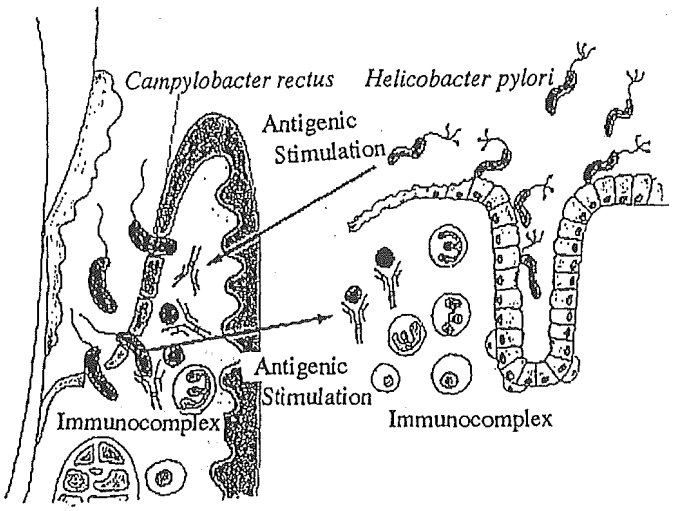

Figure 5.
Cross-reacted antigens between C. rectus and H. pylori may relate to the induction of immunopathological inflammation in the stomach and periodontal tissues infected H. pylori.

study is close to the GroEL-like protein (64 kDa). HSPs such as GroEL (HSP 60) have elicited autoreactive antibodies and have been thought to be involved in autoimmune disease.[36] As shown in Figure 5, these cross-reacted antigens between *C. rectus* and *H. pylori* may induce immunopathological inflammation in both the stomach and periodontal tissues infected with *H. pylori*.

We reported that there was a close relationship between antibody responses to HSPs in some patients with pustulosis palmaris et plantaris.[33,37] To confirm the relationship between the cross-reactivity and the responses against *H. pylori* in these patients, further analyses using sera from subjects infected with both species and with a single species are required. We concluded that the cross-reacting antigens in *C. rectus* and *H. pylori* strains may be related to the induction of immunopathological responses not only in the stomach, but also in periodontal tissues.

ACKNOWLEDGMENTS

This research was supported in part by a grant from the Oral Health Science Center, the Hightech Research Center of Tokyo Dental College.

REFERENCES

1. Konturek PC, Bielanski W, Konturek SJ, Hahn EG. *Helicobacter pylori* associated-gastric pathology. *J Physiol Pharmacol* 1999;50:695-710.
2. Dowsett SA, Archila L, Segreto VA, et al. *Helicobacter pylori* infection in indigenous families of Central America: Serostatus and oral and fingernail carriage. *J Clin Microbiol* 1999;37:2456-2460.
3. Ferguson DAJ, Li C, Patel NR, Mayberry WR, Chi DS, Thomas E. Isolation of *Helicobacter pylori* from saliva. *J Clin Microbiol* 1993;31:2802-2804.
4. Li C, Musich PR, Ha T, et al. High prevalence of *Helicobacter pylori* in saliva demonstrated by a novel PCR assay. *J Clin Pathol* 1995;48:662-666.
5. Mravak-Stipetic M, Gall-Troselj K, Lukac J, Kusic Z, Pavelic K, Pavelic J. Detection of *Helicobacter pylori* in various oral lesions by nested polymerase chain reaction (PCR). *Oral Pathol Med* 1998;27:1-3.
6. Dore-Davin C, Heitz M, Yang H, Herranz M, Blum AL, Corthesy-Theulaz I. *Helicobacter pylori* in the oral cavity reflects handling of contaminants but not gastric infection. *Digestion* 1999;60:196-202.
7. Song Q, Lange T, Spahr A, Adler G, Bode G. Characteristic distribution pattern of *Helicobacter pylori* in dental plaque and saliva detected with nested PCR. *J. Med Microbiol* 2000;49:349-353.
8. Miyabayashi H, Furihata K, Shimizu T, Ueno I, Akamatsu T. Influence of oral *Helicobacter pylori* on the success of eradication therapy against gastric *Helicobacter pylori*. *Helicobacter* 2000;5:30-37.
9. Mapstone NP, Lynch DA, Lewis FA, et al. Identification of *Helicobacter pylori* DNA in the mouths and stomachs of patients with gastritis using PCR. *J Clin Pathol* 1993;46:540-543.
10. Jaskowski TD, Martins TB, Hill HR, Litwin CM. Immunoglobulin A antibodies to *Helicobacter pylori*. *J Clin Microbiol* 1997;35:2999-3000.
11. Krajden S, Fuksa M, Anderson J, et al. Examination of human stomach biopsies, saliva, and dental plaque for *Campylobacter pylori*. *J Clin Microbiol* 1989;27:1397-1398.
12. Bickley J, Owen RJ, Fraser AG, Pounder RE. Evaluation of the polymerase chain reaction for detecting the urease C gene of *Helicobacter pylori* in gastric biopsy samples and dental plaque. *J Med Microbiol* 1993;39:338-344.
13. Asikainen S, Chen C, Slots J. Absence of *Helicobacter pylori* in subgingival samples determined by polymerase chain reaction. *Oral Microbiol Immunol* 1994;9:318-320.
14. Oshowo A, Gillan D, Botha A, et al. *Helicobacter pylori:* The mouth, stomach, and gut axis. *Ann Periodontol* 1998;3:276-280.
15. Shankaran K, Desai HG. *Helicobacter pylori* in dental plaque. *J Clin Gastroenterol* 1995;21:82-84.
16. Marshall B, Howat AJ, Wright PA. Oral fluid antibody detection in the diagnosis of *Helicobacter pylori* infection. *J Med Microbiol* 1999;48:1043-1046.
17. Fallone CA, Elizov M, Cleland P, et al. Detection of *Helicobacter pylori* infection by saliva IgG testing. *Am J Gastroenterol* 1996;91:1145-1149.
18. Luzza F, Oderda G, Maletta M, et al. Salivary immunoglobulin G assay to diagnose *Helicobacter pylori* infection in children. *J. Clin Microbiol* 1997;35:3358-3360.
19. Bogstedt AK, Nava S, Wadstrom T, Hammarstrom L. *Helicobacter pylori* infections in IgA deficiency: Lack of role for the secretory immune system. *Clin Exp Immunol* 1996;105:202-204.
20. Johansen HK, Norgaard A, Andersen LP, Jensen P, Nielsen H, Hoiby N. Cross-reactive antigens shared by *Pseudomonas aeruginosa, Helicobacter pylori, Campylobacter jejuni,* and *Haemophilus influenzae* may cause false-positive titers of antibody to *H. pylori*. *Clin Diagn Lab Immunol* 1995;2:149-155.
21. Tanner A, Maiden MF, Macuch PJ, Murray LL, Kent RL Jr. Microbiota of health, gingivitis, and initial periodontitis. *J Clin Periodontol* 1998;25:85-98.
22. Ishihara K, Miura T, Ebihara Y, Hirayama T, Kamiya S, Okuda K. Shared antigenicity between *Helicobacter*

pylori and periodontopathic *Campylobacter rectus* strains. *FEMS Microbiol Lett* 2001;197:23-27.
23. Costerton JW, Cheng KJ, Gessey GG, et al. Bacterial biofilms in nature and disease. *Annu Rev Microbiol* 1987; 41:435-464.
24. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: A common cause of persistent infections. *Science* 1999;284:1318-1322.
25. Kolenbrander PE. Oral microbial communities: Biofilms, interactions, and genetic systems. *Annu Rev Microbiol* 2000;54:413-437.
26. Kolenbrander PE, Anderson RN, Moore LV. Coaggregation of *Fusobacterium nucleatum, Selenomonas fleggei, Selenomonas infelix, selenomonas noxia,* and *Selenomonas sputigena* with strains from 11 genera of oral bacteria. *Infect Immun* 1989;57:3194-3203.
27. Ishihara K, Miura T, Kimizuka R, Ebihara Y, Mizuno Y, Okuda K. Oral bacteria inhibit *Helicobacter pylori* growth. *FEMS Microbiol Lett* 1997;152:355-361.
28. Mentis A, Tzouvelekis L, Spiliadis C, Blackwell CC, Weir DM. Inhibition of *Helicobacter pylori* hemagglutination by human salivary mucins. *FEMS Microbiol Immunol* 1990;2:125-127.
29. Okuda K, Ishihara K, Miura T, Katakura A, Noma H, Ebihara Y. *Helicobacter pylori* may have only a transient presence in the oral cavity and on the surface of oral cancer. *Microbiol Immunol* 2000;44:385-388.
30. Correa P, Fox J, Fontham E, et al. *Helicobacter pylori* and gastric carcinoma, serum antibody prevalence in populations with constructing cancer risks. *Cancer* 1990;66:2569-2574.
31. Karvar S, Burghardt W, Gross U. Detecting *Helicobacter pylori.* Immunoblot assay for detection of salivary antibodies directed against *Helicobacter pylori. Gut* 1998;42:900.
32. Yamaguchi H, Osaki T, Taguchi H, Hanawa T, Yamamoto T, Kamiya S. Production and characterization of monoclonal antibodies to heat-shock protein 60 of *Helicobacter pylori. J Med Microbiol* 1997;46:819-824.
33. Ando T, Kato T, Ishihara K, Ogiuchi H, Okuda K. Heat shock proteins in the human periodontal disease process. *Microbiol Immunol* 1995;39:321-327.
34. Okuda K, Ebihara Y. Relationship between chronic infectious diseases and systemic diseases. *Bull Tokyo Dent Coll* 1998;39:165-174.
35. Hinode D, Yoshioka M, Tanabe S, Miki O, Masuda K, Nakamura R. The GroEL-like protein from *Campylobacter rectus:* Immunological characterization and interleukin-6 and -8 induction in human gingival fibroblast. *FEMS Microbiol Lett* 1998;167:1-6.
36. Kilidireas K, Latov N, Strauss DH, et al. Antibodies to the human 60 kDa heat-shock protein in patients with schizophrenia. *Lancet* 1992;340:569-572.
37. Ishihara K, Ando T, Kosugi M, et al. Relationships between the onset of pustulosis palmaris et plantalis, periodontitis, and bacterial heat shock proteins. *Oral Microbiol Immunol* 2000;15:232-237.

Correspondence: Dr. Katsuji Okuda, Tokyo Dental College, Department of Microbiology, 1-2-2, Masago, Mihama-Ku, Chiba City, 261-8502 Japan. Fax: 81-43-270-3674; e-mail: kokuda@tdc.ac.jp.

Accepted for publication May 24, 2002.

Relationship Between Transmission of *Porphyromonas gingivalis* and *FimA* Type in Spouses

Hiroyuki Asano,* Kazuyuki Ishihara,† Taneaki Nakagawa,‡ Satoru Yamada,* and Katsuji Okuda†

Background: *Porphyromonas gingivalis* is one of the major microbial pathogens associated with chronic periodontitis. To eradicate such pathogens by periodontal therapy, it is essential to clarify the source of infection. Recent findings suggest that the genotype of the fimbriae is one of the important factors in infection by *P. gingivalis*. The objectives of the present study were to investigate the transmission of *P. gingivalis* between spouses and to determine the relationship between *P. gingivalis* *fimA* type and colonization.

Methods: A total of 14 couples were selected to investigate the transmission of *P. gingivalis* and its association with the *fimA* types. To examine the distribution of *fimA* type in the general population, 32 subgingival plaque samples from 47 patients with periodontitis were also tested. The transmission of *P. gingivalis* strains was determined by using pulsed field gel electrophoresis (PFGE). *P. gingivalis* strains isolated from the couples and subgingival dental plaque samples were studied for *fimA* classification.

Results: The PFGE patterns of *P. gingivalis* strains from matched husbands and wives were identical for six of the 14 couples. In five of these six couples (83.3%), *P. gingivalis* strains harboring the type II *fimA* gene were present. The proportion of type II *fimA* in the strains isolated from couples with probable intrafamilial transmission was significantly higher than that in patients with periodontitis or in the group of samples isolated from one member of a couple.

Conclusion: This study suggests that *fimA* type II, even though widely distributed in patients with periodontitis, may be an important factor in the transmission of *P. gingivalis* between spouses. *J Periodontol 2003;74:1355-1360.*

KEY WORDS

Fimbriae, bacterial; periodontitis/etiology; *Porphyromonas gingivalis*; spouses.

* Department of Periodontics, Oral Health Science Center, Tokyo Dental College, Chiba, Japan.
† Department of Microbiology, Tokyo Dental College.
‡ Department of Dentistry and Oral Surgery, Keio University School of Medicine, Tokyo, Japan.

Periodontitis is a chronic disease that appears to be due to a Gram-negative, bacterial-mixed infection.[1,2] *Porphyromonas gingivalis*, in particular, is closely associated with chronic (adult) periodontitis.[3-6] Family members with severe periodontitis, who are infected with *P. gingivalis*, may be a source of infection for other members of the family, and information regarding such transmission may be important in preventing further infections.

P. gingivalis expresses several pathogenic factors, including lipopolysaccharides, cysteine proteases, and fimbriae, all of which play important roles in adherence to host cells and induction of inflammatory cytokines.[7-9] In recent studies, the genotype of the fimbriae has been shown to be one of the important factors in infection by this microorganism, and type II fimbriae can bind to epithelial cellular receptors more efficiently than fimbriae from other types.[10,11] Because various pathogenetic properties of the type II *fimA* strains have previously been demonstrated, we hypothesized that *fimA* type II might play a role in the transmission of *P. gingivalis*.

In order to investigate the transmission of periodontal pathogens, several typing methods have been used to distinguish individual clones within bacterial species. Previous studies have used ribotyping, restriction endonuclease analysis (REA), and arbitrarily primed polymerase chain reaction (AP-PCR) to compare *P. gingivalis* strains obtained from married couples and have suggested that *P. gingivalis*

can be transmitted between spouses and family members.[12-14]

However, there is less information regarding the possible relationship between pathogenic factors of bacteria and their transmission. To confirm the transmission of *P. gingivalis* between spouses, we used pulsed field gel electrophoresis (PFGE), which is a powerful tool for analyzing outbreaks of infectious disease. PFGE has been used for analysis of other epidemiological data such as those from nosocomial infections.[15,16]

The objectives of this study were to investigate the transmission of *P. gingivalis* between spouses by using PFGE and to ascertain any possible relationship to *P. gingivalis fimA* type.

MATERIALS AND METHODS

Subject Population and Sampling

A total of 111 subjects were examined for this study. All subjects gave informed consent according to guidelines established by the Institutional Ethics Committee of Tokyo Dental College.

For evaluation of *P. gingivalis* transmission, 25 of 32 patients who initially visited the Tokyo Dental College Hospital with periodontitis and who agreed to have their spouses examined were selected on the basis of the presence of cultivable *P. gingivalis* and recruited as probands. The inclusion criteria for probands were as follows: 1) each subject had at least four pockets with probing depth >4 mm; 2) the subject did not have systemic infectious disease; and 3) the radiographs showed the presence of at least one intrabony defect, and the probing depths of the sites with an intrabony defect were >4 mm. The mean age of these 25 patients was 57.1 years (range, 30 to 70 years), and the mean probing depth was 6.4 mm (range, 4 to 10 mm). Samples also were collected from their spouses. Samples from 11 of 25 spouses did not produce *P. gingivalis* by culture. Fourteen couples were finally examined in this study. The mean age of all 28 subjects was 55.6 years (range, 30 to 70 years), and the mean probing depth was 6.2 mm (range, 3 to 10 mm).

To compare the distribution of the *fimA* type between patients with periodontitis and these 14 couples, we tested subgingival plaque samples from another group of 47 unrelated patients with periodontitis who visited the Tokyo Dental College Hospital. To detect the presence of *P. gingivalis* in subgingival plaque, we used the polymerase chain reaction (PCR) method described by Watanabe and Frommel.[17] Thirty-two subgingival plaque samples in which we detected *P. gingivalis* by PCR were finally tested in order to determine the distribution of *fimA* type. The mean age of the 32 positive patients was 49.7 years (range, 24 to 70 years), and the mean probing depth was 7.1 mm (range, 4 to 10 mm). No couples were included in the group.

Subgingival plaque samples were obtained with sterile paper points from the three deepest pockets or gingival sulci of each subject after removal of supragingival plaque; samples were individually tested for the isolation of *P. gingivalis*.

Identification of P. gingivalis *and Determination of* fimA *Type*

Plaque samples from the couples were immediately suspended in reduced transport fluid (RTF) as described by Syed and Loesche[18] and dispersed by a vortex mixer for 30 seconds. The samples were then serially diluted in 10-fold increments. Diluted samples were plated on blood agar plates containing 40 g/l of trypticase soy agar,§ 5% defibrinated horse blood, 5 μg/ml of hemin, and 0.5 μg/ml of menadione. All plates were cultured in an atmosphere of 10% CO_2, 10% H_2, and 80% N_2 at 37°C. After 7 to 10 days, black-pigmented colonies were isolated and purified by repeated cultivation on the agar plates. *P. gingivalis* was identified on the basis of arg-gingipain activity, and non-glycosidic activity by an enzyme assay kit.‖

The plaque samples from the 47 periodontal patients were placed in phosphate buffered saline (PBS) and stored at –20C° until they were used for *fimA* classification.

FimA classification was performed according to the methods of Amano et al.[19] and Nakagawa et al.[20] Colonies of *P. gingivalis* strains isolated from couples or subgingival plaque samples were analyzed for classification.

Pulsed Field Gel Electrophoresis (PFGE)

Preparation of the samples for PFGE in agarose was performed according to the method of Nakayama.[21] Briefly, grown cells of *P. gingivalis* were suspended in 10 mM Tris (pH 7.6), 1 M NaCl. The suspension was mixed with 1% low-melting agarose¶ and solidified at 4°C. Gel blocks were treated at 37°C for 15 hours with a solution containing 6 mM Tris HCl buffer (pH 7.6), 1 M NaCl, 0.1 M EDTA, 0.5% Briji-58,# 0.2% sodium deoxycholate,# 0.5% sodium lauroyl sarcosinate,# lysozyme (1 mg/ml),** and 20 μg/ml RNase A.** The blocks were then treated at 50°C for 48 hours with a solution containing 1 mM EDTA, SDS (1%), and 2 mg/ml proteinase K.** The blocks were dialyzed three times with 1 ml of dialysis buffer (10 mM Tris HCl [pH 7.5] containing 0.1 mM EDTA) for 2 hours each. *Not* I†† treatment of each plug was performed at 37°C for 15 hours.

PFGE was performed with a PFGE apparatus¶ using 1% agarose gels and electrophoresed in 0.5 × TBE buffer

§ Becton Dickinson, Sparks, MD.
‖ BioMerieux SA, Marcy l'Etoile, France.
¶ Bio-Rad Laboratories, Hercules, CA.
# Wako, Osaka, Japan.
** Sigma Chemical Co., St. Louis, MO.
†† New England Biolabs, Beverly, MA.

at 6 V/cm, pulse time, 5.3 to 49.9 seconds, pulse angle, 120° for 20 hours. *Saccharomyces cerevisiae* chromosomal DNA[¶] provided the molecular weight markers.

Statistical Analysis

The association of colonization of *P. gingivalis* between probands and spouses was analyzed by Fisher's exact test. The infection rates of *P. gingivalis* strains possessing type II *fimA* in the 32 patients with periodontitis and 14 couples were compared by Fisher's exact test.

RESULTS

The results of the culture of plaque samples from the 32 probands and their spouses are shown in Table 1; *P. gingivalis* was isolated from 39 individuals. We isolated *P. gingivalis* strains from both the husband and wife in 14 of the 32 couples. The association of colonization of *P. gingivalis* between probands and spouses was analyzed by Fisher's exact test. We found that the infection of either the proband or spouse was statistically significantly related to infection of the other spouse in these 32 couples ($P<0.05$).

PFGE banding patterns pretreated with *Not* I of various *P. gingivalis* strains are shown in Figure 1. The PFGE banding patterns were readily distinguishable. The banding pattern shows that the strain from W83 (lane 4) is almost identical to that from W50 (lane 5). The PFGE patterns of *P. gingivalis* strains that were isolated from more than two sites within a single individual were identical. No identical PFGE patterns were found in the 14 couples. Figure 2 illustrates the PFGE banding patterns of *P. gingivalis* strains isolated from two married couples. In couple A, the banding patterns of the strains from husband A were identical to those of his wife. In couple B, the banding patterns for husband B's isolates were distinctly different from those of his wife.

The clinical parameters of the 14 couples and the results of PFGE analysis of the *P. gingivalis* isolates are summarized in Table 2. We found identical PFGE patterns of the *P. gingivalis* strains in six of the 14 married couples. The average probing depth in these six couples

Table 1.

Number of *P. gingivalis* Culture-Positive Individuals Examined

	Probands (32)	
Spouses	Positive 25	Negative 7
Positive	14*	0
Negative	11	7

* $P<0.05$, Fisher's exact test.

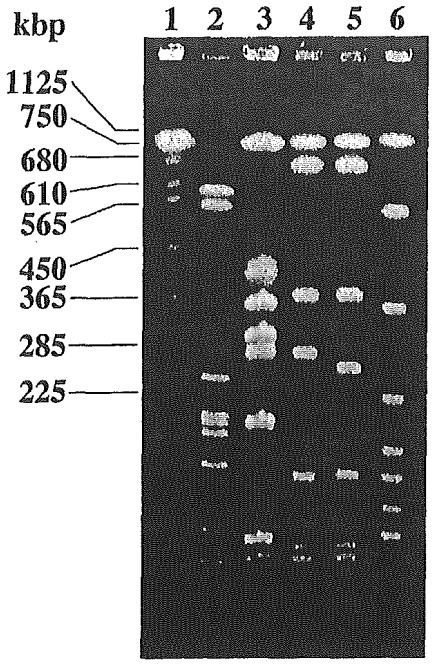

Figure 1.
PFGE banding patterns of various P. gingivalis *strains pretreated with* Not *I. Lane 1, molecular size marker; lane 2, ATCC 53977; lane 3, ATCC 33277; lane 4, W83; lane 5, W50(ATCC 53978); lane 6, 16-1.*

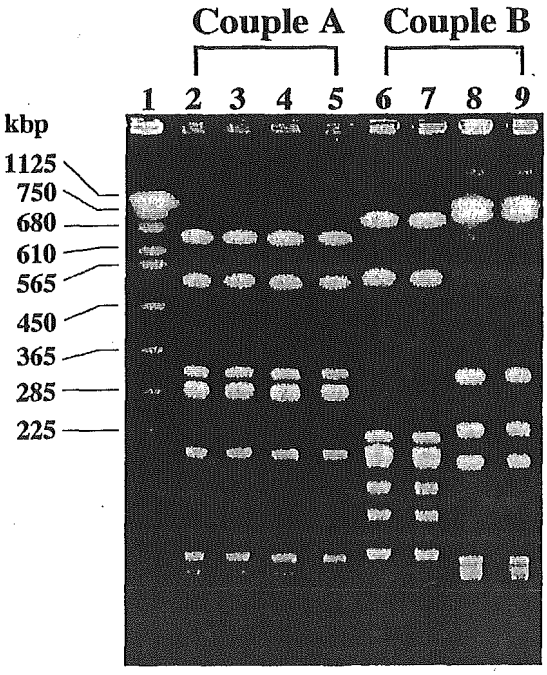

Figure 2.
PFGE banding patterns of P. gingivalis *strains isolated from two married couples. Lane 1, molecular size marker; lane 2, husband A clone 1; lane 3, husband A clone 2; lane 4, wife A clone 1; lane 5, wife A clone 2; lane 6, husband B clone 1; lane 7, husband B clone 2; lane 8, wife B clone 1; lane 9, wife B clone 2.*

with identical PFGE patterns of *P. gingivalis* strains was 6.3 mm and that of the eight couples without identical patterns was 5.4 mm; this difference was not statistically significant.

Table 2.

Analysis of PFGE Patterns of *P. gingivalis* Strains Isolated from 14 Couples

	Proband			Spouse			
Couple	Gender	Age	PD*	Gender	Age	PD*	Identical Pattern
A	F	55	6.5	M	56	6.5	+
B	F	60	6	M	60	8	–
C	M	54	6	F	40	3.5	+
D	M	57	5	F	53	6	–
E	M	65	5	F	57	3	–
F	M	54	7	F	45	7.5	–
G	M	61	8.5	F	57	7	+
H	F	52	7	M	50	3	–
I	M	47	5	F	50	5	–
J	M	50	5	F	52	5	+
K	F	61	7	M	64	7.5	+
L	F	53	8.5	M	53	5	+
M	M	63	5	F	64	5	–
N	M	64	5	F	66	3.5	–

* Mean probing depth.

The distribution of the *fimA* types of *P. gingivalis* isolated from 14 couples and 32 dental plaque samples (from 47 periodontitis patients whose plaque samples were *P. gingivalis*-positive by PCR) is summarized in Table 3. The frequency of *fimA* type II in isolates from the six couples with identical PFGE patterns was 83.3%, which was higher than that for the periodontitis patients (46.8%), for couples without evidence of transmission (62.5%), and for samples isolated from 11 probands with a negative spouse (27.3%). The infection rate of type II *fimA P. gingivalis* in couples colonized with identical PFGE strains was higher than that in 32 patients with periodontitis or in the group of samples isolated from 11 probands with a negative spouse ($P<0.05$).

DISCUSSION

We analyzed the PFGE banding patterns of different *P. gingivalis* strains from various origins using *Not* I. Each banding pattern was distinguishable from the others. The banding pattern of the W83 strain was almost identical to that of W50, but differed from those of the other strains. This is consistent with previous reports.[22,23]

We found that the PFGE banding patterns of *P. gingivalis* isolates were identical in six of 14 couples. Transmission of *P. gingivalis* strains between spouses might be a likely explanation for these results. Previous studies have investigated the likelihood of transmission of *P. gingivalis* between spouses by using other typing methods. Saarela et al.[12] showed that *P. gingivalis* ribotypes were identical in two of four couples. Using REA, van Steenbergen et al.[13] demonstrated that *P. gingivalis* DNA patterns were identical in six of eight couples. Asikainen et al.[14] also found that *P. gingivalis* AP-PCR types were identical in two of seven couples. The similar results from these studies suggest that the rate of intrafamilial infection is similar in different populations. On the other hand, previous studies and the present

Table 3.

Distribution of *P. gingivalis fimA* Type in 39 Strains Isolated from Couples and Subgingival Plaque Samples from 32 Patients with Periodontitis

	fimA Type						
Category (n subjects)	I	II	III	IV	V	Comb*	NI†
Strain isolated							
from 6 couples with identical PFGE (12)	0 (0.0%)	10 (83.3%)	0 (0.0%)	0 (0.0%)	2 (16.7%)	0 (0.0%)	0 (0.0%)
from 8 couples with non-identical PFGE (16)	2 (12.5%)	10 (62.5%)	0 (0.0%)	1 (6.2%)	2 (12.5%)	0 (0.0%)	1 (6.2%)
from 11 probands with a negative spouse (11)	2 (18.1%)	3 (27.3%)	0 (0.0%)	4 (36.4%)	1 (9.1%)	0 (0.0%)	1 (9.1%)
Subgingival plaque samples							
from periodontitis patients (32)	3 (9.3%)	15 (46.8%)	2 (6.3%)	5 (15.6%)	2 (6.3%)	3 (9.3%)	2 (6.3%)

* Isolated from more than one *fimA* type.
† Not identified.

results also failed to detect evidence of transmission of *P. gingivalis* within some couples. Therefore, it is possible that some *P. gingivalis* strains are more transmissible and/or colonize more efficiently than other strains.

Fimbriae of *P. gingivalis* are major adherence factors for these organisms,[9] and Amano et al.[10] found that the most prevalent *fimA* type was type II in subgingival plaque samples from patients with periodontitis and suggested that the genotype of fimbriae is an important factor in colonization. More recently, Nakagawa et al.[11] reported that type II *FimA* can bind to epithelial cells efficiently through specific host receptors. Various pathogenicities of type II *fimA* have been demonstrated, revealing that *fimA* type II plays a role in transmission of *P. gingivalis*. In the present study, we investigated the distribution of the *P. gingivalis fimA* types from isolates of various groups. The *fimA* gene of *P. gingivalis* strains isolated from both husbands and wives belonged to type II in five of the six couples whose PFGE patterns were identical. The high detection rate of type II in couples with identical PFGE suggests that type II fimbriae are important factors in colonization. The infection rates of *P. gingivalis* strains possessing type II in 32 patients with periodontitis and couples were compared by Fisher's exact test. It was found that the infection rates of type II *P. gingivalis* in couples colonized with an identical PFGE strain were higher than those of 32 patients with periodontitis and in the group of samples isolated from 11 probands with a negative spouse ($P<0.05$).

The ratio of *P. gingivalis* with type II fimbriae was decreased by 56% in couples with only one spouse infected by *P. gingivalis*. These data suggest that type II fimbriae might be important factors in transmission of *P. gingivalis* between spouse and proband. However, of the eight couples infected by different clonal types of *P. gingivalis*, 62.5% of the spouses harbored *P. gingivalis* with type II fimbriae, and about 27% of the spouses in the group that did not show transmission still had the type II *fimA*. In the former eight couples, it is possible that colonization of one clonal type prevented colonization by another clonal type of the same species. In this study, no transmission was found in a couple in whom the proband had type II *fimA* and the spouse had type I. Several reports have indicated that occupation of an ecological niche by one clonal type inhibits colonization by genotypically different members of the same species.[24,25] In the latter 11 couples, it is possible that the length of marriage, age of subjects, and amount of *P. gingivalis* in saliva from probands affected the frequency of the transmission. Petit et al.[26] reported that the salivary counts of *P. gingivalis* were higher in spouses who had the same species than in couples in which only one spouse was infected. Further longitudinal study of these factors is required to clarify the relationship between fimbriae type and transmission.

We isolated type IV more often than type II from 11 probands with a negative spouse. However, type IV is significantly less common in isolates from couples without identical PFGE patterns and was not found in couples with identical PFGE patterns. This suggests that type IV is less transmissible. Type II, on the other hand, appears to be highly transmissible. Our results suggest that type II *fimA* plays an important role not only in colonization by *P. gingivalis* but also in transmission. Other factors such as the clinical periodontal status may play a role in this difference. In the present study, the average probing depth of the six couples in the *P. gingivalis* transmission group was 6.3 mm and that of the couples without transmission was 5.4 mm; this difference was not statistically significant. However, it is possible that probing depth also might be related to the frequency of intrafamilial transmission of *P. gingivalis*.

In summary, although direct proof of the possibility that type II *fimA* can influence transmission remains to be clarified, the present findings suggest that *P. gingivalis fimA* type II may be an important factor in *P. gingivalis* transmission between spouses.

ACKNOWLEDGMENTS

The authors thank Dr. H. Kuramitsu, Department of Oral Biology, University at Buffalo, Buffalo, New York, for critical review of this manuscript. We appreciate the efforts of Dr. Barbara Lee Pierce in correcting the English of this manuscript. This study was supported in part by a grant from the Oral Health Science Center of Tokyo Dental College.

REFERENCES

1. Tanner AC, Haffer C, Bratthall GT, Visconti RA, Socransky SS. A study of the bacteria associated with advancing periodontitis in man. *J Clin Periodontol* 1979;6:278-307.
2. Savitt ED, Socransky SS. Distribution of certain subgingival microbial species in selected periodontal conditions. *J Periodont Res* 1984;19:111-123.
3. Slots J, Genco RJ. Black-pigmented *Bacteroides* species, *Capnocytophaga* species, and *Actinobacillus actinomycetemcomitans* in human periodontal disease: Virulence factors in colonization, survival, and tissue destruction. *J Dent Res* 1984;63:412-421.
4. Mayrand D, Holt SC. Biology of asaccharolytic black-pigmented *Bacteroides* species. *Microbiol Rev* 1988;52: 134-152.
5. Grossi SG, Zambon JJ, Ho AW, et al. Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. *J Periodontol* 1994;65:260-267.
6. Kimura S, Nagai A, Onitsuka T, et al. Induction of experimental periodontitis in mice with *Porphyromonas gingivalis*-adhered ligatures. *J Periodontol* 2000;71: 1167-1173.
7. Travis J, Potempa J, Maeda H. Are bacterial proteinases pathogenic factors? *Trends Microbiol* 1995;3:405-407.
8. Tanamoto K, Azumi S, Haishima Y, Kumada H, Umemoto T. The lipid A moiety of *Porphyromonas gingivalis* lipopolysaccharide specifically mediates the activation of C3H/HeJ mice. *J Immunol* 1997;158:4430-4436.

9. Hamada S, Amano A, Kimura S, Nakagawa I, Kawabata S, Morisaki I. The importance of fimbriae in the virulence and ecology of some oral bacteria. *Oral Microbiol Immunol* 1998;13:129-138.
10. Amano A, Kuboniwa M, Nakagawa I, Akiyama S, Morisaki I, Hamada S. Prevalence of specific genotypes of *Porphyromonas gingivalis fimA* and periodontal health status. *J Dent Res* 2000;79:1664-1668.
11. Nakagawa I, Amano A, Kuboniwa M, Nakamura T, Kawabata S, Hamada S. Functional differences among *fimA* variants of *Porphyromonas gingivalis* and their effects on adhesion to and invasion of human epithelial cells. *Infect Immun* 2002;70:277-285.
12. Saarela M, von Troil-Linden B, Torkko H, et al. Transmission of oral bacterial species between spouses. *Oral Microbiol Immunol* 1993;8:349-354.
13. van Steenbergen TJ, Petit MDA, Scholte LH, van der Velden U, de Graaff J. Transmission of *Porphyromonas gingivalis* between spouses. *J Clin Periodontol* 1993;20: 340-345.
14. Asikainen S, Chen C, Slots J. Likelihood of transmitting *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in families with periodontitis. *Oral Microbiol Immunol* 1996;11:387-394.
15. Arbeit RD, Arthur M, Dunn R, Kim C, Selander RK, Goldstein R. Resolution of recent evolutionary divergence among *Escherichia coli* from related lineages: The application of pulsed field electrophoresis to molecular epidemiology. *J Infect Dis* 1990;161:230-235.
16. Goering RV, Winters MA. Rapid method for epidemiological evaluation of Gram-positive cocci by field inversion gel electrophoresis. *J Clin Microbiol* 1992;30:577-580.
17. Watanabe K, Frommel TO. *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans* and *Treponema denticola* detection in oral plaque samples using the polymerase chain reaction. *J Clin Periodontol* 1996;23: 212-219.
18. Syed SA, Loesche WJ. Survival of human dental plaque flora in various transport media. *Appl Microbiol* 1972;24: 638-644.
19. Amano A, Nakagawa I, Kataoka K, Morisaki I, Hamada S. Distribution of *Porphyromonas gingivalis* strains with *fimA* genotypes in periodontitis patients. *J Clin Microbiol* 1999; 37:1426-1430.
20. Nakagawa I, Amano A, Kimura RK, Nakamura T, Kawabata S, Hamada S. Distribution and molecular characterization of *Porphyromonas gingivalis* carrying a new type of *fimA* gene. *J Clin Microbiol* 2000;38:1909-1914.
21. Nakayama K. Determination of the genome size of the oral anaerobic bacterium *Porphyromonas gingivalis* by pulsed field gel electrophoresis. *Dent Japan* 1995;32: 25-28.
22. Genco RJ, Loos BG. The use of genomic DNA fingerprinting in studies of the epidemiology of bacteria in periodontitis. *J Clin Periodontol* 1991;18:396-405.
23. Chen C, Slots J. Clonal analysis of *Porphyromonas gingivalis* by the arbitrarily primed polymerase chain reaction. *Oral Microbiol Immunol* 1994;9:99-103.
24. Zambon JJ, Sunday GJ, Smutko JS. Molecular genetic analysis of *Actinobacillus actinomycetemcomitans* epidemiology. *J Periodontol* 1990;61:75-80.
25. Loos BG, Dyer DW, Genco RJ, Selander RK, Dickinson DP. Natural history and epidemiology. In: Shah HN, ed. *Biology of the Species* Porphyromonas gingivalis, vol. 20. Boca Raton, FL: CRC Press; 1993:436-442.
26. Petit MDA, van Steenbergen TJ, Timmerman MF, de Graaff J, van der Velden U. Prevalence of periodontitis and suspected periodontal pathogens in families of adult periodontitis patients. *J Clin Periodontol* 1994;21: 76-85.

Correspondence: Dr. Hiroyuki Asano, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan. E-mail: asano@tdc.ac.jp.

Accepted for publication March 10, 2003.

J Anesth (2003) 17:84–91

Journal of Anesthesia

Reduction of potential respiratory pathogens by oral hygienic treatment in patients undergoing endotracheal anesthesia

MINORI OKUDA[1], YUZURU KANEKO[1], TATSUYA ICHINOHE[1], KAZUYUKI ISHIHARA[2], and KATSUJI OKUDA[2]

[1]Oral Health Science Center and Department of Dental Anesthesiology, Tokyo Dental College, 1-2-2 Masago, Chiba 261-8502, Japan
[2]Oral Health Science Center and Department of Microbiology, Tokyo Dental College, Chiba, Japan

Abstract

Purpose. This study was conducted to evaluate the usefulness of mechanical and chemical prophylactic oral cleansing treatments for reducing potential respiratory pathogens existing in the oral cavity.

Methods. Thirty-two patients scheduled to undergo oral and maxillofacial surgery that required endotracheal anesthesia were randomly allocated to one of the two groups, the oral cleansing group ($n = 16$) or the noncleansing group ($n = 16$). Culture and polymerase chain reaction (PCR) methods were used to detect and enumerate pathogens. Oral cleansing was carried out with an electric toothbrush capable of automatically supplying and aspirating povidone-iodine solution before surgery, followed by rinsing twice a day after surgery. Cephazolin (3 g·day^{-1}) was given to all patients for 5 days after surgery.

Results. The PCR detection rates of *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Porphyromonas gingivalis* in gargle samples before treatment were 87.5%, 68.8%, 53.1%, and 40.6%, respectively. Oral cleansing reduced the detection rates and numbers of methicillin-sensitive Staphylococcus species, *S. pneumoniae*, and *H. influenzae*. In contrast, there was no significant reduction of methicillin-resistant Staphylococcus species, *S. pneumoniae, H. influenzae*, or *P. aeruginosa* in subjects who underwent systemic cephazolin administration without oral cleansing.

Conclusion. The combination of mechanical and chemical oral cleansing resulted in a significant reduction of potential respiratory pathogens in the oral cavity.

Address correspondence to: M. Okuda
Received: September 5, 2002 / Accepted: January 15, 2003

Key words Respiratory pathogens · Ventilator-associated pneumonia · Oral prophylactic cleansing

Introduction

The prevention of ventilator-associated pneumonia in patients receiving endotracheal anesthesia is a major challenge, because this complication is frequent and, once present, carries a high morbidity and mortality, even with adequate treatment [1,2]. Infectious respiratory diseases such as pneumonia resulting from salivary bacterial aspiration during mechanical ventilation are common [3–6]. *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, Staphylococcus species, and *Candida albicans* are found in samples from the human oral cavity as well as in nasopharyngeal secretions, and all of these bacteria have the potential to cause infectious respiratory diseases [7–10].

It is well known that various kinds of microorganisms form biofilms in which organisms are intimately associated with each other and the solid substratum through binding and inclusion within an ex-polymer matrix; such systems are widely distributed in nature and disease [11–15]. In the oral cavity, multispecies biofilms form well-ordered structures on dental plaque, the tongue, and other oral soft-tissue surfaces [16]. These biofilms may serve as reservoirs for respiratory pathogens, especially in higher-risk patients with poor oral hygiene [4,17–19]. Such biofilms are resistant to antibiotic and chemical attack [20–23]. Povidone-iodine (polyvinylpyrrolidone) is an effective broad-spectrum disinfectant that is widely used in medicine for topical applications [24,25].

The present study evaluated the effect of mechanical and chemical oral prophylactic cleansing using povidone-iodine solution on the reduction of the

biofilm-forming respiratory pathogens in patients receiving endotracheal anesthesia.

Materials and methods

A total of 32 patients classified as American Society of Anesthesiolosists Physical Status I or II (mean age and SD, 28.9 + 8.4 years; range, 19–55 years) at Chiba Hospital, Tokyo Dental College, who were scheduled to undergo oral surgery requiring endotracheal intubation, were enrolled after written informed consent had been obtained. None of the subjects had been given any antibiotic during the 3 weeks prior to the study. No finding of infectious disease, such as sinusitis or respiratory infections, was shown in the hemanalysis or the chest X-ray photograph. The patients underwent total intravenous anesthesia with propofol and fentanil. Nasotracheal intubation was performed on all patients. The Blue Line (Portex, Hythe Kent, UK) with a cuff was used. Five percent povidone-iodine solution (Isodine, Meiji, Tokyo) at an iodine concentration of 5 mg·ml^{-1} was used for preoperative decontamination of the oral cavity. After the operation, extubation was performed immediately, and the intubation time was not prolonged. All patients received nutritional support by nasogastric tube for 3 days after the operation and were not given antacids or H_2-blockers during the investigation period. All patients were given cephazolin (3 g·day^{-1}) starting 12 h before the induction of anesthesia until 5 days after surgery. The surgical procedures mainly consisted of orthognathic surgery; procedures involving infectious diseases were excluded. We enrolled 32 subjects and randomly assigned them to two groups: the cleansing group (n = 16) and the non-cleansing group (n = 16).

To collect samples for microbial studies, the patients gargled with 5-ml samples of distilled phosphate-buffered saline (PBS, pH 7.4) for 20 s on the day before surgery. For mechanical and chemical oral cleansing, we used an Electronic Toothbrush System (Dento-Erac 910, Ozkqa, Lion, Tokyo, Japan) capable of automatically supplying and aspirating solution. The cleansing, including brushing the teeth; scrubbing the periodontium, buccal mucosa, and tongue; and rinsing, was carried out 24 h before surgery using 200 ml of 0.5% povidone-iodine solution (Isodine-Gargle, Meiji, Tokyo, Japan) at an iodine concentration of 0.5 mg·ml^{-1}. To avoid contamination of the gargle sample by povidone-iodine solution, all procedures were completed with a thorough rinse with tap water. Thirty minutes later, we examined the effect of cleansing on the total number of colony-forming-units (CFUs) of anaerobic organisms and *C. albicans* cells. All patients in the oral cleansing group performed oral rinsing with 50 ml of 0.5% povidone-iodine solution twice a day after surgery [24]. Seven days after surgery, between 9:00 and 10:00 A.M. and before oral rinsing with povidone-iodine solution, gargle samples were obtained for microbial examination.

After obtaining the gargle samples, we carried out the microbial analysis as a non-double-blind study. The samples were diluted with PBS in a gradient of 1:10 steps down to 1:10^{-5}, and 100 µl of each dilution was inoculated onto duplicate trypticase soy agar plates (Becton Dickinson, Cockeysville, MD, USA) supplemented with hemin (5 µg·ml^{-1}), menadione (0.5 µg·ml^{-1}), and 10% defibrinated horse blood. These inoculated plates were incubated at 37°C for 1 week in an anaerobic chamber containing 10% CO_2, 10% H_2, and 80% N_2. The total CFUs of anaerobic bacteria in each sample were counted.

For detection of Staphylococcus species, a selective medium (No. 110 Medium, Difco Laboratories, Detroit, MI, USA) was used. For detection of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* (MRSE), a selective medium containing 16 g·l^{-1} of phenol-red broth base (Difco), 10 µg·ml^{-1} of polymyxicin-B sulfate (Pfizer, Tokyo, Japan), and 6 µg·ml^{-1} of oxacillin sodium salt (Stacillin, Banyu Pharmaceutical, Tokyo, Japan) was used [26]. Colonies isolated from this selective medium were confirmed to harbor the *mec*A gene by polymerase chain reaction (PCR) [27]; their biochemical characteristics were examined, and then they were identified as MRSA and MRSE as described previously [28]. For detection of *P. aeruginosa* by examination of culture, we used a modified selective medium, Cetrimide agar (Nippon Pharmaceutical, Tokyo, Japan) as reported by Fonseca et al. [29] and Campbell et al. [30]. For detection of *C. albicans*, Candida GE agar (Nippon Pharmaceutical) was used. After examination of the biochemical characteristics of the colonies on selective medium, we identified *C. albicans* as described previously [28].

PCR was used to detect *S. pneumoniae* [31], *H. influenzae* [32], *P. aeruginosa* [33], *Porphyromonas gingivalis* [34], *Actinobacillus actinomycetemcomitans* [34], and *Chlamydia pneumoniae* [35]. The primers used in these reactions are listed in Table 1 and have been described in previous studies [31–35]. The reagents used in this experiment were purchased from Wako Pure Chemical Industries, Tokyo, Japan. Each 2-ml aliquot sample of gargled fluid was centrifuged at 10000×g for 15 min. The precipitate was resuspended by vortex mixing in 200 µl of 10 mM Tris-HCl buffer (pH 7.8) containing 5 mM EDTA, 0.5% sodium dodecylsulfate (SDS), and 80 µg of proteinase K and lysed by incubation at 56°C for 120 min. This was followed by phenol extraction and precipitation of DNA by ethanol. The DNA

Table 1. List of PCR primers used in this study

Species	Nucleotide sequence 5′→3′	No. of bases amplified
Streptococcus pneumoniae		
SP I	AGGATAAGGAACTGCG	
SP II	CTTATTTTCTGACCTTTCA	247
Haemophilus influenzae		
F 1	AACTTTTGGCGGTTACTCTG	
R 2	CTAACACTGCACGACGGTTT	351
Pseudomonas aeruginosa		
PAL 1	ATGGAAATGCTGAAATTCGGC	
PAL 2	CTTCTTCAGCTCGACGCGACG	504
Porphyromonas gingivalis		
PG I	ATAATGGAGAACAGCAGGAA	
PG II	TCTTGCCAACCAGTTCCATTGC	131
Actinobacillus actinomycetemcomitans		
Aa I	CAGCAAGCTGCACAGTTTGCAAA	
Aa II	CATTAGTTAATGCCGGGCCGTCT	238
Chlamydia pneumoniae		
Cpn A	TGACAACTGTAGAAATACAGC	
Cpn F	GGTTGAGTCAACGACTTAAGG	446
Staphylococcus aureus		
aureusF	AATCTTTGTCGGTACACGATATTCTTCACG	
aureusR	CGTAATGAGATTTCAGTAGATAATACAACA	422
Methicillin-resistant *Staphylococcus aureus*		
MSAF	AAAATCGATGGTAAAGGTTGGC	
MSAR	AGTTCTGCAGTACCGGATTTGC	533

was then dissolved in 20 µl of distilled water. PCR was performed using a GeneAmp PCR System 9700 (Perkin-Elmer, Foster City, CA, USA). The reaction mixture (50 µl) contained 1 µl of DNA template, 50 *p*M of primers, 1.25 U of Taq DNA polymerase (Takara, Shuzo, Shiga, Japan), 5 µl of 10× reaction buffer, and 200 µM of the four deoxynucleotide triphosphates supplied with Taq polymerase. For *S. pneumoniae*, *H. influenzae*, and *P. aeruginosa,* the total number of PCR cycles, denaturation, annealing, and extension followed the recommendations in our previous reports [28].

Five microliters of each of the amplified products were analyzed by electrophoresis in 2% agarose gel in 1 × TBE buffer (90 mM Tris-borate, 2 mM EDTA; pH 8.3) at 100 V for 40 min. A low-DNA Mass Ladder (Life Technologies, Gaithersburg, MD, USA) was used as the molecular size standard. The gel was stained with ethidium bromide (0.5 $\mu g \cdot ml^{-1}$) and photographed under ultraviolet illumination with Polaroid film (Polaroid, St. Albans, UK). The optical density of the various bands was quantified with Kodak Digital Science 1D Image Analysis Software (Eastman Kodak, Rochester, NY, USA).

The differences in the rates of detection in cultures before and after surgery were evaluated by the chi-square test. One-way analysis of variance was used to compare the numbers of CFUs before and after oral cleansing and 7 days postoperatively.

Results

There were no significant differences in age or sex between the cleansing and the noncleansing groups. The detection ratios of microorganisms examined by PCR and culture methods before the cleansing treatment are summarized in Fig. 1. We found a high prevalence (87.5%) of *S. pneumoniae* in gargle samples from 32 patients undergoing surgery under endotracheal anesthesia. The DNA sequences of the amplified *S. pneumoniae* spacer regions of the 16S and 23S rRNA genes were confirmed to be identical to those of this species. The PCR detection rates of *H. influenzae*, *P. aeruginosa*, and *P. gingivalis* were 68.8%, 53.1%, and 40.6%, respectively. Neither *A. actinomycetemcomitans* nor *C. pneumoniae* was detected in any of the gargle samples by PCR.

The culture detection rates of *S. aureus*, MRSA, *S. epidermidis*, and MRSE in gargle samples from 32 patients before surgery were 34.4%, 9.4%, 56.3%, and 15.6%, respectively. We confirmed that the identified MRSA and MRSE possessed the *mec*A gene. The detection rates of *P. aeruginosa* and *C. albicans* by the culture method were 15.6% and 28.1%, respectively. The detection rates of *P. aeruginosa* by the culture method were lower than those by PCR ($P = 0.068$), but the differences were not significant.

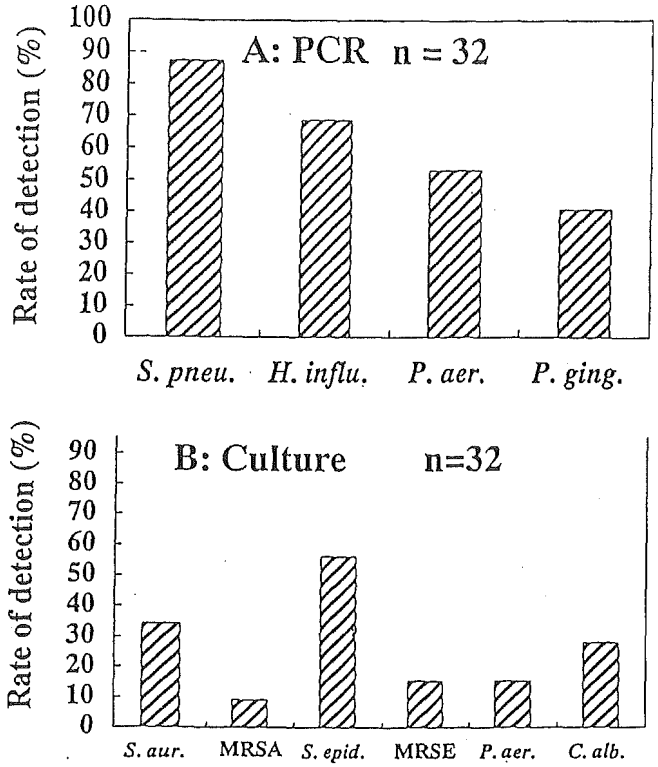

Fig. 1. Detection rates of (**A**) *Streptococcus pneumoniae* (*S. pneu.*), *Haemophilus influenzae* (*H. influ.*), *Pseudomonas aeruginosa* (*P. aer.*), and *Porphyromonas gingivalis* (*P. ging.*) by the PCR method and of (**B**) *Staphylococcus aureus* (*S. aur.*), methicillin-resistant *S. aureus* (*MRSA*), *S. epidermidis* (*S. epid.*), methicillin-resistant *S. epidermidis* (*MRSE*), and *Candida albicans* (*C. alb.*) examined by culture methods in gargle samples obtained from 32 patients before mechanical and chemical prophylactic oral cleansing. Neither *Actinobacillus actinomycetemcomitans* nor *Chlamydia pneumoniae* was detected by PCR

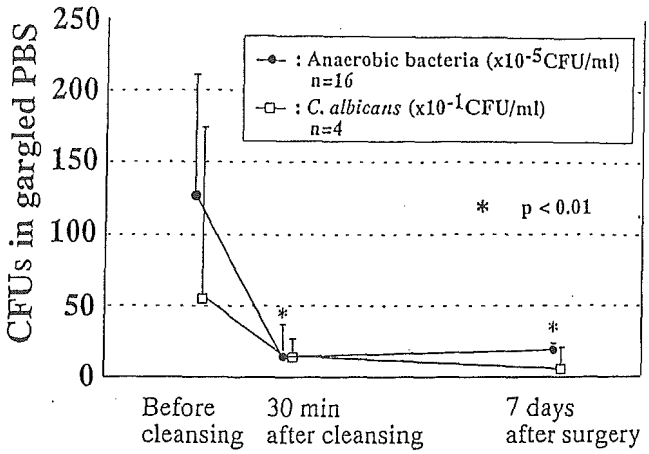

Fig. 2. Effects of mechanical and chemical prophylactic oral cleansing with povidone-iodine solution on viable cell numbers of oral anaerobic bacteria and *C. albicans* in gargle samples obtained from 16 patients. Mean number of colony-forming units (CFUs) with standard deviations examined before the cleansing treatment, 30 min after cleansing, and 7 days after surgery. * $P < 0.01$ vs. before cleansing. PBS, phosphate-buffered saline

The means and standard deviations of the number of anaerobic bacterial CFUs grown on blood agar plates and CFUs of *C. albicans* on Candida GE medium obtained before cleansing, 30 min, after cleansing, and 7 days after surgery from 16 patients are shown in Fig. 2. The average number of CFUs in the gargle samples obtained after oral cleansing using povidone-iodine solution were significantly reduced 30 min after cleansing and 7 days after surgery ($P < 0.01$). We detected *C. albicans* cells in only 4 samples from the 16 patients. Reduction in the mean number of *C. albicans* was also noted 30 min after cleansing ($P = 0.108$) and 7 days after surgery ($P = 0.068$), but these reductions were not significant.

The oral cleansing effects on microorganisms examined by PCR and culture methods are summarized in Fig. 3. We compared pre- and posttreatment findings among 16 subjects in a nonrandomized study. It was found by the PCR method that the combination of mechanical and chemical oral prophylactic cleansing resulted in significant reductions in the detection rates of *S. pneumoniae* ($P < 0.05$) and *H. influenzae* ($P < 0.05$), but not of *P. aeruginosa*. Culturing demonstrated that the treatment including cephazolin administration reduced both methicillin-sensitive *S. aureus* ($P < 0.01$) and *S. epidermidis* ($P < 0.05$), but not MRSA, MRSE, *P. aeruginosa*, or *C. albicans*. However, no reduction in *S. pneumoniae*, *H. influenzae*, or *P. aeruginosa* was found by the PCR method in the group taking cephazolin for 5 days without oral prophylactic cleansing (Fig. 4). Cephazolin administration without the oral cleansing treatment significantly reduced methicillin-sensitive *S. epidermidis* ($P < 0.01$). However, we unexpectedly found increases in the detection rates of *P. aeruginosa* ($P = 0.068$) and *C. albicans* ($P < 0.05$) in the group without oral cleansing.

The densities of amplified DNA bands of *S. pneumoniae* in samples obtained before prophylactic oral cleansing were compared with those obtained 7 days after the cleansing treatment (Fig. 5). The net intensity ranged from 0 to 87719 in the samples examined. As shown in Fig. 5, prophylactic oral cleansing resulted in either an intensity of 0 or a decreased net intensity of *S. pneumoniae* PCR bands in gargle samples obtained from 16 patients 7 days after surgery. However, there was no significant reduction of the intensity in 11 of 16 patients who had not undergone mechanical and chemical oral cleansing treatment. There was no diagnosis of postoperative infectious disease, including respiratory infection, in any of the 32 patients.

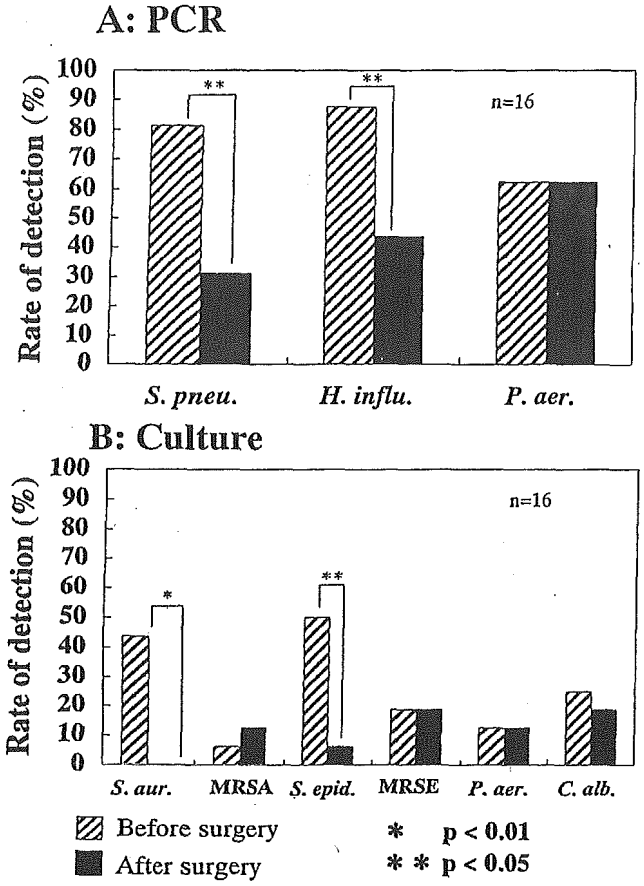

Fig. 3. Comparison of detection rates between gargle samples obtained from 16 patients before mechanical and chemical prophylactic oral cleansing and those obtained 7 days after the cleansing treatment. **A** *S. pneumoniae* (*S. pneu.*), *H. influenzae* (*H. influ.*), *P. aeruginosa* (*P. aer.*), and *P. gingivalis* (*P. ging.*) were examined by the PCR method. **B** *S. aureus* (*S. aur.*), *MRSA*, *S. epidermidis* (*S. epid.*); *MRSE*, and *C. albicans* (*C. alb.*) were examined by culture methods

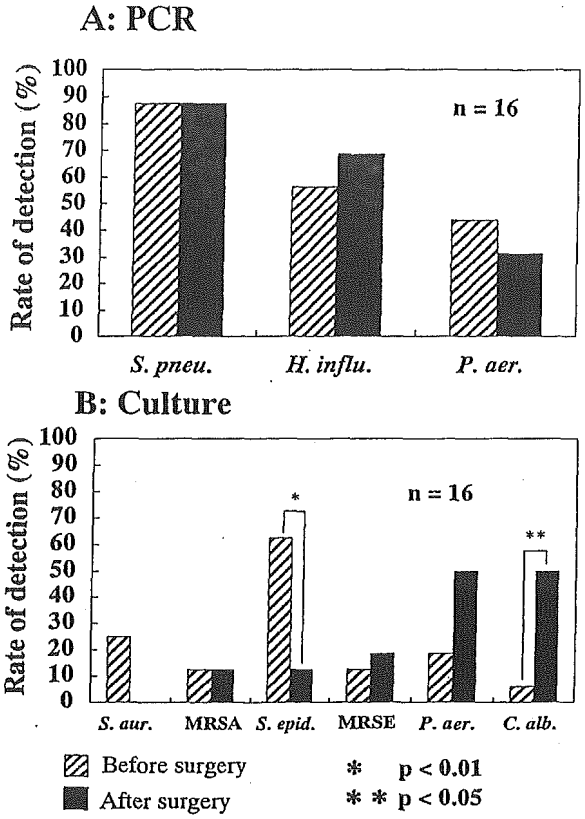

Fig. 4. Comparison of detection rates between gargle samples obtained from 16 patients who had not received any chemical oral cleansing treatment before surgery and those obtained from the same group 7 days after surgery. **A** *S. pneumoniae* (*S. pneu.*), *H. influenzae* (*H. influ.*), *P. aeruginosa* (*P. aer.*), and *P. gingivalis* (*P. ging.*) were examined by the PCR method. **B** *S. aureus* (*S. aur.*), *MRSA*, *S. epidermidis* (*S. epid.*), *MRSE*, and *C. albicans* (*C. alb.*) were examined by culture methods

Discussion

Pneumonia that develops within 48 h in critically ill patients under mechanical ventilation is thought to be associated with the ventilation process [3,7,8,36]. It has been reported that poor oral hygiene and severe periodontal disease increase the colonization of oral bacteria in the oropharynx and lung [17,18,28]. In the present study, we found high detection rates of *S. pneumoniae*, *H. influenzae*, and *P. aeruginosa* by the PCR method in gargle samples from patients undergoing oral and maxillofacial surgery that required endotracheal intubation. In our previous study, we found a high prevalence of *S. pneumoniae* in gargle samples from healthy young and elderly persons [28]. To confirm the presence of *S. pneumoniae* in these gargle samples, we examined them by an immunofluorescence method. Fluorescent cells stained with antiserum against *S.*

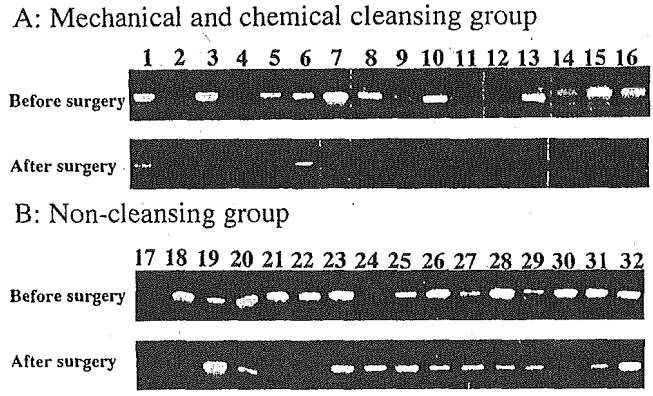

Fig. 5. Comparison of densities of amplified *S. pneumoniae* DNA bands between the gargle samples obtained before and after surgery in the mechanical and chemical prophylactic oral cleansing group (**A**) and in the noncleansing group (**B**)

pneumoniae (Biogenesis, Poole, UK) were found only in strongly PCR-positive gargle samples (data not shown).

More than 500 bacterial species are found in the human oral cavity [37]. *S. pneumoniae* is a member of the *Streptococcus mitis* group that is predominant in the human oral cavity. Haemophilus species are also endogenous in the human oral cavity. We used the PCR method, which can detect low cell numbers of both species in gargle samples. In this study, we first tried to detect and enumerate *S. pneumoniae* and *H. influenzae* by culture methods. Although we isolated many colonies, we could not identify most isolates as *S. pneumoniae* or *H. influenzae*. It is possible that various oral bacterial species interact competitively with each other in an attempt to survive and prevent other bacterial growth [38,39]. These antagonistic actions make it difficult to detect and enumerate *S. pneumoniae* and *H. influenzae* species by culture methods.

We detected Staphylococcus species, including MRSA and MRSE, *C. albicans*, and *P. aeruginosa* by culture methods. These microorganisms are known to form biofilms and to be resistant to many antibiotics [11–13,23,40,41]. Oral microorganisms grown in biofilms have also been shown to be more resistant to various antibacterial agents than their planktonic counterparts [16,23,42]. We did not find any significant reductions in *S. pneumoniae*, *H. influenzae*, *P. aeruginosa*, MRSA, or MRSE in gargle samples obtained from surgical patients administered systemic cephazolin for 5 days without undergoing mechanical and chemical oral prophylactic cleansing with povidone-iodine solution. Unexpectedly, the detection rates of *P. aeruginosa* and *C. albicans* after cephazolin administration were higher than those before surgery. These results indicated that systemic cephazolin administration for 5 days was not effective in elimination of either microorganism and might have induced superinfection by *P. aeruginosa* and *C. albicans* in some patients.

Previous clinical trials have examined the possibility of eliminating nosocomial respiratory pathogens by topical antimicrobial prophylaxis rather than systemic antibiotic administration. Pugins et al. [2] demonstrated, by a randomized, placebo-controlled, double-blind clinical trial, that topical oropharyngeal antibiotic application of polymyxin B, neomycin sulfate, and vancomycin hydrochloride produced a significant reduction in aerobic gram-negative bacterial colonization and ventilator-associated pneumonia. Rodriguez-Roldan et al. [43] also showed that nosocomial pneumonia, which is a frequent complication in critically ill patients on mechanical ventilation, could be prevented by local application of nonabsorbable antibiotics containing tobramycin, amphotericin B, and polymyxin E to the oropharynx. In addition, DeRiso et al. [44] investigated whether a chlorhexidine oral rinse would be useful in preventing pneumonia in patients scheduled for heart surgery. The results showed a 65% reduction in pneumonia, which included a significant reduction in respiratory infections caused by bacteria. The authors concluded that the use of chlorhexidine might have fewer side effects than broad-spectrum antibiotic administration in selective decontamination for the prevention of pneumonia. Bergmans et al. [45] showed the significance of oral decontamination in the prevention of nosocomial infections in critically ill patients and of ventilator-associated pneumonia. In the present study, we found that mechanical and chemical prophylactic oral cleansing using povidone-iodine effectively reduced the numbers of anaerobic bacteria. This oral hygienic treatment also resulted in the elimination or reduction of *S. pneumoniae* and *H. influenzae*. The detection rates of *H. influenzae* before cleansing with mechanical and chemical oral hygienic treatment were slightly higher than those in the noncleansing group, but the difference was not significant by the chi-square test. However, further analysis in the larger series is essential to completely eliminate sampling bias.

In conclusion, mechanical and chemical oral cleansing using povidone-iodine appears to have been effective in eliminating these microorganisms in oral biofilms such as dental plaque. All the patients in the oral cleansing group carried out oral rinsing with povidone-iodine twice a day after surgery. It is possible that continuous oral cleansing with povidone-iodine solution could effectively eliminate these pathogens from the oral cavity. In fact, we and other groups have shown that continuous professional mechanical cleansing did reduce the cell numbers of potential respiratory pathogens [28,45–47]. Based on these findings, we emphasize that mechanical and chemical prophylactic cleansing of the oral cavity with povidone-iodine is an effective protocol for reducing potential respiratory pathogens in patients undergoing endotracheal anesthesia.

Acknowledgments. We thank Dr. B.L. Pierce for assistance with the English of this manuscript. This study was supported in part by a grant from the Oral Health Center, Tokyo Dental College.

References

1. Andrews CP, Coalson JJ, Smith JD, Johanson WG Jr (1981) Diagnosis of nosocomial bacterial pneumonia in acute, diffuse lung injury. Chest 80:254–258
2. Pugin J, Auckenthaler R, Lew DP, Suter PM (1991) Oropharyngeal decontamination decreases incidence of ventilator-associated pneumonia. A randomized, placebo-controlled, double-blind clinical trial. JAMA 265:2704–2710

3. Estes RJ, Meduri GU (1995) The pathogenesis of ventilation-associated pneumonia: I. Mechanisms of bacterial transcolonization and airway inoculation. Intensive Care Med 21:365–383
4. Scannapieco FA, Stewart EM, Mylotte JM (1992) Colonization of dental plaque by respiratory pathogens in medical intensive care patients. Crit Care Med 20:740–745
5. Marik PE (2001) Aspiration pneumonitis and aspiration pneumonia. N Engl J Med 344:665–671
6. Levine SA, Niederman MS (1991) The impact of tracheal intubation on host defenses and risks for nosocomial pneumonia. Clin Chest Med 12:523–543
7. Van Uffelen R, Rommes JH, Van Saene HKF (1987) Preventing lower airway colonization and infection in mechanically ventilated patients. Crit Care Med 15:99–102
8. Allaouchiche B, Jaumain H, Chassard D, Bouletreau P (1999) Gram stain of bronchoalveolar lavage fluid in the elderly diagnosis of ventilator-associated pneumonia. Br J Anaesth 83:845–849
9. Mimoz O, Karim A, Mazoit JX, Edouard A, Leprince S, Nordmann P (2000) Gram staining of protected pulmonary specimens in the elderly diagnosis of ventilator-associated pneumonia. Br J Anaesth 85:735–739
10. Finegold SM (1999) Aspiration pneumonia. Rev Infect Dis 13: S737–742
11. Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ (1987) Bacterial biofilms in nature and disease. Annu Rev Microbiol 41:435–464
12. Costerton JW, Lewandowski Z, DeBeer D, Caldwell D, Korber D, James G (1994) Biofilms, the customized microniche. J Bacteriol 176:2137–2142
13. Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284: 1318–1322
14. Whitehead NA, Bernard AML, Slater H, Simpson NJL, Salmond GPC (2001) Quorum-sensing in gram-negative bacteria. FEMS Microb Rev 25:364–404
15. Miller MB, Bassler B (2001) Quorum-sensing in bacteria. Annu Rev Microbiol 55:165–199
16. Kolenbrander PE (2000) Oral microbial communities: biofilms, interactions, and genetic systems. Annu Rev Microbiol 54:413–437
17. Scannapieco FA, Papandonatos GD, Dunford RG (1998) Associations between oral conditions and respiratory disease in a national sample survey population. Ann Periodontol 3:251–256
18. Russell SL, Boylan RJ, Kaslick RS (1999) Respiratory pathogen colonization of dental plaque of institutionalized elders. SCD Special Care in Dentistry 19:128–134
19. Gilbert P, Das J, Foley I (1997) Biofilm susceptibility to antimicrobials. Adv Dent Res 11:160–167
20. Kobayashi H (2001) Airway biofilm disease. Int J Antimicrob Agents 17:351–356
21. Budhani PK, Struthers JK (1998) Interactions of *Streptococcus pneumoniae* and *Moraxella catarrahalis*: investigation of the indirect pathogenic role of beta-lactamase-producing moraxellae by use of a continuous-culture biofilm system. Antimicrob Agents Chemother 42:2521–2526
22. Xu KD, McFeters GA, Stewart PS (2000) Biofilm resistance to antimicrobial agents. Microbiology 146:547–549
23. Mah TF, O'Toole GA (2001) Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 9:34–39
24. Addy M, Griffiths C, Isaac R (1997) The effect of povidone-iodine on plaque and salivary bacteria. A double-blind crossover trial. J Periodontol 48:730–732
25. Okuda K, Adachi M, Iijima K (1998) The efficacy of antimicrobial mouth rinses in oral health care. Bull Tokyo Dent Coll 39:7–14
26. Van Enk R, Tompson KD (1992) Use of primary isolation medium for recovery of methicillin-resistant Staphylococcus. J Clin Microbiol 30:504–505
27. Murakami K, Minamide W, Wada K, Nakamura E, Teraoka H, Watanabe S (1996) Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction. J Clin Microbiol 29:2240–2244
28. Abe S, Ishihara K, Okuda K (2001) Prevalence of potential respiratory pathogens in the mouth of elderly patients and effects of professional oral care. Arch Gerontol Geriatr 32:45–55
29. Fonseca K, MacDougall J, Pitt T (1986) Inhibition of *Pseudomonas aeruginosa* from cystic fibrosis by selective media. J Clin Pathol 39:220–222
30. Campbell ME, Farmer SW, Speert DP (1988) New selective medium for *Pseudomonas aeruginosa* with phenanthroline and 9-chloro-9 [4-(diethylamino)Phenyl]-9, 10-dihydro-10-phenylacridine hydrochloride (C-390). J Clin Microbiol 26:1910–1912
31. Saruta K, Matsunaga T, Hoshina S, Kono M, Kitahara S, Kanemoto S, Sakai O, Machida K (1995) Rapid identification of *Streptococcus pneumoniae* by PCR amplification of ribosomal DNA spacer region. FEMS Microbiol Lett 132:165–171
32. Ueyama T, Kurono Y, Shirabe K, Takeshita M, Mogi G (1995) High incidence of *Haemophilus influenzae* in nasopharyngeal secretions and middle ear effusions as detected by PCR. J Clin Microbiol 33:1835–1838
33. De Vos D, Lim A Jr, Pirnay JP, Struelens M, Vandenvelde C, Duinslaeger L, Vandelkelen A, Cornelis P (1997) Direct detection and identification of *Pseudomonas aeruginosa* in clinical samples such as skin biopsy specimens and expectorations by multiplex PCR based on two outer membrane lipoprotein genes. *oprI* and *oprL*. J Clin Microbiol 35:1295–1299
34. Watanabe K, Frommel TO (1996) *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, and *Treponema denticola* detection in oral plaque samples using the polymerase chain reaction. J Clin Periodontol 23:212–219
35. Jantos CA, Roggendorf R, Wuppermann FN, Hegemann JH (1998) Rapid detection of *Chlamydia pneumoniae* by PCR-enzyme immunoassay. J Clin Microbiol 36:1890–1894
36. Adair CG, Gorman SP, Feron BM, Byers LM, Jones DS, Goldsmith CE, Moore JE, Kerr JR, Curran MD, Hogg G, Webb CH, McCarthy GJ, Milligan KR (1999) Implications of endotracheal tube biofilm for ventilator-associated pneumonia. Intensive Care Med 25:1072–1076
37. Moore WEC, Moore HLV (1994) The bacteria of periodontal diseases. Periodontology 2000 5:66–77
38. Takazoe I, Nakamura T, Okuda K (1984) Colonization of subgingival area by *Bacteroides gingivalis*. J Dent Res 63:422–427
39. Ishihara K, Miura T, Kimizuka R, Ebihara Y, Mizuno Y, Okuda K (1997) Oral bacteria inhibit the *Helicobacter pylori* growth. FEMS Microbiol Lett 159:355–361
40. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA (2001) Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. J Bacteriol 183:5385–5394
41. Wilson M (1996) Susceptibility of oral bacterial biofilms to antimicrobial agents. J Med Microbiol 44:79–87
42. Roberts MC (1998) Antibiotic resistance in oral/respiratory bacteria. Crit Rev Oral Biol Med 9:522–540
43. Rodriguez-Roldan JM, Altuna-Cuesta A, Lopez A, Carrillo A, Garcia J, Leon J, Martinez-Pelles AJ (1990) Prevention of nosocomial lung infection in ventilated patients: use of an antimicrobial pharyngeal nonabsorbable paste. Crit Care Med 18: 1239–1242
44. DeRiso AJ 2nd, Ladowski JS, Dillon TA, Justice JW, Peterson AC (1996) Chlorhexidine gluconate 0.12% oral rinse reduces the incidence of total nosocomial respiratory infection and nonprophylactic systemic antibiotic use in patients undergoing heart surgery. Chest 18:1556–1561
45. Bergmans DC, Bonten MJ, Gaillard CA, Paling JC, van der Geest S, van Tiel FH, de Leeuw PW, Stobberingh EE (2001) Prevention of ventilator-associated pneumonia by oral decontamination: a prospective, randomized, double-blind, placebo-controlled study. Am J Respir Crit Care Med 16:382–388

46. Fourrier F, Cau-Pottier E, Boutingny H (2000) Effect of dental plaque antiseptic decontamination on bacterial colonization and nosocomial infections in critically ill patients. Intensive Care Med 26:1239–1247

47. Adachi M, Ishihara K, Abe S, Okuda K, Ishikawa T (2002) Effect of professional oral health care on elderly living in nursing homes. Oral Surg Oral Med Oral Pathol Oral Radiol Endodontics 94: 191–195

Volume 74 • Number 10

Antibody Responses of Periodontitis Patients to Gingipains of *Porphyromonas gingivalis*

Satoru Inagaki,* Kazuyuki Ishihara,† Yuriko Yasaki,* Satoru Yamada,* and Katsuji Okuda†

Background: Arginine- and lysine-specific cysteine proteinases (arg-gingipain: Rgp, lys-gingipain: Kgp) are major virulence factors of *Porphyromonas gingivalis*. Recent reports have suggested that antibodies against gingipains can play a protective role against infection by *P. gingivalis*. The purpose of this study was to evaluate the IgG responses of patients with periodontitis to functional domains of gingipains.

Methods: A group of 29 periodontitis patients and 10 periodontally healthy subjects (control group) were recruited into this study. We prepared three recombinant fragments of *rgp A* (catalytic domain; r-Rgp CAT) and two hemagglutinin domains (r-Rgp 44, and r-Rgps 15-27) corresponding to amino acid residues 228 to 719, 720 to 1136, and 1137 to 1704, respectively. One fragment of the Kgp catalytic domain (r-Kgp CAT) corresponding to amino acid residues 229 to 737 and expressed in *Escherichia coli* was also used. IgG antibody levels to these recombinant proteins in sera from the subjects were determined by an enzyme-linked immunosorbent assay (ELISA).

Results: We found that IgG levels against r-Rgp 44 and r-Rgps 15-27 in sera obtained from the patients were significantly higher than those in the healthy group ($P < 0.01$). In contrast, no significant differences in IgG levels against r-Rgp CAT and r-Kgp CAT were found between the control and patient groups. The IgG responses to *P. gingivalis* sonic extracts, r-Rgp 44 and r-Rgps 15-27, were related to probing depth in sera from patients, but those to r-Rgp CAT and r-Kgp CAT were not.

Conclusion: The present findings suggest that the low responsiveness of IgG antibody against the catalytic domains of gingipain, r-Rgp CAT, and r-Kgp CAT is a key factor in infection by *P. gingivalis*. *J Periodontol 2003;74:1432-1439.*

KEY WORDS

Antibody response; gingipain; periodontitis/microbiology; *Porphyromonas gingivalis*.

* Department of Periodontics, Tokyo Dental College, Chiba, Japan.
† Department of Microbiology, Tokyo Dental College.

Porphyromonas gingivalis, a Gram-negative anaerobic bacterium, has been isolated frequently from subgingival lesions in patients with chronic periodontitis and is thought to be a major pathogen in the disease.[1] The pathogenicity of *P. gingivalis* has been attributed to a number of virulence factors including LPS, fimbriae, hemagglutinin, and proteases.[2] The extracellular cysteine proteases named arg-gingipain (Rgp) and lys-gingipain (Kgp) have been isolated from culture supernatants, vesicle membrane fractions, and cell extracts of *P. gingivalis*.[3-5] Arg- and Lys-gingipain play a major role in disease pathogenesis by dysregulating the host immune and inflammatory responses.[6,7]

Rgp activity is encoded by *rgp A* and *rgp B* genes. The *rgp A* gene encodes a prepropeptide, a catalytic domain, and an adhesin-hemagglutinin domain (hag-domain). The initial polyprotein is apparently subject to post-translational processing. Rgp B is expressed as a preproenzyme missing the majority of the hag-domain. The N-terminal two-thirds of the primary structure of mature Rgp B containing the active-site cysteine residue and putative histidine residue of the catalytic dyad is identical to the catalytic domain of Rgp A.[8] The hag-domains of Rgp A and Kgp are similar in amino acid sequence. The catalytic domains of both Rgp A and Kgp share only limited identity (27%) scattered throughout the polypeptide chain, but the 30-amino acid residue located in the C-terminal part of the molecule is identical in the two mole-

cules.[9,10] The major forms of high molecular mass gingipains, Rgp A and Kgp, are non-covalently bound polypeptide chains consisting of the nascent hag-domains produced following putative proteolytic processing of the proenzymes at peptide bounds containing one lysine and three arginine residues.[11-13]

These enzymes may participate in pathogenicity by activating host proenzymes such as plasminogen or by exposing host cell cryptotopes, as well as by altering blood clotting.[14-16] In addition, the protease can degrade a variety of host proteins such as connective tissue components.[17] The host immune responses in these regions may also be dysregulated by the protease.[18]

It has been reported that the antibody levels in sera from chronic periodontitis patients against *P. gingivalis* whole cells and outer membrane preparations are higher than those in sera from healthy subjects.[19,20] The severity of periodontitis has been found to be associated with an increased IgG response to *P. gingivalis*.[21] Patients with destructive periodontal disease frequently display an elevated IgG response to *P. gingivalis*; however, these antibodies are apparently ineffective at limiting disease progression.[22,23] Trials for induction of protective immune responses against *P. gingivalis* have been performed using Rgp A.[24,25] However, the mechanism of antibody production has not been elucidated. To clarify the antibody responses against the functional domains of gingipains, we prepared recombinant proteins of the Rgp A functional domains as well as the catalytic domain of Kgp and determined the IgG antibody responses to these domains in sera from patients with chronic periodontitis and in healthy subjects.

MATERIALS AND METHODS

Subject Population

After obtaining informed consent according to guidelines established by the Institutional Ethics Committee of Tokyo Dental College, patients with chronic periodontitis who were scheduled for treatment in the Department of Periodontics, Tokyo Dental College, Chiba Hospital, were randomly selected. Twenty-nine patients (15 males and 14 females, mean age 48.2 years old, range from 35 to 68 years) were selected based upon radiographical evidence of bone loss and the presence of four or more sites with pocket depths >5 mm. Patients were assigned to the "*P. gingivalis*-infected" group if they had subgingival plaque samples positive for *P. gingivalis* as determined by the polymerase chain reaction (PCR) as described by Watanabe et al.[26] Ten periodontally healthy individuals (six males and four females, mean age 36.9 years old, range from 30 to 44 years) were selected because they had no detectable alveolar bone loss and mostly healthy gingiva with only mild gingivitis. All subjects were Japanese and had no history of systemic antibiotic administration during the previous 6 months.

Sampling

Samples of subgingival plaque and venous blood were collected and diagnostic examinations including probing depth (PD) were performed at the first visit to the hospital. After clotting, serum was separated by centrifugation at 1,500 × g for 20 minutes, and stored at –20°C until analysis. Subgingival plaque samples were collected with three sterilized paper points‡ (#40) and transferred to 100 µl of phosphate buffered saline (PBS, pH 7.2).

Preparation of Antigens for Enzyme-Linked Immunosorbent Assay

P. gingivalis ATCC 33277 was incubated in Trypticase soy broth§ supplemented with 5 µg of hemin and 0.5 µg of menadione per ml under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) at 37°C until late log phase. Harvested cells were washed three times with PBS (pH 7.2), and then resuspended in 20 ml PBS. Cell suspensions were sonicated on ice for 5 minutes at 100 W using a sonicator.‖ Unbroken cells were removed by centrifugation at 20,000 × g for 30 minutes.

Preparation of Recombinant Gingipains

The genomic DNA of *P. gingivalis* ATCC 33277 was isolated as described previously.[27] This genomic DNA was used as a template for amplifying the *rgp* and *kgp* genes by PCR. The primers for amplification of the fragments are listed in Table 1. The catalytic domain of Rgp A, the hag-domain of Rgp A, and the catalytic domain of Kgp were amplified by primers pRgp CAT, pKgp CAT, pRgp 44, and pRgps 15-27, respectively. We prepared amplified fragments by pRgp CAT, pRgp 44, and pRgps 15-27 coding for amino acid residues 228 to 719, 720 to 1136, and 1137 to 1704 of RgpA, respectively, and an amplified fragment by pKgp CAT encoding amino acid residues 229 to 737 of Kgp (Fig. 1). The amplification was performed using a thermal cycler¶ under the following conditions: 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 60 seconds, and primer extension at 72°C for 3 minutes. The PCR product was electrophoresed on 1% agarose gel and visualized under UV light after staining with ethidium bromide.

Subsequent ligation of the *rgp A* gene into an expression vector# was carried out by using the pET32 Xa LIC vector kit according to the manufacturer's instructions. The obtained plasmid was transformed into *Escherichia coli* Novablue. Isolation of recombinant clones was performed on Luria-Bertani (LB) agar plates or in LB broth containing 60 µg/ml of ampicillin. Confirmation of the identity of the cloned fragment was

‡ Pierce, Tokyo, Japan.
§ BBL Microbiological Systems, Becton Dickinson, Cockeysville, MD.
‖ SONIFIER 250, Branson, Dunbery, CT.
¶ Perkin-Elmer Biosystems, Foster City, CA.
# Novagen, Madison, WI.

Table I.
Primers Used in Study

	Primers (5′-3′)
pRgp CAT	5′-GGTATTGAGGGTCGCATGTACACACCGGTAGAGGAAAAACAAAATGGTCG-3′ 5′-AGAGGAGAGTTAGAGCCTTAGCGAAGAAGTTCGGGGGCATCGCTG-3′
pRgp 44	5′-GGTATTGAGGGTCGCATGAGCGGTCAGGCCGAGATTGTTCTTG-3′ 5′-AGAGGAGAGTTAGAGCCTTAGCGCTTGCCGTTGGCCTTGATTCTCAA-3′
pRgps 15-27	5′-GGTATTGAGGGTCGCATGCAGCGAGTTTCTCTACGTAAG-3′ 5′-AGAGGAGAGTTAGAGCCTTACAGCGAGTTTCTCTACGTAAG-3′
pKgp CAT	5′-GGTATTGAGGGTCGCGATGTTTATACAGATCATGGCGACT-3′ 5′-AGAGGAGTTAGAGTTAGAGCGTTAACGTACATCGTTTGCAGGTTCGATCG-3′
P. gingivalis	5′-ATAATGGAGAACAGCAGGAA-3′ 5′-TCTTGCCAACCAGTTCCATTGC-3′

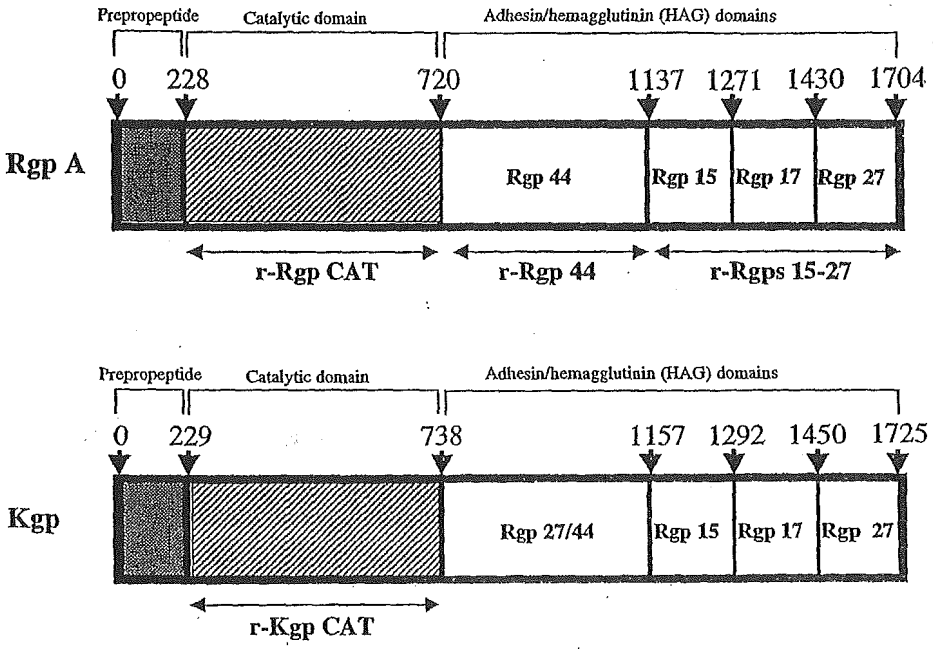

Figure 1.
Structures of Rgp A and Kgp polyproteins used. Number indicates the number of amino acid residues.

determined following nucleotide sequencing with the dideoxy chain termination method using a capillary sequencer.**

The expression and isolation of gingipain were performed using Ni-NTA kits.†† Briefly, an overnight culture of *E. coli* BL21 harboring a recombinant plasmid was inoculated into 500 ml of fresh LB broth containing 60 µg/ml ampicillin. The broth was incubated at 37°C with vigorous shaking until the optical density at 590 nm reached 0.8; then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM. After an additional 3 hours of incubation, the cells were harvested and washed three times with 50 mM Tris-HCl (pH 8.0). The cell pellet was suspended in the 0.01 M Tris-HCl buffer (pH 8.0) containing 8 M urea and 0.1 M NaH_2PO_4 and incubated with agitation for 1 hour at room temperature. The supernatant was applied to Ni-NTA (nitrilotriacetic acid) affinity chromatography with a Ni-NTA SUPERFLOW (1.6 by 5 cm), which was equilibrated with the same buffer.

The column was washed with 0.01 M Tris-HCl buffer (pH 6.3) containing 8 M urea and 0.1 M NaH_2PO_4 and eluted with 0.01 M Tris-HCl buffer (pH 4.5) containing 8 M urea and 0.1 M NaH_2PO_4. Eluted r-Rgp A and r-Kgp proteins were examined for purity following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

Measurement of Serum IgG Levels

Enzyme-linked immunosorbent assays (ELISA) were performed according to the method of Moughton et al.[19] Briefly, 100 µg/ml of *P. gingivalis* sonic extract or each recombinant protein was coated on flat-bottomed polystyrene microtiter plates‡‡ at 37°C for 2 hours. Each well was blocked with 2% of bovine serum albumin (BSA) for 60 minutes at room temperature. After washing, 1/1,000 dilution of the sera from each subject suspended in PBS containing 0.5% BSA was added to each well and incubated for 1 hour at 37°C. After washing, peroxidase-conjugated goat anti-human IgG (1/3,000 dilution) was reacted at 37°C for 60 minutes. After washing, development was performed by adding 0.1 M citric acid buffer containing o-phenylene diamine dihydrochloride and 0.02 µl of 30% H_2O_2. After developing for 10 minutes, an aliquot of 50 µl 6N H_2SO_4 was

** Kurabou, Osaka, Japan.
†† QIAGEN, Valencia, CA.
‡‡ Corning, Corning, NY.

added to stop the reaction. The absorbance at 490 nm was determined using a microplate reader. In a preliminary experiment, we confirmed that the antibody response against histidine-tag was at a negligible level. The antibody levels obtained by ELISA were expressed as ELISA units (EU). In a preliminary experiment, checkerboard assays were conducted to identify human sera having reactivity of the IgG classes of antibody against each domain of the gingipains. A standard titration curve was obtained by plotting the mean absorbance of the reference serum, and a straight line equation of the regression was developed. EU of all samples were calculated by relating optical density values from each experimental sample to the reference serum, which was assigned a value of 100 EU (optical density 1.2 in each antigen at 490 nm). The appropriate dilution of subject sera used in the ELISA was determined from preliminary experiments involving serial dilutions of sera and measurement of antibody responses.

Statistical Analysis

Differences in serum antibody levels between the periodontitis patient group and the healthy subject group were assessed by Mann-Whitney U test. The Spearman's rank correlation coefficient was used to determine the correlations between serum antibody responses of the 29 periodontal patients against each recombinant protein and mean probing depth.

RESULTS

Purification of Recombinant Gingipains

We constructed plasmids containing each of the three functional domains of Rgp A (r-Rgp CAT, r-Rgp 44, r-Rgps 15-27) and a catalytic domain of Kgp (r-Kgp CAT). The DNA fragments inserted into the expression vector were confirmed by sequencing. These recombinant proteins were expressed in *E. coli* as fusion proteins of the histidine-tag protein with each domain. The recombinant protein was purified using a nickel chelate affinity column and confirmed by SDS-PAGE and immunoblotting. As shown in Figure 1, the four recombinant proteins isolated, which corresponded to residues 228-719, 720-1136, 1137-1704 of Rgp A, and 229-737 of Kgp, were designated as r-Rgp CAT, r-Rgp 44, r-Rgps 15-27, and r-Kgp CAT, respectively. The results of SDS-PAGE and immunoblotting of these recombinant proteins are shown in Figure 2. The molecular masses of the purified proteins were 62, 61, 76, and 65 kDa for r-Rgp CAT, r-Rgp 44, r-Rgps 15-27, and r-Kgp CAT, respectively. These molecular masses of the recombinant proteins corresponded well to those of the histidine-tag protein and each domain of Rgp A or Kgp. Rabbit antiserum against *P. gingivalis* whole cells reacted against all of the recombinant gingipain proteins.

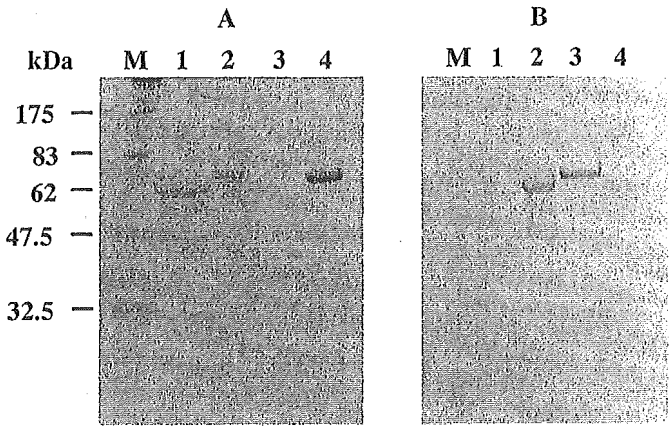

Figure 2.
A) *SDS-PAGE CBB staining of recombinant proteins used in this study. M: molecular weight standards; Lane 1: r-Rgp CAT; 2: r-Rgp 44; 3: r-Rgps 15-27; 4: r-Kgp CAT.* ***B)*** *Immunoblot analysis of recombinant proteins used in this study. M: molecular weight standards; Lanes 1: r-Rgp CAT; 2: r-Rgp 44; 3: r-Rgps 15-27; 4: r-Kgp CAT.*

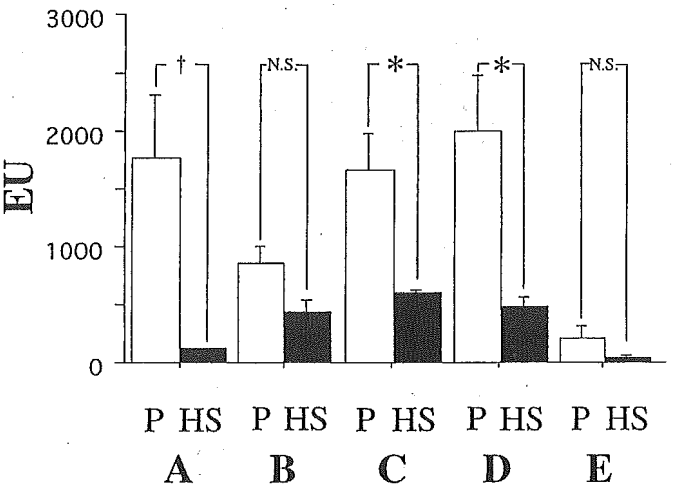

Figure 3.
*Comparison of serum IgG levels against functional domains of gingipains in sera obtained from periodontitis patients with those from healthy subjects. Antigen A: P. gingivalis sonic extract; B: r-Rgp CAT; C: r-Rgp 44; D: r-Rgps 15-27; E: r-Kgp CAT. P: periodontitis patients; HS: healthy subjects. *P <0.01; †P <0.001.*

IgG Responses to Recombinant Gingipains

The IgG responses in sera from the control and patient groups are shown in Figure 3. The patient group had significantly higher IgG responses to the *P. gingivalis* sonic extract ($P < 0.001$), r-Rgp 44, and r-Rgp 15-27 ($P < 0.01$) than those of the control group. Interestingly, no significant differences in IgG responses to r-Rgp CAT and r-Kgp CAT were observed between the control and patient groups.