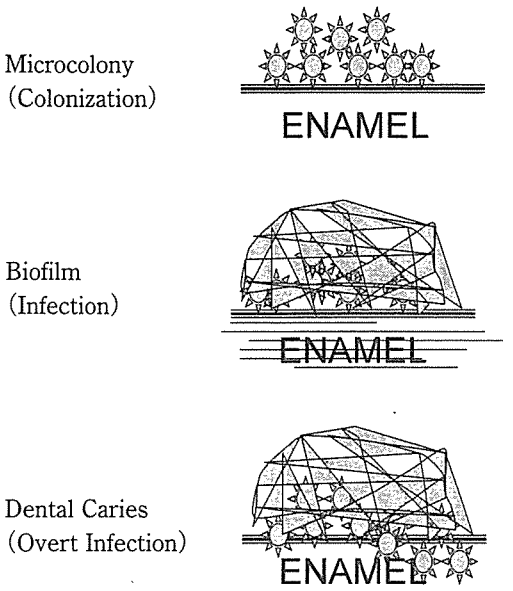

Fig. 1 Models of development of an MS biofilm
MS initially adhere to the dental pellicle via PAc (transmission). This is followed by microbial aggregation, leading to the formation of a microcolony (colonization). When sucrose intake is frequent, MS synthesize a water insoluble glucan catalyzed by GTF using sucrose as a substrate to form a cariogenic biofilm (infection). It strongly adheres to the tooth surface and aids in the storage of bacterial organic acids, which lead to dental caries (overt infection).

tice, even in developed countries.

MS initially adhere to the dental pellicle via a surface protein antigen (PAc)[6]. This is followed by microbial aggregation, leading to the formation of a microcolony. At this stage, MS elimination from the tooth surface is simple. When sucrose intake is frequent, MS synthesize a water insoluble glucan catalyzed by glucosyltransferase (GTF), using sucrose as a substrate to form a cariogenic biofilm[1]. This strongly adheres to the tooth surface and aids in the storage of bacterial metabolites (organic acids). This biofilm also aids in the drug resistance of MS.

At this stage, MS elimination from the tooth surface is complicated. (Fig. 1).

Several immunological control methods for MS have been reported[7,8]. However, whether the purpose of immunological control methods was to eliminate the source of contagion or to block the infection route is unclear. Immunological prevention methods for the dental caries epidemic can be classified into two categories : active immunity[9] that provides an antigen to the host, and passive immunity[10] that involves the direct incorporation of specific antibodies produced by other organisms. If there were a specific antibody the against the adhesive proteins such as PAc and GTF that is effective for immunological treatment in the oral cavity, it would be possible to inhibit the external adhesion of the MS, thereby blocking the route of infection. However, after MS form a microcolony, the effectiveness of a specific antibody appears questionable[11]. After MS has formed a biofilm on the tooth surface leading to a persistent infection, MS elimination cannot be accomplished even with a specific antibody coating. A biofilm infection is difficult to cure because of its low susceptibility to medication. Antibiotics and antibody molecules cannot effectively penetrate the biofilm. In particular, the susceptibility of biofilm-forming bacteria to antibiotics is extremely low due to their lowered metabolic activity within the biofilm[11]. Since full-mouth disinfection (FMD) advocated by Quirynen, *et al.*[12,13], using a nonspecific disinfectant, is more effective in killing commensal bacteria such as *Streptococcus mitis, S. oralis*, and *S. salivarius*, rather than the pathogenic biofilm, its use against dental caries is not possible. Therefore, once a biofilm has formed over the tooth surface, the elimination of the source of contagion is complicated[14].

Immunological Mechanisms for Bacterial Elimination

Cellular immunity does not function optimally in eliminating MS ; therefore, humoral immunity is the main route of acquired immunity. Humoral immunity against MS differs from the usual process of antigen phagocytosis where IgG is secreted into the blood. MS and the relevant antigen are taken into microfold cells (M cell) present in the mucous membrane, processed by antigen processing cells (APC), and recognized by T cells through MHC class II molecules. This causes B cells to recognize the antigen and multiply, and precursor cells to move into glandular tissues such as salivary glands, resulting in the production of

antigen-specific secretory immunoglobulin A (sIgA). Among the types of humoral immunity, mucous membrane immunity by the sIgA specific antibody from the salivary glands plays a particularly important role for dental caries prevention. The oral immune reaction mainly involves mucous membrane immunity, which primarily involves sIgA naturally present at 200 μg/mL in saliva.

There are two methods for immunologically eliminating MS : (1) an active immunity method which induces IgA (consisting of 60% of the immunoglobulins) by the direct presentation of the antigen derived from MS through a mucosal immunization, and (2) a passive immunity method, where a specific antibody against MS is produced externally and applied to the oral cavity.

The possible prevention of dental caries in humans using active immunization was first suggested in 1978[15,16]. A series of reports stated that the antibody titer of sIgA against *S. mutans* in saliva clearly increases when formalin-killed cells of *S. mutans* are orally administered[17,18]. Since then, the inhibition of *S. mutans* adhesion, the production of a GTF-inhibiting sIgA antibody by oral administration of killed cells[19] as well as adhesion inhibition by oral administration of GTF[20] have been reported. Childers, *et al.*[21] have also reported the production of inhibiting sIgA with GTF oral administration.

However, no case of eliminating MS by active immunity alone has thus far been reported. In 1998, Lehner's group[22] in England passively immunized a human oral cavity with Guy's 13 monoclonal antibody derived, genetically altered, plant-originated, anti-*S. mutans* sIgA. This study captured the attention of many researchers in the world, since it clearly indicated the elimination of *S. mutans*. The passive immunity method, wherein an effective specific antibody is applied externally, has low risks of side effects and is easy to administer due to its safety.

A Follow-up Clinical Test

Lehner's group concluded that passive immunity that involves the use of a specific antibody is essential for eliminating MS. To confirm this, we conducted a follow-up clinical test[23] in humans with regard to passive immunotherapy, using monoclonal antibodies (mAb) specific to *S. mutans*. Three types of anti *S. mutans* mAb (p126, P136 and KH5)[24,25] were used. The follow-up clinical test[23] was carried out by seven subjects who are engaged in research in this field. A crossover study design was adopted. The test consisted of a first trial in which saline was applied instead of antibody, and a second trial in which anti *S. mutans* mAb was applied. The test evaluated the numbers and ratios of MS in the saliva of each subject. Prior to coating the tooth surfaces with antibodies, we performed a biofilm destruction and chemical elimination procedure identical to that used in the study by Lehner's group[22]. These pre-treatment procedures included professional mechanical tooth cleaning (PMTC) and local application of a disinfectant on the tooth surfaces using custom trays. Reasons for performing pre-treatment procedures are : (1) to minimize the amount of antigen (*S. mutans* epitope), which tends to exist in overwhelming proportions in comparison with the amount of antibody, and (2) to convert the antigen into a planktonic state through the mechanical destruction of the biofilm with PMTC, since the antibody molecules themselves cannot couple with bacteria in the biofilm[11]. The mAb application (passive immunity) protocol used in our follow-up clinical test included the use of mAb P126 and P136[24] that inhibit the activity of GTF. The test also included the use of mAb KH5[25] against the PAc molecules associated with the recolonization of MS to tooth surfaces. It is recognized that KH5 is effective for an over 60% adhesion inhibition of MS on rat tooth surfaces, and that P126 and P136 are effective for inhibition of dental caries in rats[24]. To avoid contamination by mouse hybridoma-originated unknown virus pathogens, safety precautions were taken by treating the antibodies with gamma ray irradiation following a multi-step purification process. Three types of antibodies, anti-PAc antibody and two types of anti-GTF mAb, were mixed at a ratio of KH5 : P136 : P126 = 1 : 1 : 1 (V/V) (Table 1) and dissolved in a PBS buffer to yield a final concentration of 1.55 mg/mL, anticipating a synergetic effect by differing inhibition mechanisms. In this experiment, a custom

Table 1 mAb (KH5, P126, P136) used in the follow-up clinical test was mixed at a ratio of KH5 : P136 : P126 = 1 : 1 : 1 (V/V) and dissolved in PBS buffer to yield a final concentration of 1.55 mg/mL

KH5 (anti PAc) in PBS	5.67 mg/mL
P136 (anti GTF) in PBS	5.03 mg/mL
P126 (anti GTF) in PBS	3.25 mg/mL

tray termed as a drug retainer was used to allow 1,400 μL of mAb solution to react over the tooth surfaces for 5 minutes. Eating and drinking was prohibited for two hours after the treatment. This treatment was given once every three days, for 6 times in total. A total of 12.6 mg of protein was used in the drug retainer for each person. The results show[23], the amount of MS in the saliva of the subjects reduced through the first and second trial. An unexpected finding was observed in the first trial. After the first trial, in 4 out of 7 subjects, the amount of MS dropped below the detection limit sooner than we expected[12,23].

The result of this follow-up clinical test demonstrates that MS are eliminated with physicochemical treatment alone. Therefore, an antibody is not a prerequisite in eliminating MS but rather plays a secondary role.

A New Clinical Inspection System of MS

In a follow-up clinical test, we initially used an MSB agar plate[26], which is a commonly used selective medium for the examination of MS. However, specificity is poor when used with to clinical saliva samples as the MSB agar allows growth of other bacteria and necessitates colony counting based on the difference seen in colony configuration by eye. For a systematic bacterial test, it is necessary to make the distinction using an automatic colony counter. This problem was solved by adding gramicidin into the MSB agar (BML Co. Ltd., Patent No. JP2002027975 ; modified MSB agar ; Tables 2, 3). The modified MSB agar is more reliable than the MSB agar (see Table 3 (C, D). The inspection system of MS calculates the ratio between MS and total streptococci by using a stimulated saliva sample. Based on these data, a new clinical inspection system of MS for general practice has been developed by BML Co. Ltd in Japan.

Dental Drug Delivery System (3DS)

An MS elimination system has not yet been established despite many studies because previous tests have not incorporated a protocol that includes the intensive administration of a simultaneous physicochemical method. As a physical elimination method, Axelsson's group[27] in Sweden attempted to physically control MS with an approach centering on PMTC. They divided 187 subjects (13-year-old) with a high level of the MS in their saliva into three groups to under go a program focusing on a physical elimination method. However, the difference among the test groups was temporary, and it had disappeared six months later[28]. With the treatment focusing on a physical treatment, the reduction in the number of the MS was transient. On the other hand, Sandham's group[28] performed a chemical elimination method with 33 adult subjects with chlorhexidine varnish. This resulted in the reduction of the MS to below the detection limit in 21 subjects (64%). The average period of time that the MS remained below the detection limit was 34.6 months. Chlorhexidine varnish application was administered an average of 3.14 times, and the elimination of the MS was unsuccessful in 12 subjects (36%). In this study, the application of very concentrated chlorhexidine (40%) is necessary due to its dilution by saliva. This application may result in mucous membrane sensitivity and may destroy any commensal bacterial flora[29]. We attempted to create a protocol by summarizing Axelsson's group[27], Sandham's group[28] and our follow-up clinical test findings without using antibodies. The careful destruction of the biofilm with PMTC, followed by a disinfectant regionally applied with custom trays to the tooth surfaces, eliminated MS from the oral cavity without decreasing the population of oral commensal bacteria (see Table 2). These physicochemical methods described above have been designated as the Dental Drug Delivery System (3DS)[14].

Table 2 Development of modified MSB—Bacterial changes over the whole period of the first trial (2 of 7 subjects (A—G) ; raw data G, F)

Results of the first trial of subject G (stimulated saliva samples)

	Blood agar (A)		MS agar (B)			MSB agar (C)			Modified MSB agar (D)		
	CFU/mL	log	CFU/mL	log	B/A (%)	CFU/mL	log	C/B (%)	CFU/mL	log	D/B (%)
D1-1(6/12)	7.36E+08	8.87	2.66E+08	8.42	36.1	4.01E+05	5.60	0.15	4.28E+05	5.63	0.16
D1-2(6/14)	4.37E+08	8.64	2.65E+08	8.42	69.6	1.14E+06	6.06	0.43	1.12E+06	6.05	0.42
D1-3(6/15)	2.09E+08	8.32	1.57E+08	8.20	75.1	7.06E+05	5.85	0.45	5.38E+05	5.80	0.41
D1-4(6/19)	1.04E+09	9.02	2.95E+08	8.47	28.4	1.24E+06	6.09	0.42	1.18E+06	6.07	0.40
D1-5(6/20)	5.54E+08	8.74	2.14E+08	8.33	38.6	8.66E+05	5.94	0.41	1.05E+06	6.02	0.50
D2 (6/29)	5.61E+08	8.75	2.19E+08	8.34	39.0	2.00E+02	2.30	0.00009	1.00E+02	2.00	0.00005
D3 (7/21)	4.11E+08	8.61	2.76E+08	8.44	60.3	1.30E+03	3.11	0.0005	1.00E+03	3.00	0.0004
D4 (7/31)	7.23E+08	8.86	3.36E+08	8.53	46.5	1.80E+03	3.26	0.0005	2.50E+03	3.40	0.0007
D5 (8/14)	3.64E+08	8.56	1.59E+08	8.20	43.7	5.10E+02	2.71	0.0003	5.60E+02	2.75	0.0004
D6 (9/6)	2.99E+08	8.48	1.23E+08	8.09	41.1	1.02E+03	3.01	0.0008	1.32E+03	3.12	0.0011
D7 (10/18)	1.59E+09	9.20	1.00E+09	9.00	62.9	1.27E+04	4.10	0.0013	9.84E+03	3.99	0.0010

Results of the first trial of subject F (stimulated saliva samples)

	Blood agar (A)		MS agar (B)			MSB agar (C)			Modified MSB agar (D)		
	CFU/mL	log	CFU/mL	log	B/A (%)	CFU/mL	log	C/B (%)	CFU/mL	log	D/B (%)
D1-1(6/12)	7.70E+08	8.89	2.96E+08	8.47	38.4	7.50E+04	4.88	0.028	7.50E+04	4.88	0.028
D1-2(6/14)	5.62E+08	8.75	1.39E+08	8.14	24.7	8.40E+04	4.92	0.032	1.50E+04	4.18	0.006
D1-3(6/15)	6.87E+08	8.84	2.25E+08	8.35	32.8	3.20E+04	4.51	0.020	4.50E+04	4.65	0.035
D1-4(6/19)	8.50E+08	8.93	1.51E+08	8.18	17.7	6.20E+04	4.79	0.021	6.00E+04	4.78	0.020
D1-5(6/20)	3.27E+08	8.51	1.47E+08	8.17	45.0	8.50E+04	4.93	0.040	1.16E+05	5.06	0.054
D2 (6/29)	2.73E+08	8.44	1.29E+08	8.11	47.2	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D3 (7/21)	3.06E+08	8.49	1.79E+08	8.25	58.5	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D4 (7/31)	1.02E+09	9.01	1.28E+08	8.11	12.5	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D5 (8/14)	4.65E+08	8.67	8.21E+07	7.91	17.7	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D6 (9/6)	1.97E+08	8.29	5.02E+07	7.70	25.5	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D7 (10/18)	4.28E+08	8.63	2.02E+08	8.31	57.3	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00

Blood agar ; total bacteria, MS agar ; total streptococci, MSB and modified MSB agar ; mutans streptococci (MS). B/A ; total streptococci/total bacteria. C/B ; MS/total streptococci. D/B ; MS/total streptococci. Bacterial examination before physicochemical treatment ; D1-1 to D1-5. Bacterial examination after physicochemical treatment ; D2 to D7. Actual days are indicated in the parenthesis.

This consists of two phases : (1) physical destruction of the biofilm, and (2) chemical elimination of the remaining bacteria using custom trays (a drug retainer). Since the detection of MS is very rare in the predentate group, MS are considered to proliferate over the tooth surfaces rather than mucous membranes and saliva[30]. Therefore, MS have an ecological localization in the oral cavity. If a non specific disinfectant is delivered regionally to the tooth surfaces, it is possible to selectively reduce the MS population without damaging the commensal bacteria on the mucous membrane and saliva. Based on the follow-up clinical test, we concluded that it was possible to eliminate MS using commercial disinfectants. Our finding has become the foundation for the development of a more practical system (3DS unit). 3DS differs from FMD which does not utilize a custom tray. FMD for periodontal diseases should not be used in the prevention

Table 3 Development of modified MSB—Bacterial changes over the whole period of the second trial (2 of 7 subjects (A—G) ; raw data of A, D)

Results of the second trial of subject A (stimulated saliva samples)

	Blood agar (A)		MS agar (B)			MSB agar (C)			Modified MSB agar (D)		
	CFU/mL	log	CFU/mL	log	B/A (%)	CFU/mL	log	C/B (%)	CFU/mL	log	D/B (%)
D1-1 (7/2)	8.20E+07	7.91	3.20E+07	7.51	39.02	5.10E+02	2.71	0.00	1.50E+03	3.18	0.00
D1-2 (7/5)	6.10E+07	7.79	2.20E+07	7.34	36.07	3.10E+03	3.49	0.01	3.40E+03	3.53	0.02
D1-3 (7/6)	3.40E+07	7.53	1.80E+07	7.26	52.94	4.50E+03	3.65	0.03	5.00E+03	3.70	0.03
D1-4 (7/7)	1.80E+07	7.26	7.80E+06	6.89	43.33	1.40E+03	3.15	0.02	4.60E+03	3.66	0.06
D1-5 (7/8)	4.00E+07	7.60	9.40E+06	6.97	23.50	2.70E+03	3.43	0.03	4.70E+03	3.67	0.05
D2 (6/27)	2.50E+07	7.40	1.40E+07	7.15	56.00	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D3 (7/18)	4.57E+07	7.66	1.69E+07	7.23	36.98	0.00E+00	0.00	0.00	5.10E+01	1.71	0.00
D4 (7/28)	3.50E+07	7.54	1.10E+07	7.04	31.43	0.00E+00	0.00	0.00	1.52E+02	2.18	0.00
D5 (8/15)	3.82E+07	7.58	8.33E+06	6.92	21.81	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D6 (9/4)	6.00E+07	7.78	1.46E+07	7.16	24.33	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D7 (10/16)	4.52E+07	7.66	9.82E+06	6.99	21.71	2.54E+02	2.40	0.00	1.52E+02	2.18	0.00

Results of the second trial of subject D (stimulated saliva samples)

	Blood agar (A)		MS agar (B)			MSB agar (C)			Modified MSB agar (D)		
	CFU/mL	log	CFU/mL	log	B/A (%)	CFU/mL	log	C/B (%)	CFU/mL	log	D/B (%)
D1-1 (7/2)	5.60E+07	7.75	1.80E+07	7.26	32.14	1.80E+03	3.26	0.01	2.80E+03	3.45	0.02
D1-2 (7/5)	8.70E+07	7.94	3.20E+07	7.51	36.78	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D1-3 (7/6)	6.50E+07	7.81	1.70E+07	7.23	26.15	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D1-4 (7/7)	7.90E+07	7.90	2.90E+07	7.46	36.71	0.00E+00	0.00	0.00	5.00E+01	1.70	0.00
D1-5 (7/8)	7.20E+07	7.86	2.00E+07	7.30	27.78	0.00E+00	0.00	0.00	5.00E+01	1.70	0.00
D2 (6/27)	9.10E+06	6.96	6.80E+06	6.83	74.73	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D3 (7/18)	1.22E+08	8.09	6.61E+07	7.82	54.18	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D4 (7/28)	5.60E+07	7.75	1.40E+07	7.15	25.00	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D5 (8/15)	4.27E+07	7.63	1.55E+07	7.19	36.30	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D6 (9/4)	9.86E+07	7.99	2.80E+07	7.45	28.40	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00
D7 (10/16)	1.25E+80	8.10	2.17E+07	7.34	17.37	0.00E+00	0.00	0.00	0.00E+00	0.00	0.00

See Table 2 for explanation

of a dental caries epidemic.

Clinical Use of 3DS (3DS Unit)

Outpatients at risk of having a high ratio of MS received a clinically modified 3DS treatment, called a 3DS unit. The 3DS unit is indicated in the diagram (Fig. 2). 41 subjects who had consented to receive a 3DS unit treatment were involved. The MS population and their ratio were evaluated before and after 1cycle of the 3DS unit. The conditions for the initiation of the 3DS unit treatment were an MS number greater than 1×10^5 CFU/mL and/or an MS ratio (number of MS/total streptococci $\times$ 100) of over 2%. The conditions for terminating the treatment were an MS number less than 1×10^4 CFU/mL and/or an MS ratio of less than 0.2% (data in preparation). PMTC was performed prior to the application of 0.2% chlorhexidine gel (Fig. 3). Subjects were instructed to refrain from sucrose ingestion immediately after the administration of 3DS. After 1cycle of 3DS unit treatment, MS number and MS ratio were suppressed to

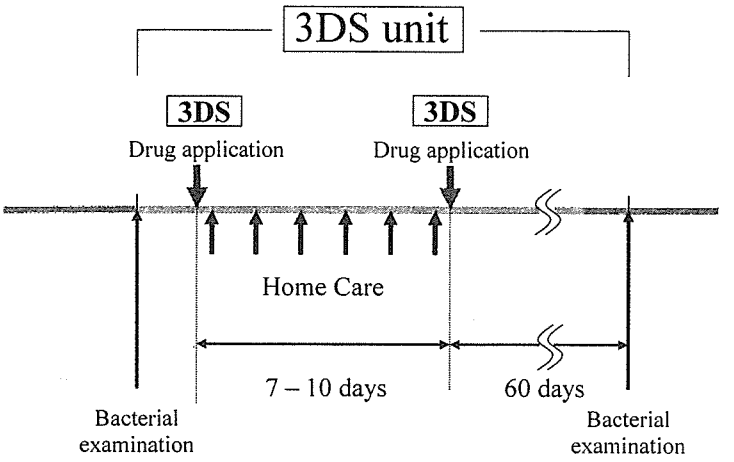

Fig. 2 Clinical protocol of 3DS

At first, bacterial examination must be carried out to decide on appropriate application of 3DS. Second, PMTC is carried out for the physical elimination of biofilm. Third, as a chemical treatment, 0.2% chlorhexidine gel is applied via a customized tray called a drug retainer that fits onto the dentition for 5 minutes. A 3DS unit involves 2 chemical treatments over approximately 1 week. The subjects use the drug retainer once a day for 5 minutes. After 60 days, bacterial examination was reperformed.

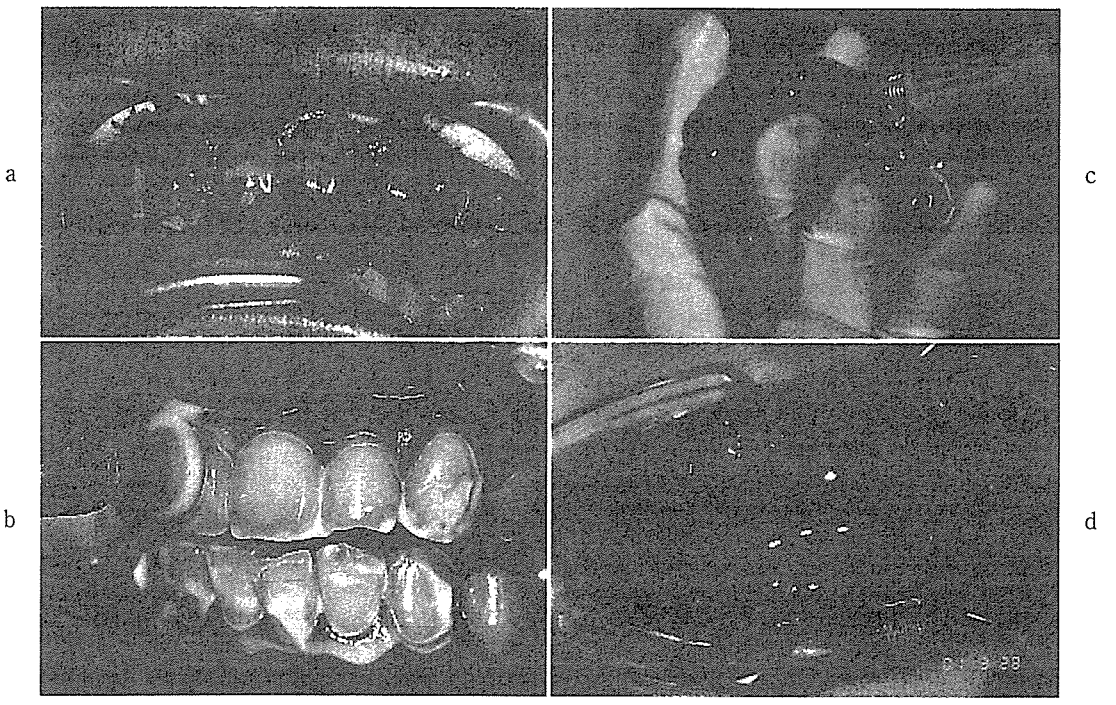

Fig. 3 Partial view of 3DS

a : Disclosing tooth surfaces. b : PMTC. The device destroys dominant biofilms and makes bacteria susceptible to contact with drugs. c : A custom tray (drug retainer) with 0.2% chlorhexidine gel. d : Fitting the tray onto tooth surfaces for 5 minutes. The drug retainer delivers the disinfectant directly on to tooth surfaces.

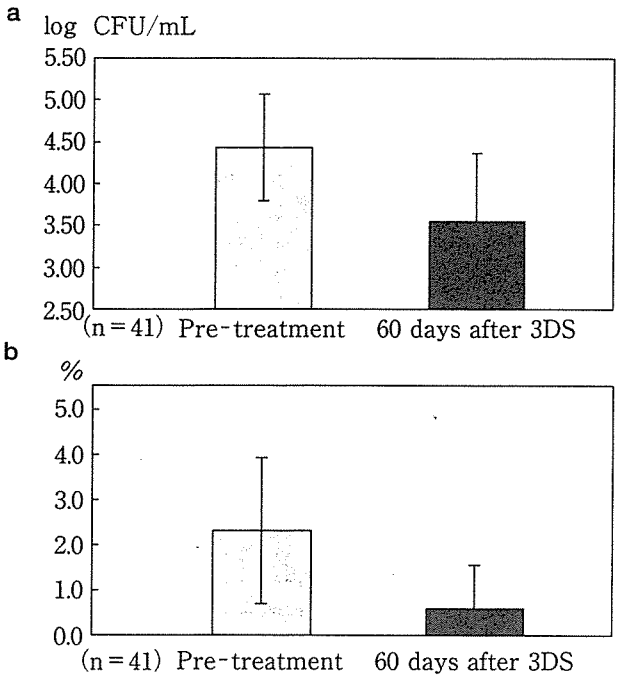

Fig. 4 Bacterial changes before and after the 3DS unit

Numbers (a) and ratios (b) of MS at the pre-treatment stage and 60 days after the final treatment of 3DS.

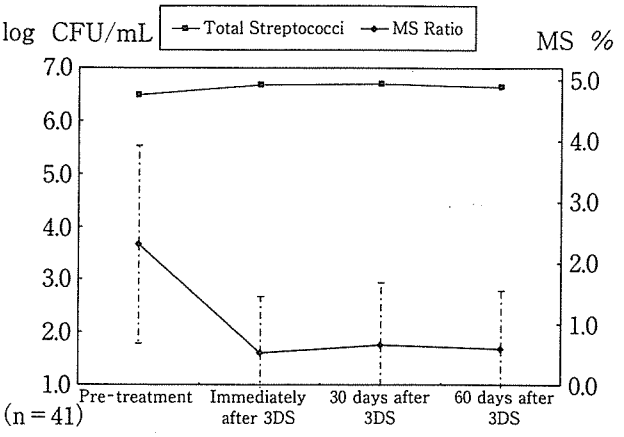

Fig. 5 Bacterial changes over the whole period of 3DS unit

This graph shows the clinical result of 3DS treatment for MS. The salivary bacterial samples were taken at each stage of pre-treatment, immediately after 3DS, 20 days after 3DS, and after 60 days. The y-axis on the left side shows the amount of total streptococci (CFU/mL), and on the right side shows the ratio of MS (%). The ratio of MS was apparently reduced after the 3DS treatment, and was still at a low level after 60 days. However, the total number of streptococci remained at the same level during the whole stage.

below the risk values ($p<0.05$) as compared to the values pre-treatment (Figs. 4, 5). However, the number of total streptococci before and after a cycle of 3DS unit treatment commonly remained at the same level (Fig. 5). Destruction of commensal bacteria and unpleasant oral symptoms were not observed in any of the subjects.

A Paradigm Shift in the Role of Caries Vaccine

Although the ability of initial attachment of MS is lower than for oral commensal bacteria, it is possible for MS in saliva to resettle over tooth surfaces. Since it is beyond the scope of 3DS to prevent a resettlement of MS, the development of immunological methods has become necessary. Specific antibodies can effectively bind and block this kind of process. Dental caries is not a lethal illness, and any side effects of the vaccine will always pose a problem to the use of an active immunity method. In 1976, a cross reaction between human cardiac muscles and antibody derived from the immunity against whole MS cells was reported[31]. In the active immunity approach, a peptide antigen, which is an antigenic epitope and the smallest unit of a adhesive protein molecule, is a safe consideration. After suppressing the formation of biofilm over the tooth surfaces by using 3DS, a steady secretion of inhibitory antibodies can suppress the process of resettlement of MS to the tooth surfaces and colonization. Antibody proteins remain effective for 72 hours by non-specifically coupling to the surface layer of the enamel via the pellicle, with intermolecular and van der Waals forces[26]. During this process, MS are specifically bound and absorbed by mAb[22]. However, the current viewpoint states that antibodies themselves may prevent the initial adhesion of planktonic bacteria. Therefore, antibodies should be present in the saliva at all times to adhere to and absorb planktonic MS to prevent their adhesion to tooth surfaces. Antibody characteristics were

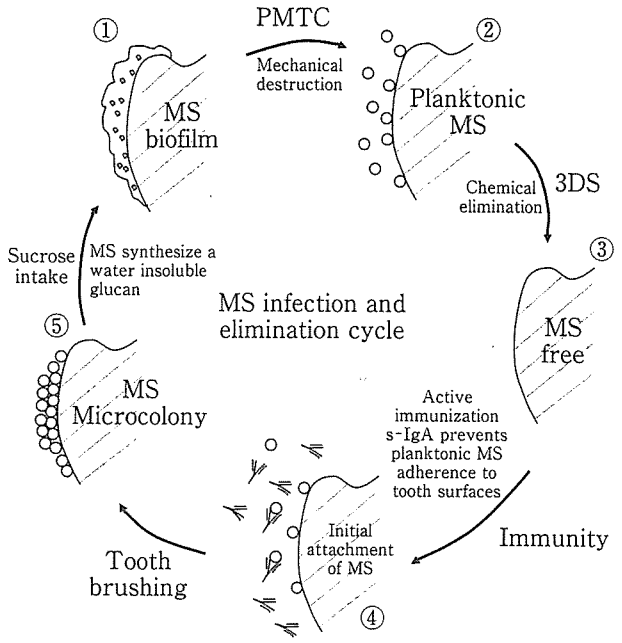

Fig. 6 A schematic diagram of the MS infection and elimination cycle
The MS biofilm covering tooth surfaces is destroyed by PMTC (1). Planktonic MS remains on the tooth surface (2). 3DS eliminates the remaining MS (3). Antibodies absorb against planktonic MS and protect the tooth surface from resettlement of the MS (4). The MS escape from the antibodies to form microcolonies (5). MS synthesize a water insoluble glucan catalyzed by glucosyltransferase (GTF) using sucrose as a substrate to form a cariogenic biofilm (1).

not utilized to their full potential because they were used inside a tray. On the other hand, a disinfectant should only be administered regionally to the tooth surface, and not to the full mouth. Since we have developed an approach to remove the biofilm formed over the tooth surfaces using 3DS, we have succeeded in creating an environment where antibodies can work effectively. The next strategy is to prevent the initial adhesion of MS. If 3DS becomes widely used, the actual need to develop a vaccine will become more pressing as antibodies work more effectively against planktonic bacteria (Fig. 6). A combination of passive immunity as well as active immunity will be most desirable.

Conclusion

We have shown that it is possible to eliminate the MS that have adhered to tooth surfaces using 3DS. However, to effectively eliminate MS altogether, it is important to prevent the resettlement of MS to tooth surfaces. In particular, the synergistic effect of combined physicochemical and immunological treatments is desirable for the effective inhibition of MS.

References

1) Hanada, N. : Current understanding of cause of dental caries. Jpn. J. Infect. Dis. **53** : 1—5, 2000.
2) Pihlanto-Leppala, A., Soderling, E. and Makinen, K. K. : Expulsion mechanism of xylitol 5-phosphate in *Streptococcus mutans*. Scand. J. Dent. Res. **98** : 112—119, 1990.
3) Isokangas, P., Soderling, E., Pienihakkinen, K. and Alanen, P. : Occurrence of dental decay in children aftermaternal consumption of Xylitol chewing gum, a follow-up from 0 to 5 years of age. J. Dent. Res. **79** : 1885—1889, 2000.
4) Iijima, Y. and Koulourides, T. : Mineral density and fluoride content of in vitro remineralized lesions. J. Dent. Res. **67** : 577—581, 1988.
5) Köhler, B. and Andreen, I. : Influence of caries-preventive measures in mothers on cariogenic bacteria and caries experience in their children. Arch. Oral Biol. **39** : 907—911, 1994.
6) Senpuku, H., Miyauchi, T., Hanada, N. and Nisizawa, T. : An antigenic peptide inducing cross-reacting antibodies inhibiting the interaction of *Streptococcus mutans* PAc with human salivary components. Infect. Immun. **63** : 4695—4703, 1995.
7) Smith, D. J. : Caries vaccines for the twenty-first century. J. Dent. Educ. **67** : 1130—1139, 2003.
8) Russell, M. W., Childers, N. K., Michalek, S. M., Smith, D. J. and Taubman, M. A. : A caries vaccine? —The state of the science of immunization against dental caries—. Caries Res. **38** : 230—235, 2004.
9) Michalek, S. M., MacGhee, J. R., Mestecky, J. and Bozzo, L. : Ingestion of *Streptococcus mutans* induces secretory immunoglobulin A and caries immunity. Science **192** : 1238—1240, 1976.
10) Ma, J. K., Hunjan, M., Smith, R., Kelly, C. and Lehner, T. : An investigation into the mechanism of protection by local passive immunization with mono-

clonal antibodies against *Streptococcus mutans*. Infect. Immun. **58** : 3407—3414, 1990.
11) Costerton, J. W., Stewart, P. S. and Greenberg, E. P. : Bacterial biofilms : A common cause of persistent infections. Science **284** : 1318—1322, 1999.
12) Quirynen, M., Mongardini, C. and van Steenberghe, D. : The effect of a 1-stage full-mouth disinfection on oral malodor and microbial colonization of the tongue in periodontitis. A pilot study. J. Periodontol. **69** : 374—382, 1998.
13) De Soete, M., Mongardini, C., Peuwels, M., Haffajee, A., Socransky, S., van Steenberghe, D. and Quirynen, M. : One-stage full-mouth disinfection. Long-term microbiological results analyzed by checkerboard DNA-DNA hybridization. J. Periodontol. **72** : 374—382, 2001.
14) Takeuchi, H., Senpuku, H., Matin, K., Kaneko, N., Yusa, N., Yoshikawa, E., Ida, H., Imai, S., Nisizawa, T., Abei, Y., Kono, Y., Ikemi, T., Toyoshima, Y., Fukushima, K. and Hanada, N. : New dental drug delivery system for removing mutans streptococci from the oral cavity : effect on oral microbial flora. Jpn. J. Infect. Dis. **53** : 211—212, 2000.
15) McGhee, J. R., Mestecky, J., Arnold, R. R., Michalek, S. M., Prince, S. J. and Babb, J. L. : Induction of secretory antibodies in humans following ingestion of *Streptococcus mutans*. Adv. Exp. Med. Biol. **107** : 177—184, 1978.
16) Krasse, B., Gahnberg, L. and Bratthall, D. : Antibodies reacting with *Streptococcus mutans* in secretions from minor salivary glands in humans. Adv. Exp. Med. Biol. **107** : 349—354, 1978.
17) Gahnberg, L. and Krasse, B. : Salivary immunoglobulin A antibodies and recovery from challenge of *Streptococcus mutans* after oral administration of *Streptococcus mutans* vaccine in humans. Infect. Immun. **39** : 514—519, 1983.
18) Cole, M. F., Emilson, C. G., Hsu, S. D., Li, S. H. and Bowen, W. H. : Effect of peroral immunization of humans with *Streptococcus mutans* on induction of salivary and serum antibodies and inhibition of experimental infection. Infect. Immun. **46** : 703—709, 1984.
19) Gregory, R. L. and Filler, S. J. : Protective secretory immunoglobulin A antibodies in humans following oral immunization with *Streptococcus mutans*. Infect. Immun. **55** : 2409—2415, 1987.
20) Smith, D. J. and Taubman, M. A. : Oral immunization of humans with *Streptococcus sobrinus* glucosyltransferase. Infect. Immun. **55** : 2562—2569, 1987.
21) Childers, N. K., Zhang, S. S. and Michalek, S. M. : Oral immunization of humans with dehydrated liposomes containing *Streptococcus mutans* glucosyltransferase induces salivary immunoglobulin A2 antibody responses. Oral Microbiol. Immunol. **9** : 146—153, 1994.
22) Ma, J. K-C., Hikmat, B. Y., Wycoff, K., Vine, N. D., Chargelegue, D., Yu, L., Hein, M. B. and Lehner, T. : Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans. Nat. Med. **4** : 601—606, 1998.
23) Takeuchi, H., Fukushima, K., Senpuku, H., Nomura, Y., Kaneko, N., Yano, A., Morita, E., Imai, S., Nisizawa, T., Kono, Y., Ikemi, T., Toyoshima, Y. and Hanada, N. : Clinical study of mutans streptococci using 3DS and monoclonal antibodies. Jpn. J. Infect. Dis. **54** : 34—36, 2001.
24) Fukushima, K., Okada, T. and Ochiai, K. : Production, characterization and application of monoclonal antibodies which distinguish three glucosyltransferases from *Streptococcus mutans*. Infect. Immun. **61** : 323—328, 1993.
25) Senpuku, H., Kato, H., Takeuchi, H., Noda, A. and Nisizawa, T. : Identification of core B cell epitope in the synthetic peptide inducing cross-inhibiting antibodies to a surface protein of *Streptococcus mutans*. Immunol. Invest. **26** : 531—548, 1997.
26) Gold, O. G., Jordan, H. V. and van Houte, J. : A selective medium for *Streptococcus mutans*. Arch. Oral Biol. **18** : 1357—1364, 1973.
27) Axelsson, P., Kristoffersson, K., Karlsson, R. and Bratthall, D. : 30-month longitudinal study of the effects of some oral hygiene measures on *Streptococcus mutans* and approximal dental caries. J. Dent. Res. **66** : 761—765, 1987.
28) Sandham, H. J., Brown, J., Chan, K. H., Phillips, H. I., Burgess, R. C. and Stokl, A. J. : Clinical trial in adults of an antimicrobial varnish for reducing mutans streptococci. J. Dent. Res. **70** : 1401—1408, 1991.
29) Smith, D. J., Anderson, J. M., King, W. F., van Houte, J. and Taubman, M. A. : Oral streptococcal colonization of infants. Oral Microbiol. Immunol. **8** : 1—4, 1993.
30) Smith, R. E., Badner, V. M., Morse, D. E. and Freeman, K. : Maternal risk indicators for childhood caries in an inner city population. Community Dent. Oral Epidemiol. **30** : 176—181, 2002.
31) Van de Rijn, I., Bleiweis, A. S. and Zabriskie, J. B. : Antigens in *Streptococcus mutans* cross reactive with human heart muscle. J. Dent. Res. **55** : C59—64, 1976.

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Original

Effect of 3.8% $Ag(NH_3)_2F$ Solution as an Anti-caries Agent on Dentin in Artificial Mouth Model System using *Actinomyces naeslundii*

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Nobuhiro HANADA** and Hiroshi UEMATSU*

Abstract：The object of this study was to evaluate the effect of 3.8% and 38% $Ag(NH_3)_2F$ solutions as a caries-preventive agent on dentin surfaces in the artificial mouth model system. A total of 36 bovine dentin slabs were divided into three groups：3.8% $Ag(NH_3)_2F$ and 38% $Ag(NH_3)_2F$ application groups and a control group. *Actinomyces naeslundii*, selected as having an important role in dentin caries, was used in this experiment. Several parameters concerning caries development were evaluated by the following methods：the amount of artificial biofilm, the changes in pH, the changes in dentin microhardness measured by a hardness tester, and element quantitative analysis for silver and fluorine elements by an energy dispersive X-ray micro analyzer. The dentin specimens in the 3.8% and 38% $Ag(NH_3)_2F$ application groups had significantly less hardness reduction than the specimens in the control group ($p<0.05$). Little difference, however, was observed for the dentin hardness reduction between the two test solution groups. The amounts of silver and fluorine elements on the specimen surface between the two groups were not significantly different after a lapse of 60 hours. The outcomes in this study suggest that 3.8% $Ag(NH_3)_2F$ solution might have similar effectiveness as 38% $Ag(NH_3)_2F$ solution as a preventive agent for dentin caries.

Key words：**$Ag(NH_3)_2F$, *Actinomyces naeslundii*, Dentin microhardness, Element quantitative analysis, Artificial mouth system**

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Introduction

With the increase of the dentate elderly, root caries has become an important clinical issue in dentistry. In spite of emerging as a common cause of tooth loss in the elderly, the initiation and progression of root caries remains unclear[1,2]. In contrast, the caries lesion on enamel has been studied thoroughly. Moreover, many studies have shown that various fluoride compound applications prevent caries and retard caries lesion progress[3-6]. However, the prevention of root caries by fluorides has been supported by little evidence in past studies, due to compositional and anatomical complexities of dentin[7,8].

The 3.8% and 38% of silver diamine fluoride solutions, $Ag(NH_3)_2F$, were developed in Japan[9]. The 38% $Ag(NH_3)_2F$ solution has been studied mostly in Japan and used in Asian countries as a caries arresting agent[10] and a dentin desensitizing agent[11]. The 3.8% $Ag(NH_3)_2F$ solution, however, was approved only as a root canal treatment agent by the Ministry of Health, Labour and Welfare in Japan in 1979[12,13].

Therefore, we decided to examine of the anticariologic effect of two different concentrated $Ag(NH_3)_2F$ solutions on dentin caries *in vitro*. The purpose of this study was to evaluate and to compare the anticaries effect of the 3.8% and 38% $Ag(NH_3)_2F$ solutions in an artificial mouth model system.

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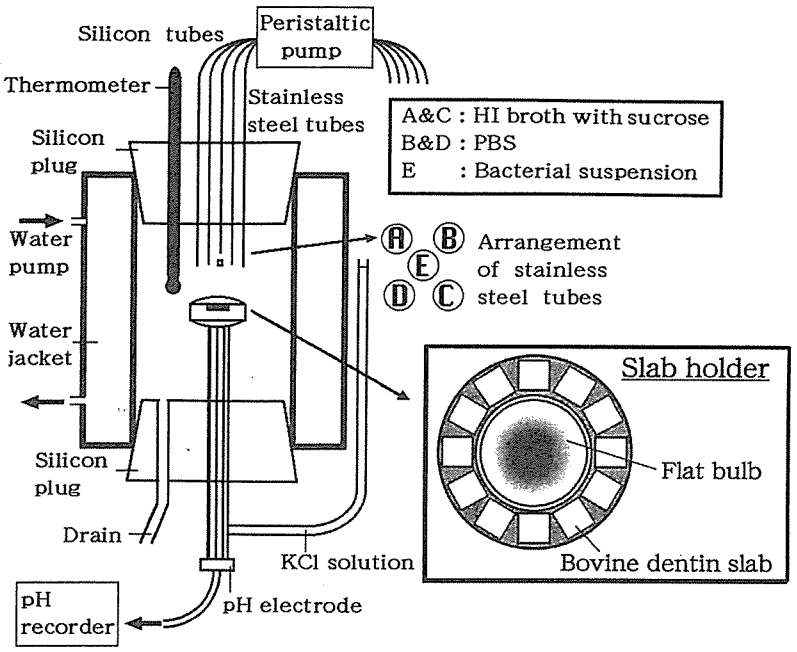


Fig. 1 A diagram of the artificial mouth model system

Materials and Methods

1. Reagent

The 3.8% and 38% of silver diamine fluoride solutions, $Ag(NH_3)_2F$ (Saforide RT and Saforide, Bee Brand Medico Dental Co. Ltd., Osaka, Japan), were used in this experiment. The 38% $Ag(NH_3)_2F$ solution contains 30 w/v % of AgF and approximately 8 w/v% of NH_3 [9].

2. Preparation of bacterial broths

Actinomyces naeslundii JCM 8349 was selected. Following pre-culture, *A. naeslundii* was anaerobically incubated in a brain heart infusion broth (BHI : Difco Laboratories, Detroit, MI) at 37℃ for 24 hours. The cell suspension of bacteria in 0.1 M phosphate-buffered saline (PBS) solution was prepared to optical density at 500 nm (OD_{500}) = 2.0 (5.0×10^8 colony forming units/m*l*).

3. Preparation of dentin slabs

Bovine dentin slabs, 3.5 × 3.5 × 1.2 mm in size, with flat surfaces polished by a 1 micron grade polishing film, were prepared from bovine unerupted lower incisors cleaned of soft tissue debris. All slabs were sonicated at 11 W for 20 seconds for removal of debris from the dentinal tubules. Then, all slabs were measured for hardness at nine symmetrical points on the test surface of each slab with a diamond Vickers indenter on a microhardness tester loaded with 50 g or 100 g (Hardness Tester MVK-E, AKASHI, Japan). The mean and standard deviation of dentin hardness of each slab were calculated as a baseline. Dentin slabs of 55 to 65 in average Vickers hardness number were used in this experiment. Finally, 36 dentin slabs were selected.

4. Treatment groups

A total of 36 dentin slabs were equally divided into two experimental groups and a control group. One application of 38% $Ag(NH_3)_2F$ for 10 seconds by a cotton-tipped swab was applied to 12 dentin slabs dried out by an air-syringe prior to placement in the artificial mouth. In the same preparation, three applications of 3.8% $Ag(NH_3)_2F$ at 12 hour intervals were applied to 12 dentin slabs prior to placement in the artificial mouth. The remaining 12 dentin slabs served as controls and did not receive application of 38% $Ag(NH_3)_2F$ solution.

5. Artificial mouth model system and sampling

Three identical artificial mouths were used. Figure 1 shows a diagram of an artificial mouth. Each artificial mouth consisted of an 18 mm diameter Teflon holder around a 9 mm flat bulb of a pH electrode. The pH electrode was set upside down in the center of the artificial

mouth constantly supplied with Heart Infusion broth medium (HI : Difco Laboratories, Detroit, MI) supplemented with 2.5% sucrose, PBS, and *A. naeslundii* suspension at 37℃ for 60 hours, continuously dropped at 3 m*l* per hour per tube. There were five tubes in the artificial mouth system ; two for HI medium, two for PBS, and one for bacterial suspension. The prepared dentin slabs and control slabs were seated on the Teflon holder. Change in pH underneath artificial biofilm was automatically recorded. All procedures were carried out under aseptic conditions. Three slabs at each interval were sampled for post-examinations at 24, 36, 48, and 60 hours.

6. Determination of total amount of artificial biofilm

The specimens were sonicated in 1 m*l* of PBS at 11 w for 10 seconds for removal of artificial biofilm. Optical density of artificial biofilm at 540 nm was measured to assess the accumulated artificial biofilm on surface of dentin specimens (Double-beam Spectrophotometer, UV-190, SHIMADZU, Japan).

7. Measurement of microhardness

The change in microhardness for each dentin specimen after exposure to the bacterial environment for each interval was measured as already described. The change in dentin hardness (ΔDH) between baseline and post-experiment hardness values for the control and test specimens was calculated [ΔDH = (baseline-hardness) − (post-hardness)]. Next, the percentage of changed hardness (%CDH) was calculated [%CDH = ΔDH × 100/ (baseline-hardness)].

8. Scanning electron microscopy (SEM) and energy-dispersive X-ray analysis

After sonication with 11 W for 10 seconds, 24 specimens were prepared for scanning electron microscopy and element quantitative analysis. They were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate-buffered saline solution (pH 7.4) for 1 hour at room temperature. Each fixed specimen was then dehydrated through a graded ethanol series until a value of 99.5% was reached, and dried in a critical point dryer (HCP-2, Hitachi, Japan). Next, they were osmium plasma coated by a Neoc Pure Osmium Coater (Meiwa Shoji, Japan) for element quantitative analysis using an energy dispersive X-ray micro analyzer (EMAX EX-220, Horiba, Japan) and for scanning electron microscope observation (S-5200, Hitachi, Japan). Energy dispersive X-ray micro analyzer was performed at an acceleration voltage of 10 kV and magnification of × 5000.

9. Statistical analysis

A One-Way Analysis of Variance and Tukey Post-hoc Test were performed to assess the differences found in the outcomes among control group and two treatment groups at each interval. Moreover, Student's t-test was done for the results of the element quantitative analysis (Statcel, 2nd ed., OMS, Saitama, Japan).

Results

1. The change in pH

Figure 2 expresses the change in pH on an electrode covered with accumulated artificial biofilm in each artificial mouth. Compared to the control whose terminal pH was 5.93, the terminal pHs of the 3.8% and 38% Ag $(NH_3)_2F$ solution groups had inhibited decline, and were 6.09 and 6.44 respectively.

2. The change in total amount of artificial biofilm

Figure 3 shows the amount of artificial biofilm accumulation on specimens. There was statistically significantly less artificial biofilm accumulation in the experimental groups compared to the control group. On the other hand, there was no statistical difference between 3.8% and 38% Ag $(NH_3)_2F$ solution groups.

3. The change in dentin microhardness

Figure 4 shows the change in microhardness of dentin at each interval in the artificial mouth system. Terminal changed dentin hardness of specimens in the control group became more than 75% (75.87 ± 2.52%), while the figures for the 3.8% and 38% Ag $(NH_3)_2F$ solution groups were more than 50% (51.94 ± 3.77%) and 30% (32.53 ± 1.76%) respectively. For specimens treated with Ag $(NH_3)_2F$ solutions, there was statistically significantly less reduction of dentin hardness than in the control group. However, there was no statistically significant difference at most of the intervals between the specimens treated with 3.8% $Ag(NH_3)_2$ and 38% $Ag(NH_3)_2$ solutions, except for the value of reduced hardness at 60 hours.

4. Silver and fluorine elements microanalysis

Figure 5 presents an example of the images of element quantitative analysis by the energy-dispersive X-

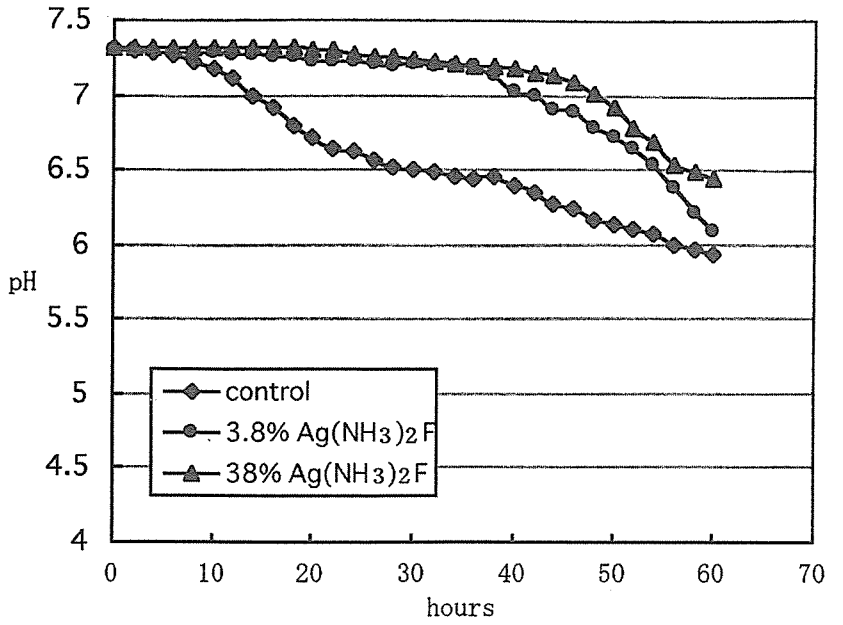


Fig. 2 The change in pH underneath artificial biofilm on an electrode in each artificial mouth.

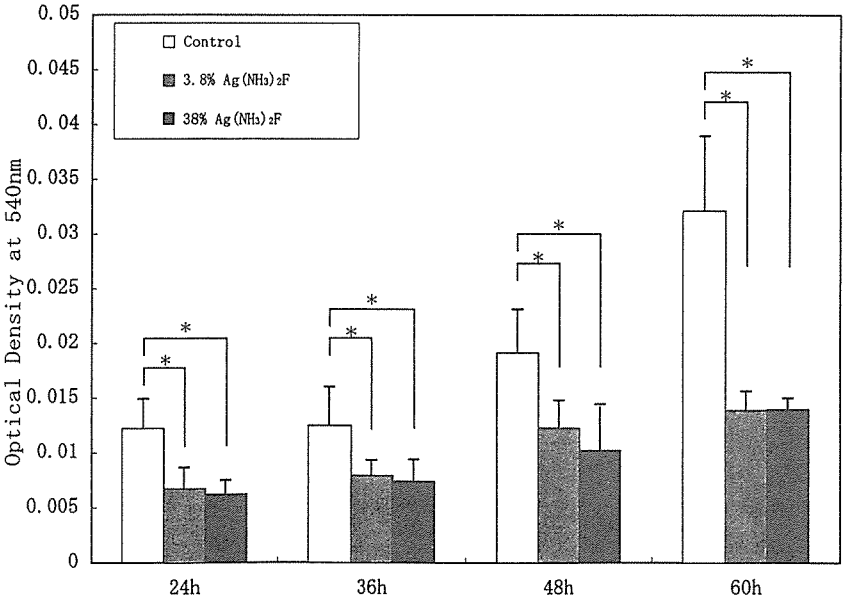


Fig. 3 Means and standard deviations of change in total amount of artificial biofilm at each interval, 24, 36, 48, and 60 hours
*Denotes a significant relationship at the 0.05 level

ray micro analyzer. Table 1 shows the percentage of weight of residual silver and fluorine elements on the specimen surfaces at 0, 24, 48, and 60 hours. The amounts of silver elements on the specimen surface of the two groups were not significantly different after a lapse of 60 hours. Also there was mostly no statistically significant amount of residual fluorine from the 3.8% and 38% $Ag(NH_3)_2F$ applications on the dentin surfaces.

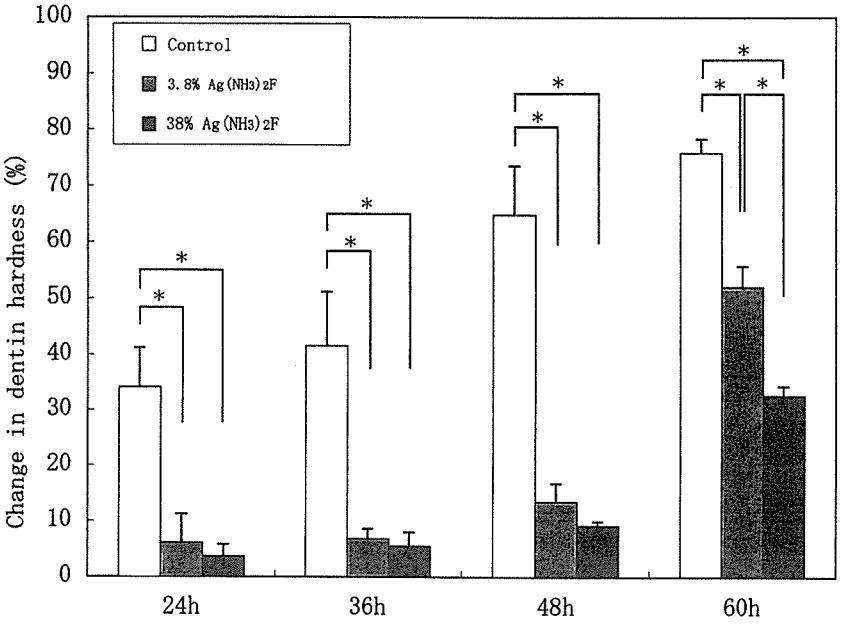
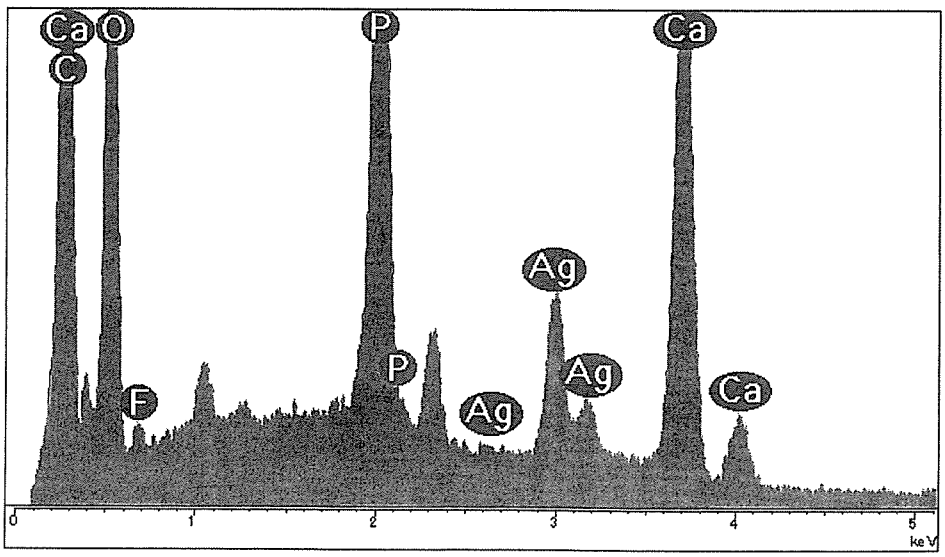


Fig. 4 The percentage of means and standard deviations of changed dentin microhardness for 3.8% $Ag(NH_3)_2F$, 38% $Ag(NH_3)_2F$, and control at each interval, 24, 36, 48, and 60 hours
*Denotes a significant relationship at the 0.05 level



Elements	C	O	Ca	P	F	Ag	total
Weight %	19.95	27.61	24.63	13.13	1.52	13.17	100

Fig. 5 An example of the images of element quantitative analysis for silver and fluorine elements by an energy dispersive X-ray micro analyzer
Specimen with 38% $Ag(NH_3)_2F$ (48 hours) : full scale 792, count cursor : 5.012 keV

Table 1 Means and standard deviations of residual silver (A) and fluorine (B) (w%) on specimens

A : Silver	0 hour	24 hours	48 hours	60 hours
3.8%Ag $(NH_3)_2F$	8.77 ± 2.52	1.78 ± 0.23	2.00 ± 1.32	1.70 ± 1.68
38%Ag $(NH_3)_2F$	16.2 ± 3.22 *	13.30 ± 5.12 *	13.32 ± 1.09 *	2.68 ± 1.49
B : Fluorine	**0 hour**	**24 hours**	**48 hours**	**60 hours**
3.8%Ag $(NH_3)_2F$	14.33 ± 4.00	10.36 ± 1.18	3.45 ± 0.47	2.56 ± 0.43
38%Ag $(NH_3)_2F$	14.43 ± 4.91	11.17 ± 3.32	2.24 ± 0.33 *	2.02 ± 1.00

* Denotes a significant relationship at the 0.05 level

Discussion

1. Artificial mouth system and bacteria used

Artificial mouth laboratory models were developed in the late 19th century. Since then, artificial mouth model systems have been investigated and improved by overcoming several problems, such as contamination of the system and difficulty to mimic complicated human oral cavity[14]. Moreover, as ethical problems related to studying dental caries in humans have recently been raised, the artificial mouth model systems have been useful not only to investigate the etiology of dental caries, but also to seek preventive methods. Thus, the use of artificial mouth systems for dental caries experimens has been extremely useful[15].

The artificial mouth model system in our laboratory was developed in 1984[16]. The characteristic of it is that the changes in pH in the artificial biofilm are constantly measured with a glass electrode. Many experiments to evaluate anti-cariogenic food and medicine with this system have been done in our laboratory. The results from the repeated experiments have had good reproducibility of the pH curves[17]. Thus, it is clearly suggested that this system has reliability as an *in vitro* system.

Actinomyces spp. has been implicated as a root caries pathogen although their initiation and progression have not been fully understood[18]. From those results, however, the control group finally reached pH 5.93, which was below a critical point of dentin decalcification, and had more than 75% (75.87 ± 2.52) of dentin hardness reduction after a lapse of 60 hours. Moreover, when pH of the control group reached below pH 6.2 after a lapse of 48 hours, more dentin hardness reduction occurred. Similarly, drastic hardness reduction started at around pH 6.3 for both experimental groups. Although those pH values might not demonstrate accurate pHs located on the specimens in our experimental model, this finding verifies previous studies that *A. naeslundii* itself is capable of producing acidic substances to decalcify dentin structure.

2. Silver diamine fluoride : $Ag(NH_3)_2F$

Silver has been used as a disinfectant with a long history. The efficacy of silver includes anti-bacterial, anti-viral and anti-fungal effects. Silver is thought to be non-toxic in humans at standard rates of consumption. Moreover, the efficacy of the silver compound for periodontal pathogens has been reported recently[19,20].

$Ag(NH_3)_2F$ was developed by Yamaga et al. in 1966. The safety and toxicity of 38% $Ag(NH_3)_2F$ solution has been well-evaluated and it has been proven to be a safe anti-caries agent[21,22].

The primary purpose of development of 38% $Ag(NH_3)_2F$ solution was to reduce deciduous tooth caries lesions clinically[23]. Chu et al. discussed the effectiveness of 38% $Ag(NH_3)_2F$ solution comparing to NaF varnish for deciduous teeth of Chinese pre-school children. They indicated that the examiner could not distinguish in post-experiment evaluation either application of NaF varnish or $Ag(NH_3)_2F$ solution, because all arrested caries lesions on the subjects were similar ; that is, the appearance of arrested dentin caries seemed to be blackening and hardening. Moreover, they concluded that an-

nual application of 38% Ag $(NH_3)_2F$ solution for preschool children was more effective in hardening or arresting dentin caries lesions in upper anterior deciduous teeth than the three-month applications of a 5% NaF varnish[24].

The clinical use of 3.8% Ag $(NH_3)_2F$ solution is a medication for root canal treatment. The studies have revealed that 3.8% Ag $(NH_3)_2F$ solution has capability to occlude dentinal tubules and to resist oral bacterial growth[25,26]. We recognized the advantage of this characteristic to treat flat dentin surfaces.

From this experiment, outcomes show that the percentage of the reduction of post-hardness of 3.8% Ag $(NH_3)_2F$ and 38% Ag$(NH_3)_2F$ groups were not significantly different although the hardness of 3.8% and 38% Ag $(NH_3)_2F$-coated specimens were remarkably maintained compared to the hardness of controls. Moreover, artificial biofilm formation with test solutions was significantly inhibited due to the anti-bacterial effect of silver. Furthermore, residual elements of silver and fluorine on the dentin surfaces with both test solutions were not significant after a lapse of time. Thus, it indicates that 3.8% Ag$(NH_3)_2F$ solution might have a similar effectiveness to 38% Ag$(NH_3)_2F$ solution as an anti-caries agent.

The overall impression from this study is that the usage of 3.8% Ag$(NH_3)_2F$ solution may be one of the solutions for the prevention of dentin caries, especially for the dentate elderly and persons with difficulty in proper home care. It might be expected that 3.8% Ag$(NH_3)_2F$ solution would be less harmful to oral soft tissue and less staining on teeth. Future investigations are expected to determine whether 3.8% Ag $(NH_3)_2F$ solution is clinically suitable for the prevention of root caries and to define the frequency of application of 3.8% Ag $(NH_3)_2F$ solution to get maximum efficacy. Moreover, its capability and limitation for the root-caries-preventive effect need to be investigated. This study, however, may help develop clinical strategies to prevent the initiation and progression of root caries.

References

1) Aamdal-Scheie A, Luan WM, Dahlen G et al. : Plaque pH and microflora of dental plaque on sound and caries root surfaces. J Dent Res 75 : 1901-1908, 1996.
2) Brailsford SR, Shah B, Simons D et al. : The predominant aciduric microflora of root-caries lesions. J Dent Res 80 : 1828-1833, 2001.
3) McDonald SP, Sheiham A : A clinical comparison of non-traumatic methods of treating dental caries. Int Dent J 44 : 465-470, 1994.
4) Sampaio FC, Nazmul Hossain ANM, von der Fehr FR et al. : Dental caries and suger intake of children from rural areas with different water fluoride levels in Paraiba, Brazil. Community Dent Oral Epidemiol 28 : 301-313, 2000.
5) Pearce EIF, Dibdin GH : The effect of pH, temperature and plaque thickness on the hydrolysis of monofluorophosphate in experimental dental plaque. Caries Res 37 : 178-184, 2003.
6) Ammari AB, Bloch-Zupan A, Ashley PF : Sysytematic review of studies comparing the anti-caries efficacy of children's toothpaste containing 600 ppm of fluoride or less with high fluoride toothpastes of 1, 000 ppm or above. Caries Res 37 : 85-92, 2003.
7) Heilman JR, Jordan TH, Warwick R et al. : Remineralization of root surfaces demineralized in solutions of differing fluoride levels. Caries Res 31 : 423-428, 1997.
8) Nyvad B, ten Cate JM, Fejerskov O : Arrest of root surface caries *in situ*. Caries Res 76 : 1845-1853, 1997.
9) Yamaga R, Higashi S, Ida K : Preventive material for dental caries. Jpn J Dent Mater 16 : 10, 1966. (in Japanese)
10) Nishino M : Studies on the topical application of ammoniacal silver fluoride for the arrestment of dental caries. J Osaka Univ Dent Sch 14 : 1-14, 1969. (in Japanese)
11) Murase M, Takai H : Effect of ammoniacal silver fluoride on cervical hypersensitivity. Nippon Dent Rev 323 : 112-116, 1969. (in Japanese)
12) Nagao K : Fundamental study of effect of 3.8% Ag$(NH_3)_2F$ solution on dentin structure. J Osaka Odnt Soc 42 : 413-429, 1979. (in Japanese)
13) Yokoyama K, Matsumoto K, Murase J : Permeability of the root canal wall and occlusion of dentinal tubules by Ag $(NH_3)_2F$: a comparison of combined use with pulsed Nd : YAG laser or iontophoresis. J Clin Laser Med Surg 18 : 9-14, 2000.
14) Tang G, Yip HK, Cutress TW et al. : Artificial mouth model system and their contribution to caries research : a review. J Dent 31 : 161-171, 2003.
15) Shu M, Wong L, Miller JH et al. : Development of multispecies consorta biofilm of oral bacteria as an enamel and root caries model system. Arch Oral Biol 45 : 27-40, 2000.
16) Hinoide M, Imai S, Nisizawa T : New artificial mouth for evaluation of plaque accumulation, pH changes underneath the plaque, and enamel demineralization. Jpn J Oral Biol 26 : 288-291, 1984.
17) Kamoda T : Effect of disaccharide xylosylfructoside on cariogenicity of sucrose in artificial mouth system. [thesis for a doctorate]. The Nippon Dental University, 2003. (in Japanese)
18) Brailsford SR, Tregaskis RB, Leftwich HS et al. : The predominant *Actinomyces* spp. Isolated from infected dentin of active root caries lesions. J Dent Res 78 : 1525-1534, 1999.
19) Spacciapoli P, Buxton D, Rothstein D et al. : Antimicrobial activity of silver nitrate against periodontal pathogens. J Periodontal Res 2 : 108-13, 2001.
20) Straub AM, Suvan J, Lang NP et al. : Phase 1 evaluation of a local delivery device releasing silver ions in periodontal pockets : safety, pharmacokinetics and bioavailability. J Periodontal Res 3 : 187-193, 2001.

21) Maehara S, Ito N, Onari S et al.：Acute and chronic toxicity test in evaluation of 38% $Ag(NH_3)_2F$ solution in rats. Bull Kanagawa Dent Univ 8：103–110, 1973. (in Japanese)
22) Yoshikawa M：The toxicity of 38% Ag $(NH_3)_2F$ solution with a series of oral administration. J Osaka Univ Dent 38：459–475, 1975. (in Japanese)
23) Nishino M, Yoshida S, Sobue S et al.：Effect of topically applied ammoniacal silver fluoride on dental caries in children. J Osaka Univ Dent Sch 9：149–155, 1969.
24) Chu CH, Lo EC, Lin HC：Effectiveness of silver diamine fluoride and sodium fluoride varnish in arresting dentin caries in Chinese pre-school children. J Dent Res 81：767–770, 2002.
25) Suzuki K, Okubo T：Root canal treatment by saforide-treatment for root caries. Dental Outlook 43：99–102, 1974. (in Japanese)
26) Katsuumi I：Basic studies of Ag $(NH_3)_2F$ as a root canal disinfectant. Jpn Dent 65：934–950, 1978. (in Japanese)

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Actinomyces naeslundii を用いた人工口腔装置における 3.8% フッ化ジアミン銀の象牙質う蝕抑制効果

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概要：有歯顎高齢者が増加している今日にあって，加齢や歯周病の進行などの原因により根面露出した歯は，潜在的に根面カリエス罹患のリスクが高くなるといわれている．*Actinomyces naeslundii* は，根面カリエスの重要な原因菌のひとつと考えられている．本研究は，*A. naeslundii* をう蝕誘発菌として使用し，人工口腔装置内における 3.8 および 38% フッ化ジアミン銀水溶液の象牙質う蝕抑制効果を比較検討することを目的とした．

36 個のウシ象牙質歯片を 3.8% フッ化ジアミン銀水溶液 3 回塗布群および 38% フッ化ジアミン銀水溶液 1 回塗布群とコントロール群の 3 つのグループに分け，人工口腔装置中で 60 時間観察した．う蝕抑制効果の評価として，pH 測定，象牙質硬度測定，，人工バイオフィルム蓄積量の測定，象牙質表面における残留銀量およびフッ素量の微小 X 線元素分析を経時的に行った．

その結果，3.8 および 38% フッ化ジアミン銀水溶液塗布群はコントロール群と比べ，著しく人工バイオフィルムの蓄積と象牙質の硬度減少を抑制することがわかった．一方，3.8 と 38% フッ化ジアミン銀水溶液塗布群間には統計学的な有意差はみられなかった．また，残留銀およびフッ素の微小 X 線元素分析の結果により，60 時間経過後の銀とフッ素の残留量は，3.8 と 38% のフッ化ジアミン銀水溶液塗布群間に有意差がないことが明らかとなった．

以上のことから，3.8% フッ化ジアミン銀水溶液に 38% フッ化ジアミン銀水溶液と同等の象牙質う蝕抑制効果が期待されることが示唆された．

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Original Articles

Relationships between Breath Odors and Odor Emission Sources in the Elderly Investigated using the Electronic Nose

Amiko Hayashida[1], Susumu Imai[2], Nobuhiro Hanada[2], Keika Hoshi[1] and Hiroshi Uematsu[1]

Abstract : The aim of this study is to compare breath odors and odor emission sources (saliva, tongue coat, and dentures in current use) of the elderly and to define the relationships between the odors. Fifteen patients visiting the outpatient clinic, Tokyo Medical and Dental University Geriatric Dentistry, were recruited. The breath, saliva, denture, and tongue coat of each individual were collected as samples. Then, the gas evaporating from each sample was saved in a sampling bag. The sampling gas was measured by an electronic nose (Odor Discrimination Analyzer FF-1, Shimazdu, Japan). In principle component analysis, principle component 1 showed significant differences between three pairs of items : breath odor (BR) and the odor of the saliva (SL), breath odor and the odor of the denture (DE), and breath odor and the odor of tongue coat (TC) ($p<0.001$). There were no significant differences between SL and TC, SL and DE, and TC and DE. The mean differences between BR and the each odor emission source showed that DE was the nearest to BR, then SL, and TC lastly. In this study, TC, previously named as one of the main causes of breath odor, has no relation with BR. Moreover, TC is related to SL and DE in the elderly. It is suggested that one of the main odor emission sources of breath odor may be the dentures.

Key words : Breath odor, Elderly, Electronic nose, Bad breath

Introduction

Organoleptic judgments and measuring concentration of particular substances known to be the causes of odors have been applied to measurement of odors. It is well known that organoleptic judgment and measurement of volatile sulfur compounds (VSC) are the most standardized method for breath odor evaluation at this time[1,2].

An electronic nose (e-nose) developed recently mimics the human nose sensing mechanism, which discriminates various odors by pattern-recognitions of the responses in olfactory receptor[3]. The e-nose used in this study (Odor Discrimination Analyzer FF-1, Shimazdu, Japan) is equipped with MOS (metal-oxcide-semiconductor) sensors as alternatives to olfactory receptors, and the e-nose recognizes the sensor patterns for discriminating odors[1]. Thus, the e-nose system enables objective assessment of the odor discrimination of the different types of odors.

The aim of this study is to compare breath odors and odor emission sources (saliva, tongue coat, and dentures in current use) of the elderly and to define the relationships among the odors.

Materials and Methods

1. Subjects

All of the subjects were recruited among the patients visiting the outpatient clinic, Tokyo Medical and Dental University Geriatric Dentistry (15 patients : male = 9, female = 6 : mean age 73.9±4.1 yrs). The inclusion criterion was to have

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communication skills for understanding instructions. The exclusion criterion was to have communication problems. All subjects signed a written informed consent document. Permission for this study was obtained from the Ethical Committee for Clinical Research of Tokyo Medical and Dental University (No. 66).

2. Methods

At the initial visit, the patients' chief complaint and medical history were obtained. Periodontal examinations and dental examinations were performed. On the second visit, breath odor was assessed by both organoleptic judgment and by e-nose measurement. Odor samples of the dentate patients were taken from 1) the breath, 2) the saliva, and 3) the tongue coat. Denture wearers provided on additional odor sample from 4) the surface of the denture (8 patients : male = 4, female = 4).

2-1. Organoleptic judgment of the breath odor

Prior to the odor assessment, the subjects were instructed to abstain from oral activities such as eating and drinking, from oral hygiene such as brushing and mouth rinsing, and from smoking on the morning of the test day[5]. Odor assessment was performed between 9 : 00 and 10 : 30 AM[1]. The subjects were instructed to keep the mouth closed and to breathe through the nose for a minute. Then, the subjects were requested to exhale slowly facing the odor judge. One trained panel performed the organoleptic judgment. Organoleptic results were recorded on a scale of 0 to 5 as follows : 0 : no appreciable odor ; 1 : barely noticeable odor ; 2 : slight, but clearly noticeable odor ; 3 : moderate odor ; 4 : strong odor ; 5 : extremely unpleasant odor [6].

2-2. Measurements by electronic nose

1) Odor sample of the breath odor (BR)

Again, subject was instructed to keep the mouth closed and to breathe through the nose for a minute. Subject was then requested to exhale into a three-liter polyester bag (Flek-Sampler® NIOIBUKURO, OMI ODORAIR SERVICE Co. Ltd., Japan). The duration of the exhaling was two to three seconds to prevent dilution of mouth odor with lung air. This procedure was repeated 10 times.

2) Odor sample of the saliva (SL)

After the breath odor assessment, each subject donated a sample of whole saliva, which was collected by chewing paraffin for five minutes. The collected saliva was injected into a three-liter polyester bag, and then the bag was filled with pure nitrogen. The saliva was kept in the polyester bag for an hour in room air (mean temperature 24.1℃, mean humidity 53.3%). The gas evaporated from the saliva was transferred into another polyester bag for the e-nose measurement.

3) Odor sample of the tongue coat (TC)

The subjects were instructed to keep the mouth wide-open and to stick the tongue out. Then, saliva on the dorsal surface of the tongue was removed with a tissue (Kim Wipe, USA). The tongue coat was removed with a small spoon from the terminal sulcus to the apex of the tongue[7]. The spoon used to scrape the tongue coat was put in a three-liter polyester bag, and then the bag was filled with pure nitrogen. The spoon used to scrape the tongue coat was kept in the polyester bag for an hour in room air (mean temperature 24.1℃, mean humidity 53.3%). The gas evaporated from the tongue coat was transferred into another polyester bag for the e-nose measurement.

4) Odor sample of the denture (DE)

The denture of the subject was put in a three-liter polyester bag, and then the bag was filled with pure nitrogen. The denture was kept in the polyester bag for an hour in room air (mean temperature 23.8℃, mean humidity 51.9%). The gas evaporated from the denture was transferred into another polyester bag for the e-nose measurement. The denture was returned to the subject immediately after the sampling gas was collected.

The operating sequence of the e-nose is as fol-

lows : (a) sampling : the gas sample is sucked into the preconcentrator (a trap tube to concentrate the sample gas). The flow rate is 165 cc/min with a the sampling time of 60 seconds, (b) top-note measurement mode : the trapped odor is driven to the sensor section and the odor is detected by the six MOS sensors, (c) drying : the gas sample trapped in the preconcentrator is dried with pure nitrogen, (d) deep-note measurement mode : the preconcentrator is heated from 40℃ to 220℃, then the trapped odor is driven to the sensor section for the six MOS sensors to detect the odor, (e) cleaning : the preconcentrator is heated to 250℃ in order to clean out the remaining odor. The top-note measurement mode of the sensors one to six is shown as channels one to six, and the deep-note measurement mode is shown as channels seven to twelve.

Each of the sample gases was measured seven times, and the first two responses were not used for data analysis because of the poor stability due to sensor performance. The means of the latter five responses were used for data analysis.

3. Statistical analysis

Principal component analysis (PCA) is a method of identifying patterns in data, and describing the data to highlight their similarities and differences. The data of the twelve sensor responses are identified their similarities and differences in terms of principal component (PC) by PCA. (SPSS. Ver11.0, USA). Therefore, the twelve sensor responses are summarized into two (PC1 + PC2) to three (PC1 + PC2 + PC3) dimensions in order to emphasize the similarities and differences of the data.

PC1 is an axis which describes the largest distribution of the data simplified by PCA. PC2 is the second largest axis of perpendicular to PC1, and PC3 is the third largest axis of perpendicular to PC2.

PC 1, 2, and 3 (Eigenvalues >1) were analyzed by one-way ANOVA with Scheffé's test for multiple comparisons. The mean distances between the odors show the similarities and differences between the odors. The farther plots in the results of the PCA shows the different patterns in the data. The nearer plots in the PCA results show the similar patterns in the data. Significance was set at $p<0.05$.

Results

The result of the organoleptic judgment of the breath odor is shown on Table 1. There are 11 subjects whose scores are 0 or 1 (no oral malodor), and there are four subjects whose scores are over 2 (presence of oral malodor).

BR and the other odors were separated. SL, TC, and DE were not clearly separated (Figure 1a). BR and the others are separated upon PC 1, but not separated on PC 2 (Figure 1b).

When data of BR and SL were extracted, two odors were clearly separated on PC 1, but not sep-

Table1. Results of the organoleptic judgement of the breath odor

scale		number of subjects
0	no appreciable odor	5
1	barely noticeable odor	6
2	slight, but clearly noticeable odor	3
3	moderate odor	0
4	strong odor	1
5	extremely unpleasant odor	0

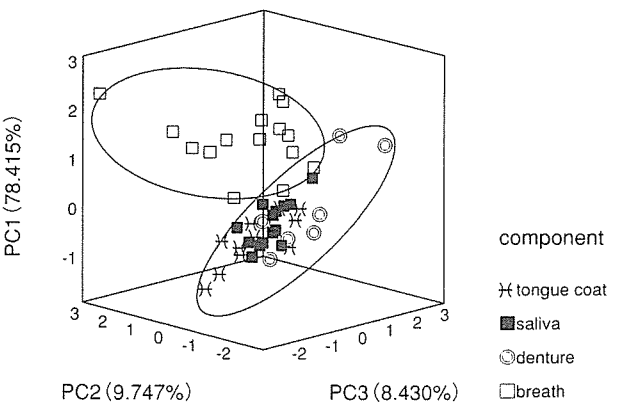

Figure 1a. Results of PCA among breath, saliva, tongue coat and denture. Y-axis : PC 1, X-axis : PC 2, Z-axis : PC 3. Contribution ratio% in parentheses.
□breath ◎denture ■saliva ⋕tongue coat