腔の病原菌感染が多く見られる疾患が他にも多々ある。口腔疾患は、直接死に至らないためにないがしろにされる場合がある。しかし、近年では、入院日数の減少、術後感染症の減少、予後が良好など口腔感染症を予防することによって多様な利点が見出されるようになってきた。医療が充実していくためには、口腔感染症の予防が大きなキーワードになる日も近いと考える。

参考文献

1) Kolenbrander PE. Oral microbial communiti-es: biofilms, interactions, and genetic systems. Annu Rev Microbiol. 54 : 413－37, 2000.

2) Rölla G, Scheie AA, Ciardi JE. Role of suc-rose in plaque formation. Scand J Dent Res. 93(2) : 105－11, 1985.

3) Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms : a common cause of persis-tent infections. Science. 284 (5418) : 1318－22, 1999.

4) Spoering, A. L. and Gilmre, M. S. Quorum sensing and DNA release in bacterial biofilms. Curr Opin Microbiol. 9 (2) : 133－7, 2006.

5) Taylor GW, Loesche WJ, Terpenning MS. Impact of oral diseases on systemic health in the elderly : diabetes mellitus and aspiration pneumonia. J Public Health Dent. 60(4) : 313－ 20, 2000.

6) Slots J, Slots H. Bacterial and viral pathoge-ns in saliva : disease relationship and infectious risk. Periodontol. 2000. 55(1) : 48－69. 2011.

7) Senpuku H, Sogame A, Inoshita E, Tsuha Y, Miyazaki H, Hanada N. Systemic diseases in association with microbial species in oral biofi-lm from elderly requiring care. Gerontology. 49(5) : 301－ 9 , 2003.

（国立感染症研究所　細菌第一部　第六室）

日歯保存誌 54（3）：193〜200，2011

歯科用ユニット給水管路の新クリーンシステムの評価

小 澤 寿 子　中 野 雅 子
木 村 泰 子　新 井 　 髙

鶴見大学歯学部歯内・歯周病学講座

抄録：歯科用ユニットの給水管路（DUWL）のバイオフィルム形成と水汚染については，1993年から報告されてきている．このDUWLの汚染対策として，新しい水回路クリーンシステム搭載の歯科用ユニットが2008年に開発された．本研究では，鶴見大学歯学部附属病院に同年11月に設置された新しい歯科用チェアユニットに内蔵されたクリーンシステムの有効性について評価した．

クリーンシステムでは，毎日診療後に過酸化水素水（1,000 ppm）をDUWL内に流し夜間滞留させてDUWLに作用させる．毎朝診療開始前に過酸化水素水を完全に排出して水道水に入れ替える．2本のハイスピードハンドピースのうちの1本（H-1）はクリーンシステムに属すが，もう1本（H-2）は結果を比較するためにクリーンシステムに属さない．定期的に，診療後H-1，H-2，コップ給水から水サンプルを採取した．サンプルはすべて，残留塩素濃度を測定し，25℃，7日間，R2A寒天培地上で培養後，CFU/m*l*数を測定した．H-2から検出された優勢菌種の発育コロニーに対して，16S rDNAの塩基配列解析を行った．さらに，DUWLチューブの一部を切断して，チューブ小片の内壁をSEM観察した．

その結果は，次のとおりである．

1．H-1およびコップ給水と比較すると，H-2では4カ月目以降，残留塩素濃度は0.1〜0.4 ppmに低下し，R2A寒天培地上で微生物のコロニーが検出されはじめた．

2．16S rDNA塩基配列解析したコロニー内の優勢菌種は，*Methylobacterium populi*, *Sphyngobium chlorophenolicum*，*Caulobacter vibrioides* であった．

3．6カ月目以降，H-1，H-2，コップ給水のチューブ内壁において，SEMによる観察で，ごく少量バイオフィルム様の形態が観察された．18カ月後も，いずれのDUWLにおいてもバイオフィルムが広がっている様子は観察されなかった．

本研究により，この新クリーンシステムはDUWLの水の汚染対策として有効であることが示唆された．

キーワード：歯科用ユニット給水管路，バイオフィルム，汚染対策

責任著者連絡先：小澤寿子
〒230-8501　横浜市鶴見区鶴見2-1-3　鶴見大学歯学部歯内・歯周病学講座
TEL：045-581-1001，FAX：045-583-8401，E-mail：ozawa-t@tsurumi-u.ac.jp
受付：平成23年3月15日/受理：平成23年4月26日

緒　言

歯科用ユニット水(歯科用チェアユニットのタービン，シリンジなどを通して排出される水）の汚染度は高く10^4〜10^7 CFU/mlに達すると報告[1-8]されている．その微生物の大部分は一般的な従属栄養性水生細菌である[5-7]が，易感染性宿主では日和見感染症を起こす可能性のある*Pseudomonas*, *Legionella*, *Mycobacterium*, *Candida* なども検出されている[9]．そのため，汚染水から起こる疾患のリスクは，高齢者，幼児，そして免疫不全性疾患患者で高くなり，また心疾患患者にも注意が必要である．

歯科用ユニット給水管路（DUWL）においては，①直径が小さく，流水量に相対して表面積が大きい，②チューブ内の水には，高圧がかからない，③水流の速度が壁近くでは遅い，という問題点がある．チューブ内の水流は，中央では流れが最も速いが外側にいくにつれて遅くなり，チューブの内壁付近では流速は0に近くなり，バイオフィルム形成が起こるという問題点がある．すなわち，流入する水の中には微生物が少なくても，持続的に存在するとバイオフィルム形成の原因となり，その中を水が流れるので，バイオフィルムから微生物を巻き込んだ汚染水として流出する．

DUWLの汚染対策の基準として，米国のAmerican Dental Associationでは歯科用ユニット水の水質基準を従属栄養細菌で200 CFU/ml[10]とし，米国疾病対策センター（Centers for Disease Control & Prevention）では，非外科的処置の場合，米国の飲料水の水質基準として従属栄養細菌500 CFU/ml以下を推奨している．また，骨削除など外科的処置時には，滅菌水を使用することを提示している[11]．しかしながら，日本では歯科用ユニット水の水質基準は提示されていないのが現状である．

一般的なDUWLの対策として，マイクロフィルターの設置やタービン回路への逆流防止装置の設置，診療前の水排出（フラッシング）が行われている．マイクロフィルターを設置することで微生物の新たな侵入のブロックは可能である．また，フラッシングとして，毎日診療開始前に数分間，さらに患者ごとに最低20〜30秒空回しをして水回路から水と空気を排出することが必要である．フラッシング量が増えるに従って微生物数は減少するものの，バイオフィルムを除去することはできない．このバイオフィルムを除去するためには，定期的なショックトリートメント[12-18]と呼ばれるDUWLの化学的洗浄消毒が必要となる．

鶴見大学歯学部附属病院では，2004年から歯科用ユニット水の微生物による汚染状況について把握するために汚染状況調査を実施しながら，DUWLの汚染対策としてショックトリートメントを実践し，その結果，チューブ内面のバイオフィルムを除去し水中の微生物数減少に効果的であったことを報告した[19]．しかしながら，導入にはDUWLの流入元にコック取付けなど，洗浄液流入には特殊な装置の設置，またショックトリートメントにより溶解排出された汚染物によるチューブの詰まりや，古いチューブへの化学的作用により発生した水漏れに対する対策が必要であった．また1台の歯科用チェアユニットに対して3日間連続して行う必要があるため労力を要し，洗浄剤の飛散などによる作業者への危険がある．さらに一度バイオフィルムを除去しても，通常使用していると約4カ月後にはまた水質汚染が認められるようになるため，定期的に（年に2〜3回）ショックトリートメントを繰り返す必要があることがわかった．

DUWLの汚染の源泉は水道水など公共水の流入にあるという観点から，米国を中心として，滅菌蒸留水を歯科用ユニット水として供給する給水ボトル装備のユニットが普及している．しかしながら，water lineの消毒方法として，週1回次亜塩素酸ナトリウム溶液を入れて洗浄することが必要で，次亜塩素酸ナトリウム溶液による錆の発生や労力がかかることが欠点であった．2003年にボトル内の滅菌蒸留水に日常的に溶解して治療中も使用することができ，DUWLの汚染を防止できる洗浄剤が発表された．人体への為害作用がなく，レジンの接着にも悪影響がないことが報告されている[20,21]．

国内では，これまでDUWL汚染対策が模索されてきていたが，2008年に新クリーンシステム搭載の歯科用ユニットが試作された．われわれは，このDUWL自動洗浄装置を組み込んだ歯科用ユニットの新クリーンシステムの有効性について，臨床現場で評価してきたので，その経過について報告する．

材料および方法

1．対象ユニット

対象は，鶴見大学歯学部附属病院保存科診療室に2008年11月15日に設置したクリーンシステム搭載の歯科用チェアユニット（スペースライン™ イムシアⅢ型，モリタ製作所）である．DUWLチューブには，内面の材質がフッ素樹脂の積層チューブが組み込まれている．また，タービンハンドピースはハンドピース単体で逆流防止効果がある機能を搭載したTwinPower™（PAR＝4HEX-0，モリタ製作所）[22]を使用し，通常どおり日常の診療に使用した．

毎日の診療後に備え付けのタンクに入った1,000 ppmの過酸化水素水をハイスピードハンドピース，マイクロモーター，3 wayシリンジ，超音波スケーラー，コップ

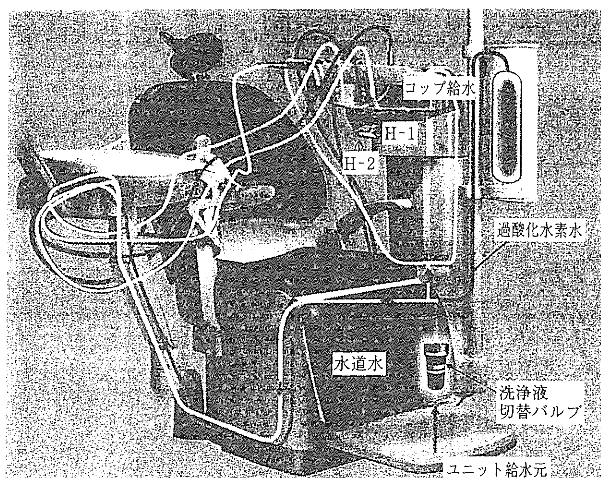


図1　クリーンシステム回路図
1,000 ppm の過酸化水素水を備え付けのタンクから，DUWL 内に流入できる．また，フラッシング装置により，過酸化水素水を排出し水道水に入れ替えることができる．2本のハイスピードハンドピース1本（H-2）は，クリーンシステムに属さない水道水のみの従来管路である．

給水の DUWL 内に流して洗浄後，夜間および休日中滞留させ，翌日以降，診療開始前に残留水排出用フラッシング装置を使用して，過酸化水素水を排出して水道水に入れ替え，診療中は水道水を使用する．過酸化水素水の供給と排出，水道水への入れ替えは，コックとボタン操作により自動的に行うことができる．

今回は，このクリーンシステムの有効性を評価するために，2本のハイスピードハンドピース（H-1，H-2）のうちの1本（H-2）には，過酸化水素水が供給されない水道水のみの従来管路を設置した（図1）．最初の3カ月間は，給水元からキャビネット内の H-1，H-2 分岐部までは過酸化水素水で洗浄する回路で，それより先のみ H-2 を洗浄システムから分離していたが，4カ月目以降は，給水元より全く分離して H-2 回路にはすべて水道水を流入するように変更した．

また，2本のハイスピードハンドピースの稼動時間は，積算タイマー記録を目安に均等になるように使用した．

2．水質検査

毎月1回，1日の診療終了後に H-1，H-2，コップ給水，ユニット給水元から流出する水を滅菌容器に採取して，残留塩素濃度を測定後，0.1 m*l* を R2A 寒天培地上で，25℃，7日間培養後にコロニー数を測定した．同時に標準寒天培地上で 37℃，48 時間の培養を行った．また，カップリング部の汚染が認められた 21 カ月以降には，カップリングを除去後にチューブ終末部からも水採取を行った．なお，その際にカップリング部の注水管路を3％過酸化水素水と綿棒を使用して洗浄した．

さらに，検出された優勢菌種の発育コロニーから細菌の DNA を抽出後，PCR 法により 16S rDNA 領域の DNA を増幅し，ABI PRISM 310 Genetic Analyzer（Applied Biosystems）を用いて塩基配列解析を行った．得られた配列を国際塩基配列データベース（DDBJ/EMBL/GenBank）に登録されている配列および MicroSeq ID Analysis Software Version 2.1（Applied Biosystems）のデータベースと相同性検索を行い同定した．

3．給水チューブの内壁への菌の付着状態の観察

3, 6, 12, 18 カ月後に H-1, H-2, コップ給水部のチューブの一部を切断して，中性緩衝ホルマリン固定し，チューブ内壁を SEM（JSM-5600LV，日本電子）観察した．

結　　果

1．水質検査

1）残留塩素濃度

当初の3カ月間は，H-1，H-2，コップ給水ともに，残留塩素濃度は 0.24～0.63 ppm で3部位の相違は明らかでなかった．H-2 回路を水道水のみに変更した4カ月目より，H-2 の残留塩素濃度は 0.10～0.37 ppm に低下し，H-1，コップ給水との相違が認められた．14～20 カ月目の間，H-1，H-2，コップ給水の残留塩素濃度の値は混在していた．21 カ月以降，H-2 の残留塩素濃度は，24 カ月目にコップ給水よりわずかに高かったが，H-1 の残留塩素濃度（0.64～0.77 ppm）より低く，0.06～0.71 ppm であった．一方，ユニット給水元から採取した水の残留塩素濃度は H-1，H-2，コップ給水よりも高い値を示した（図2）．

2）微生物学的分析結果

過酸化水素水による洗浄が行われているコップ給水に水の汚染は認められなかった．また同様に，H-1 では，10 カ月後までは汚染は認められなかったが，11 カ月以降少量のコロニーが観察された．また，カップリング部の汚染が認められた 21 カ月後に 1.1×10^3 CFU/m*l* が検出されたが，カップリング除去後の水質検査では検出限界以下となり，またカップリング部の汚染洗浄後 25 カ月まで検出限界以下であった．

一方，洗浄システムから分離した H-2 では，残留塩素濃度の低下が認められた4カ月以降，微生物のコロニーが検出されはじめ，H-1 との相違が認められたが，20 カ月までは 3.7×10^2 CFU/m*l* 以下であった．H-1 と同様にカップリング部の汚染が認められた 21 カ月後には，7.2×10^3 CFU/m*l* が検出されたが，カップリング除去後の水質検査では 6.7×10^2 CFU/m*l* となり，またカップリング部の汚染洗浄後はカップリング装着時でも 3.0×