Treponema maltophilum, Treponema medium, Treponema socranskii, Treponema vincentii, Mogibacterium timidum (formerly *Eubacterium timidum*), *Capnocytophaga ochracea*, and *Capnocytophaga sputigena* have also been recognized as pathogens of periodontitis based on their presence and relative numbers in healthy vs. diseased sites (18, 24, 48, 51). *Actinobacillus actinomycetemcomitans, Prevotella tannerae, Prevotella intermedia, Prevotella nigrescens, Centipeda periodontii*, and *Selenomonas sputigena* have been found in subgingival lesions of periodontitis patients (1, 23, 25, 36). *Dialister pneumosintes, Slackia exigua* (formerly *Eubacterium exiguum*), *Eubacterium saphenum, and Porphyromonas endodontalis* have also been considered to be associated with periodontitis (10, 18, 46).

Although all 25 bacterial species mentioned above are thought to be related to periodontitis, they have not been considered in the same study. Therefore, firstly, we undertook a comprehensive investigation of the 25 bacteria in subgingival and supragingival plaque using polymerase chain reaction (PCR) of the 16S rRNA genes. Secondly, we evaluated the relationship between periodontitis and the detection frequency of these bacteria. Thirdly, we compared the bacterial detection frequencies in subgingival plaque with the frequencies in supragingival plaque.

Material and methods

Subject population

Eighteen patients with periodontitis (mean age 63 ± 10.4 years; range 41–77 years) and 12 periodontally healthy subjects (mean age 27 ± 1.8 years; range 22–29 years) were randomly selected for this study. They had not received periodontal treatment or antimicrobial therapy for at least 6 months and were free of systemic diseases. Informed consent was obtained from each subject. Probing depths were measured in all teeth at six sites per tooth in each subject, and the teeth with the deepest probing depths were chosen as the target sites of sampling. The deepest probing depths were <4 mm (range 2.0–3.0 mm; mean 2.4 ± 0.5 mm) in periodontally healthy subjects (n = 12) and ≥4 mm (range 4.0–10.0 mm; mean 6.2 ± 2.1 mm) in subjects with periodontitis (n = 18).

Collection of samples

Supragingival plaque samples were taken with sterile explorers. For subgingival plaque samples, each of the target teeth was isolated with cotton rolls and air-dried after thorough removal of supragingival plaque with sterile cotton pellets. The samples were then collected using sterile periodontal pocket probes. In addition, alveolar mucosal samples (7 of 12 healthy subjects, 6 of 18 periodontitis subjects) were collected at the same sampling sites by swabbing 10 times with sterile swabs. All samples were immediately suspended in 1 ml of sterile distilled water and stored at −20°C before extraction of genomic DNA.

DNA extraction and nested PCR detection

After thawing, samples were centrifuged at 7740 × *g* for 5 min and the supernatants removed. Genomic DNA was then extracted from the pellets using an InstaGene Matrix Kit (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions.

In the first amplification, the 16S rRNA genes were amplified by PCR with universal primers 27F and 1492R (19, 34, 35) and Taq DNA polymerase (HotstarTaq Master mix; Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The primer sequences were: 27F, 5′- AGA GTT TGA TCC TGG CTC AG -3′; and 1492R, 5′- TAC GGG TAC CTT GTT ACG ACT T -3′. PCR mixtures were 5 µl of genomic DNA and 95 µl of reaction mixture containing 1.5 mM $MgCl_2$. PCR amplification was performed in a PCR Thermal Cycler MP (TaKaRa Biomedicals, Ohtsu, Shiga, Japan) programmed for 15 min at 95°C for initial heat activation, 35 cycles of 1 min at 94°C for denaturation, 1 min at 60°C for annealing, and 1.5 min at 72°C for extension, and 10 min at 72°C for final extension. The predicted PCR product with the universal primers was 1505 bp in length.

The 25 bacteria were identified by amplification of the first PCR amplification products using species-specific primers (Table 1) based on 16S rRNA gene sequences. PCR mixtures were 1 µl of the first PCR amplification mixture and 24 µl of reaction mixture containing 1.5 mM $MgCl_2$. PCR amplification was performed in a PCR Thermal Cycler MP (TaKaRa Biomedicals) programmed for 15 min at 95°C for initial heat activation, 35 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1.5 min at 72°C for extension, and 10 min at 72°C for final extension. The predicted sizes of PCR products with species-specific primers are listed in Table 1.

The PCR products were separated on 2% agarose gels (High Strength Analytical Grade Agarose, Bio-Rad Laboratories) in Tris-borate EDTA buffer (100 mM Tris, 90 mM borate; 1 mM EDTA, pH 8.4) stained with ethidium bromide and photographed under ultraviolet light. A 100-bp DNA Ladder (Invitrogen Corp., Carlsbad, CA) was used as a molecular size marker.

Data analysis

Fisher's exact probability tests were applied to compare the detection frequencies of the bacterial species between the healthy and periodontitis subjects, and between supragingival and subgingival plaques to evaluate significance. *P*-value was adjusted from 0.05 to 0.002 based on the Bonferroni correction for multiple comparisons.

Results

The detection frequencies of target bacteria in subgingival and supragingival plaque are shown in Fig. 1. The bacterial species were listed in order of the differences (*P*-values) in their detection frequencies in subgingival plaque between healthy and periodontitis subjects. There were no significant differences ($P \geqslant 0.05$) in the detection frequencies of the first 14 bacteria between healthy and periodontitis subjects. Eleven of the 14 species (*F. nucleatum, C. rectus, E. corrodens, P. nigrescens, C. ochracea, T. maltophilum, S. exigua, M. micros, T. forsythia* (*T. forsythensis*), *C. sputigena*, and *C. periodontii*) were frequently detected in subgingival plaque samples (≥50%) of both periodontitis and healthy subjects. *A. actinomycetemcomitans, T. amylovorum*, and *T. vincentii* were infrequently detected in both periodontitis and healthy subjects (Fig. 1).

Of the last 11 species in Fig. 1, the detection frequencies of nine bacteria (*E. saphenum, P. intermedia, T. denticola, P. tannerae, T. medium, D. pneumosintes, T. socranskii, P. endodontalis*, and *S. sputigena*) seemed to be higher in subgingival plaque samples from periodontitis subjects than in healthy subjects, although these differences were not statistically significant by multiple comparisons ($0.002 \leq P < 0.05$, Fig. 1). The detection frequencies of *M. timidum* and *P. gingivalis* of periodontitis subjects were significantly higher ($P<0.002$) than those of healthy subjects. In addition, *P. gingivalis* was not detected in the subgingival plaque of healthy subjects (Fig. 1).

Table 1. Target bacteria and their species-specific primers

Species	Sequence (5'-3')	Size	References
Actinobacillus actinomycetemcomitans	CTC AGA GAT GGG TTT GTG CC AGA TTC ACT CCC CAT CGC TG	273 bp	(47)
Campylobacter rectus	TTT CGG AGC GTA AAC TCC TTT TC TTT CTG CAA GCA GAC ACT CTT	598 bp	(1)
Capnocytophaga ochracea	AGA GTT TGA TCC TGG CTC AG GAT GCC GTC CCT ATA TAC TAT GGG G	185 bp	(4)
Capnocytophaga sputigena	AGA GTT TGA TCC TGG CTC AG GAT GCC GCT CCT ATA TAC CAT TAG G	185 bp	(4)
Centipeda periodontii	AGA GTT TGA TCC TGG CTC AG TTA CAA AGG ATT ATT CGC CC	450 bp	(36)
Dialister pneumosintes	TTC TAA GCA TCG CAT GGT GC GAT TTC GCT TCT CTT TGT TG	1105 bp	(6)
Eikenella corrodens	CGA TTA GCT GTT GGG CAA CTT ACC CTC TGT ACC GAC CAT TGT AT	410 bp	(9)
Eubacterium saphenum	TCT ACT AAG CGC GGG GTG A A CCC GAT TAA GGG TAC	430 bp	(12)
Fusobacterium nucleatum	GAA GAA ACA AAT GAC GGT AAC AAC GTC ATC CCC ACC TTC CTC CT	705 bp	(31)
Micromonas micros	TCG AAC GTG ATT TTT GTG GA TCC AGA GTT CCC ACC TCT	1074 bp	(29)
Mogibacterium timidum	AAG CTT GGA AAT GAC GC CCT TGC GCT TAG GTA A	524 bp	(12)
Porphyromonas endodontalis	GCT GCA GCT CAA CTG TAG TC CCG CTT CAT GTC ACC ATG TC	672 bp	(2)
Porphyromonas gingivalis	GCG TAT GCA ACT TGC CTT AC GTT TCA ACG GCA GGC TGA AC	518 bp	(47)
Prevotella intermedia	CGT GGA CCA AAG ATT CAT CGG TGG A CCG CTT TAC TCC CCA ACA AA	259 bp	(26)
Prevotella nigrescens	GTG TTT CAT TGA CGG CAT CCG ATA TGA AAC CA CGT CTC TGT GGG CTG CGA	828 bp	(26)
Prevotella tannerae	CTT AGC TTG CTA AGT ATG CCG AG CTG ACT TAT ACT CCC G	550 bp	(50)
Selenomonas sputigena	AGA GTT TGA TCC TGG CTC AG TC AAT ATT CTC AAG CTC GGT T	478 bp	(36)
Slackia exigua	GCC AAG CGG CCT CGT CGA AG C GGC TTT AAG GGA TTC GCT CG	697 bp	(12)
Tannerella forsythensis	AAA ACA GGG GTT CCG CAT GG C ACC GCG GAC TTA ACA GC	426 bp	(22)
Treponema amylovorum	AGA GTT TGA TCC TGG CTC AG C ACG CCT TTA TTC CGT GAG	193 bp	(48)
Treponema denticola	TAA TAC CGA ATG TGC TCA TTT ACA T TCA AAG AAG CAT TCC CTC TTC TTC TTA	316 bp	(1)
Treponema maltophilum	AGA GTT TGA TCC TGG CTC AG CT ATT GTG CTT ATT CAT CAG GC	438 bp	(48)
Treponema medium	CAC TCA GTG CTT CAT AAG GG CG GCC TTA TCT CTA AGA CC	856 bp	(33)
Treponema socranskii	AGG TAG ACA GCG GGA AAG GA AA CCC AAC ACC TCA CGG CA	902 bp	(32)
Treponema vincentii	GTC TCA ATG GTT CAT AAG AA CAA GCC TTA TCT CTA AGA CT	856 bp	(33)

There were no significant differences in the detection frequencies of the 25 bacteria between subgingival and supragingival plaque (Fig. 1). The detection frequencies in alveolar mucosal plaque samples were generally lower than those in the respective subgingival and supragingival plaque samples (Fig. 2).

The percent agreement of detection frequencies of the 25 bacteria was also evaluated. In subjects with periodontitis, the percent agreement of subgingival vs. supragingival plaque was higher (mean 81%, range 50–100%) than that of alveolar mucosal plaque vs. subgingival plaque (mean 63%, range 17–100%) and alveolar mucosal plaque vs. supragingival plaque (mean 60%, range 33–83%). This trend was also observed in healthy subjects (data not shown).

Discussion

In this study, the detection frequencies of 25 bacteria in subgingival, supragingival, and alveolar mucosal plaque of untreated periodontitis subjects and periodontally healthy subjects were obtained by nested PCR based on 16S rRNA genes. Since the subject's age is one of the most important risk factors of periodontitis, the periodontitis subjects were first divided into two groups according to age (41–64 years and 65–77 years). However, as the detection frequencies of the 25 bacterial species were quite similar in subgingival plaque samples from the two groups (data not shown), the groups were combined into one in the present study.

The first 14 bacteria in Fig. 1 showed no significant differences in detection frequencies in subgingival plaque between healthy and periodontitis subjects ($P \geq 0.05$); 11 of these bacteria were detected at high frequencies (≥50%) in both periodontitis and healthy subjects (Fig. 1). The detection rates of *C. rectus* and *E. corrodens* have been reported to be unrelated to the progression of attachment loss (28). Moreover, *C. rectus, E. corrodens, C. ochracea*, and *C. sputigena* have been frequently

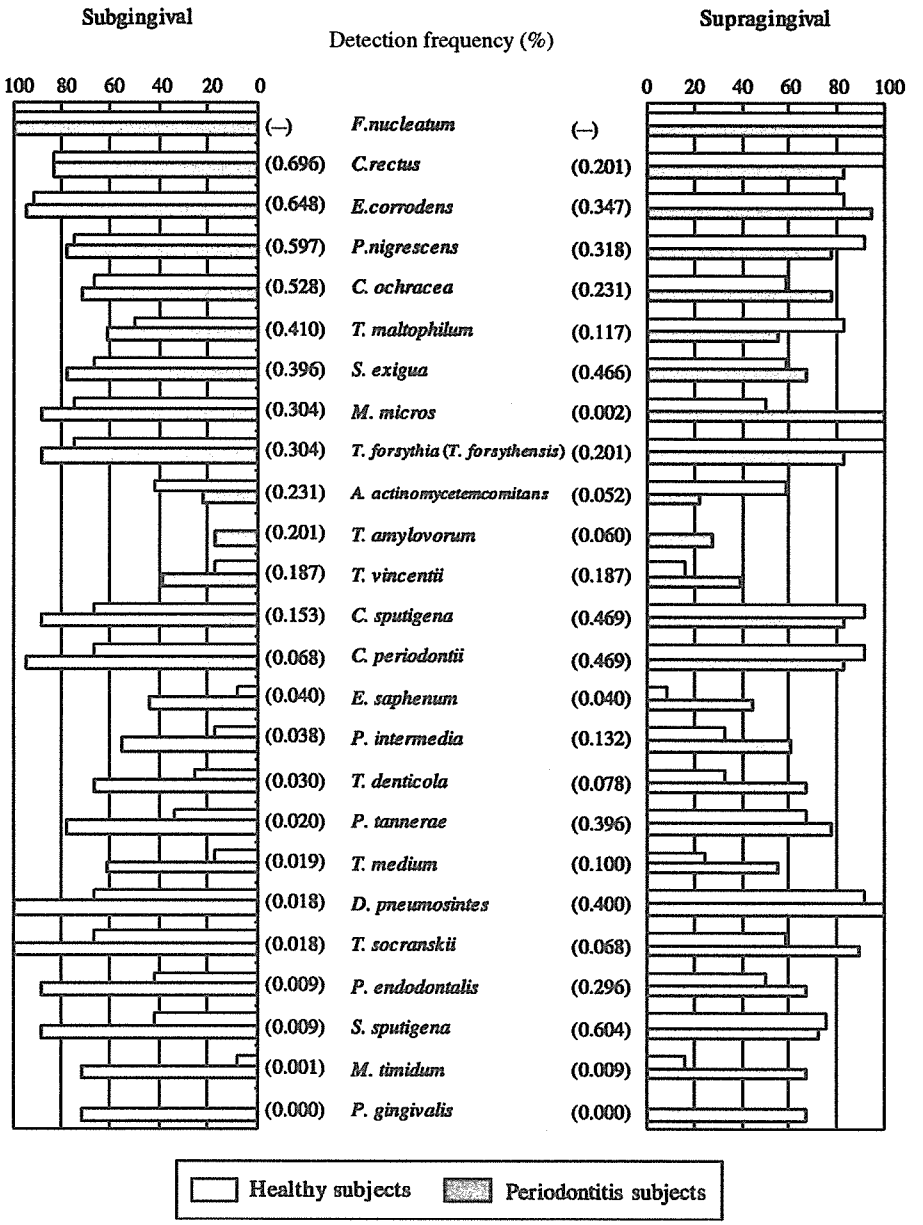

Fig. 1. Detection frequencies of target bacteria in subgingival and supragingival plaque. *P*-values of differences between healthy and periodontitis subjects are given in parentheses. Bacterial species are listed in order of *P*-values.

detected in plaque samples from healthy children (14). The results obtained in this study are therefore in accordance with those of previous studies, suggesting that these species are among the commensal bacteria of the oral cavity. *F. nucleatum* and *T. forsythia* (*T. forsythensis*) were also frequently detected in both healthy and periodontitis subjects in this study, suggesting that their relationship with periodontitis is uncertain. The *F. nucleatum* species consists of several subspecies (subspecies *nucleatum*, *polymorphum*, *fusiforme*, and *vincentii*) (7), and the *T. forsythia* (*T. forsythensis*) species has been reported to have *prtH* genetic subtypes (42). Thus, the pathogenicity of *F. nucleatum* and *T. forsythia* (*T. forsythensis*) probably varies depending on the strain (8, 30, 42). Further study will be required to determine the subspecies and subtypes of *F. nucleatum* and *T. forsythia* (*T. forsythensis*) specific to periodontitis. *A. actinomycetemcomitans* was detected at the adult periodontitis sites in less than 25%, which was lower than at healthy sites in this study (Fig. 1). This is consistent with a recent study (11), although this species has been considered to be associated with juvenile and adult periodontitis (17, 20, 52, 53). This discrepancy could be due to differing pathogenicity of the various serotypes and genotypes of *A. actinomycetemcomitans* (3, 43).

Of the remaining 11 species, the detection frequencies of nine bacteria in subgingival plaque were higher in periodontitis subjects than in healthy subjects, but this difference was not

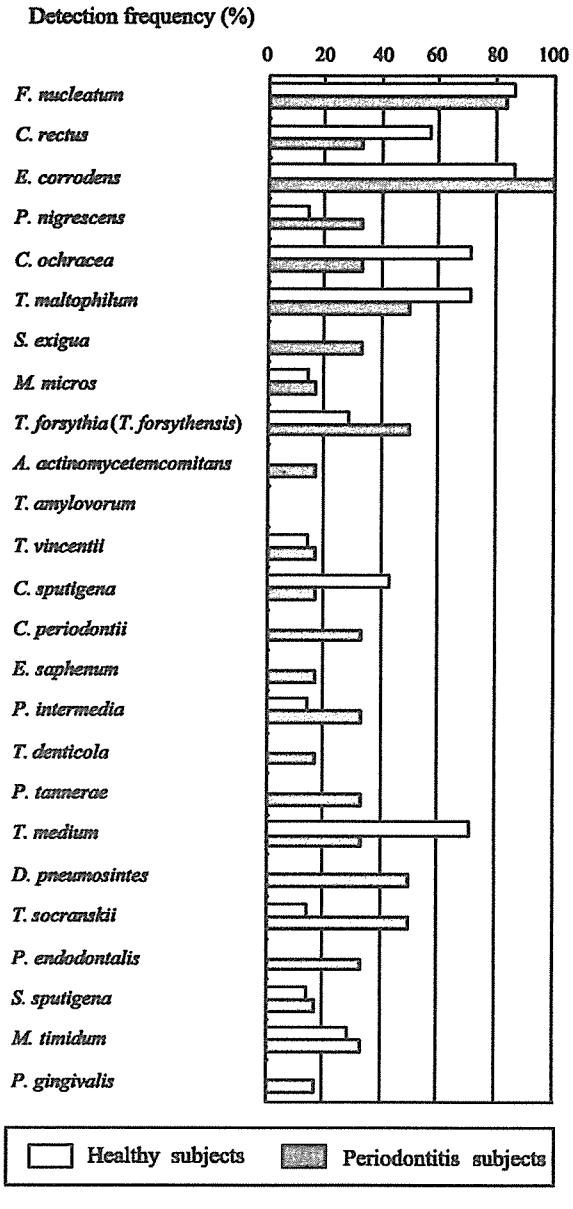

Fig. 2. Detection frequencies of target bacteria in alveolar mucosal plaque. Bacterial species are listed in the same order as in Fig. 1.

statistically significant ($0.002 \leq P < 0.05$) (Fig. 1). Two species (*M. timidum* and *P. gingivalis*) were detected significantly more frequently ($P < 0.002$) in subjects with periodontitis than in healthy subjects *P. gingivalis* was detected only in the periodontitis subjects. This is further evidence of an association of these two species with periodontitis, as reported previously for *P. gingivalis* (3, 18, 41, 51). *M. timidum* has also been isolated from deep periodontal pockets, supporting such an association (24, 45).

The detection frequencies of the bacteria in subgingival and supragingival plaque were similar in this study. A few studies have also suggested that the microbiota of subgingival and supragingival plaque were similar (16, 24, 51). Supragingival plaque may play an important role in bacterial invasion to subgingival sites, providing a reservoir of bacteria (51).

The recently proposed concept of 'biofilm' implies that bacteria establish a kind of supportive community (5). Based on this concept, bacterial species in dental plaque cooperate to make their environment anaerobic through oxygen consumption in their sugar and amino acid metabolic pathways (15, 21, 44) and maintain neutral pH via acid-neutralization by amino acid metabolism (39, 40), thus providing an environment where obligate anaerobic and acid-sensitive periodontopathic bacteria, such as *P. gingivalis*, are able to survive and grow. This may explain why *P. gingivalis* was detected at supragingival sites, which often become acidic and aerobic, in this study. From this point of view, the bacteria, frequently detected both in supragingival and subgingival plaque and at both healthy and periodontitis sites, are not individually pathogenic in the oral cavity, but rather may influence the initiation and progression of periodontitis when present together with periodontopathic bacteria.

The detection frequencies of bacteria in alveolar mucosa (Fig. 2) were generally lower than those in subgingival and supragingival plaque (Fig. 1), and the agreement rate of these bacteria at alveolar mucosa vs. subgingival (63%) or supragingival (60%) sites was also lower than those at subgingival vs. supragingival sites (81%). This suggests that alveolar mucosa has a microbiota composition that apparently differs from that of subgingival and supragingival plaque. This may be due to unique environmental factors of alveolar mucosa such as oxygen exposure and mobility, which result in less suitable environments for the target bacteria used in this study.

In conclusion, of 25 bacteria, 14 species showed no relationship with periodontitis, and 11 out of these 14 species were frequently detected (≥50%) in subgingival plaque in both periodontitis and healthy subjects. Nine bacteria seemed to be related to periodontitis, although their higher detection frequencies in subgingival plaque were not statistically significant by multiple comparisons. Only two species (*M. timidum* and *P. gingivalis*) in subgingival plaque were detected significantly more frequently in subjects with periodontitis than in healthy subjects, suggesting that the two species are closely related to periodontitis. In addition, the similarity of detection frequencies of the bacteria between subgingival and supragingival plaque suggests that analysis of supragingival plaque microbiota is useful in estimating the microbial composition of subgingival plaque.

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References

1. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. Oral Microbiol Immunol 1996: **11**: 266–273.
2. Bogen G, Slots J. Black-pigmented anaerobic rods in closed periapical lesions. Int Endod J 1999: **32**: 204–210.
3. Colombo AP, Teles RP, Torres MC, Souto R, Rosalem WJ, Mendes MC, et al. Subgingival microbiota of Brazilian subjects with untreated chronic periodontitis. J Periodontol 2002: **73**: 360–369.
4. Conrads G, Mutters R, Fischer J, Brauner A, Lutticken R, Lampert F. PCR reaction and dot-blot hybridization to monitor the distribution of oral pathogens within plaque samples of periodontally healthy individuals. J Periodontol 1996: **67**: 994–1003.
5. Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, et al. Bacterial biofilms in nature and disease. Ann Rev Microbiol 1987: **41**: 435–464.
6. Doan N, Contreras A, Flynn J, Slots J, Chen C. Molecular identification of *Dialister pneumosintes* in subgingival plaque of humans. J Clin Microbiol 2000: **38**: 3043–3047.
7. Dzink JL, Sheenan MT, Socransky SS. Proposal of three subspecies of *Fusobacterium nucleatum* Knorr 1922: *Fusobacterium nucleatum* subsp. *nucleatum* subsp. nov., comb. nov., *Fusobacterium nucleatum* subsp. *polymorphum* subsp. nov., nom. rev., comb. nov. & *Fusobacterium nucleatum* subsp. *vincentii* subsp. nov., nom. rev., comb. nov. Int J Syst Bacteriol 1990: **40**: 74–78.
8. Dzink JL, Socransky SS, Haffajee AD. The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. J Clin Periodontol 1988: **15**: 316–323.
9. Furcht C, Eschrich K, Merte K. Detection of *Eikenella corrodens* and *Actinobacillus actinomycetemcomitans* by use of the polymerase chain reaction (PCR) *in vitro* and in subgingival plaque. J Clin Periodontol 1996: **23**: 891–897.
10. Ghayoumi N, Chen C, Slots J. *Dialister pneumosintes*, a new putative periodontal pathogen. J Periodontal Res 2002: **37**: 75–78.
11. Hamlet SM, Cullinan MP, Westerman B, Lindeman M, Bird PS, Palmer J, et al. Distribution of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia* in an Australian population. J Clin Periodontol 2001: **28**: 1163–1171.
12. Hashimura T, Sato M, Hoshino E. Detection of *Slackia exigua, Mogibacterium timidum* and *Eubacterium saphenum* from pulpal and periradicular samples using the polymerase chain reaction (PCR) method. Int Endod J 2001: **34**: 463–470.
13. Kazor CE, Mitchell PM, Lee AM, Stokes LN, Loesche WJ, Dewhirst FE, et al. Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients. J Clin Microbiol 2003: **41**: 558–563.
14. Kimura S, Ooshima T, Takiguchi M, Sasaki Y, Amano A, Morisaki I, et al. Periodontopathic bacterial infection in childhood. J Periodontol 2002: **73**: 20–26.
15. Korayem M, Westbay G, Kleinberg I. Constituents of salivary supernatant responsible for stimulation of oxygen uptake by the bacteria in human salivary sediment. Arch Oral Biol 1990: **35**: 145–152.
16. Kornman KS. The role of supragingival plaque in the prevention and treatment of periodontal diseases. A review of current concepts. J Periodontal Res 1986: **21** (Suppl. 16): 5–22.
17. Kornman KS, Robertson PB. Clinical and microbiological evaluation of therapy for juvenile periodontitis. J Periodontol 1985: **56**: 443–446.
18. Kumar PS, Griffen AL, Barton JA, Paster BJ, Moeschberger ML, Leys EJ. New bacterial species associated with chronic periodontitis. J Dent Res 2003: **82**: 338–344.
19. Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, eds. Nucleic acid techniques in bacterial systematics. Chichester: John Wiley & Sons, 1991: 115–175.
20. Mandell RL, Socransky S. A selective medium for *Actinobacillus actinomycetemcomitans* and the incidence of the organism in juvenile periodontitis. J Periodontol 1981: **52**: 593–598.
21. Marquis RE. Oxygen-metabolism, oxidative stress and acid-base physiology of dental plaque biofilms. J Ind Microbiol 1995: **15**: 198–207.
22. Meurman JH, Wahlfors J, Korhonen A, Alakuijala P, Vaisanen P, Torkko H, et al. Identification of *Bacteroides forsythus* in subgingival dental plaque with the aid of a rapid PCR method. J Dent Res 1997: **76**: 1376–1380.
23. Moore LV, Johnson JL, Moore WE. Description of *Prevotella tannerae* sp. nov. and *Prevotella enoeca* sp. nov. from the human gingival crevice and emendation of the description of *Prevotella zoogleoformans*. Int J Syst Bacteriol 1994: 44: 599–602.
24. Moore WE, Holdeman LV, Cato EP, Smibert RM, Burmeister JA, Ranney RR. Bacteriology of moderate (chronic) periodontitis in mature adult humans. Infect Immun 1983: **42**: 510–515.
25. Moore WE, Moore LV. The bacteria of periodontal diseases. Periodontol 2000 1994: **5**: 66–77.
26. Okamoto M, Maeda N, Kondo K, Leung KP. Hemolytic and hemagglutinating activities of *Prevotella intermedia* and *Prevotella nigrescens*. FEMS Microbiol Lett 1999: **178**: 299–304.
27. Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, et al. Bacterial diversity in human subgingival plaque. J Bacteriol 2001: **183**: 3770–3783.
28. Renvert S, Dahlen G, Wikstrom M. Treatment of periodontal disease based on microbiological diagnosis. Relation between microbiological and clinical parameters during 5 years. J Periodontol 1996: **67**: 562–571.
29. Riggio MP, Lennon A, Smith A. Detection of *Peptostreptococcus micros* DNA in clinical samples by PCR. J Med Microbiol 2001: **50**: 249–254.
30. Roques CG, El Kaddouri S, Barthet P, Duffort JF, Arellano M. *Fusobacterium nucleatum* involvement in adult periodontitis and possible modification of strain classification. J Periodontol 2000: **71**: 1144–1150.
31. Rupf S, Merte K, Eschrich K. Quantification of bacteria in oral samples by competitive polymerase chain reaction. J Dent Res 1999: **78**: 850–856.
32. Sakamoto M, Takeuchi Y, Umeda M, Ishikawa I, Benno Y, Nakase T. Detection of *Treponema socranskii* associated with human periodontitis by PCR. Microbiol Immunol 1999: **43**: 485–490.
33. Sato T, Kuramitsu HK. Polymerase chain reaction for the detection of *fla*A-1 genes of oral spirochaetes in advanced periodontal pockets. Arch Oral Biol 2000: **45**: 921–925.
34. Sato T, Matsuyama J, Kumagai T, Mayanagi G, Yamaura M, Washio J, et al. Nested PCR for detection of mutans streptococci in dental plaque. Lett Appl Microbiol 2003: **37**: 66–69.
35. Sato T, Sato M, Matsuyama J, Hoshino E. PCR-restriction fragment length polymorphism analysis of genes coding for 16S rRNA in *Veillonella* spp. Int J Syst Bacteriol 1997: **47**: 1268–1270.
36. Sawada S, Kokeguchi S, Takashiba S, Murayama Y. Development of 16S rDNA-based PCR assay for detecting *Centipeda periodontii* and *Selenomonas sputigena*. Lett Appl Microbiol 2000: **30**: 423–426.
37. Socransky SS, Haffajee AD. Evidence of bacterial etiology: a historical perspective. Periodontol 2000 1994: **5**: 7–25.
38. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol 1998: **25**: 134–144.
39. Takahashi N. Acid-neutralizing activity during amino acid fermentation by *Porphyromonas gingivalis*, *Prevotella intermedia* and *Fusobacterium nucleatum*. Oral Microbiol Immunol 2003: **18**: 109–113.
40. Takahashi N, Saito K, Schachtele CF, Yamada T. Acid tolerance and acid-neutralizing activity of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Fusobacterium nucleatum*. Oral Microbiol Immunol 1997: **12**: 323–328.
41. Takeuchi Y, Umeda M, Sakamoto M, Benno Y, Huang Y, Ishikawa I. *Treponema socranskii, Treponema denticola*, and *Porphyromonas gingivalis* are associated with severity of periodontal tissue destruction. J Periodontol 2001: **72**: 1354–1363.
42. Tan KS, Song KP, Ong G. *Bacteroides forsythus prtH* genotype in periodontitis patients: occurrence and association with

periodontal disease. J Periodontal Res 2001: **36**: 398–403.
43. Tan KS, Song KP, Ong G. Cytolethal distending toxin of *Actinobacillus actinomycetemcomitans*. Occurrence and association with periodontal disease. J Periodontal Res 2002: **37**: 268–272.
44. Traudt M, Kleinberg I. Stoichiometry of oxygen consumption and sugar, organic acid and amino acid utilization in salivary sediment and pure cultures of oral bacteria. Arch Oral Biol 1996: **41**: 965–978.
45. Uematsu H, Hoshino E. Predominant obligate anaerobes in human periodontal pockets. J Periodontal Res 1992: **27**: 15–19.
46. Wade WG. The role of *Eubacterium* species in periodontal disease and other oral infections. Microb Ecol Health Dis 1996: **9**: 367–370.
47. Wahlfors J, Meurman JH, Vaisanen P, Alakuijala P, Korhonen A, Torkko H, et al. Simultaneous detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* by a rapid PCR method. J Dent Res 1995: **74**: 1796–1801.
48. Willis SG, Smith KS, Dunn VL, Gapter LA, Riviere KH, Riviere GR. Identification of seven *Treponema* species in health- and disease-associated dental plaque by nested PCR. J Clin Microbiol 1999: **37**: 867–869.
49. Wilson MJ, Weightman AJ, Wade WG. Applications of molecular ecology in the characterisation of uncultured microorganisms associated with human disease. Rev Med Microbiol 1997: **8**: 91–101.
50. Xia T, Baumgartner JC, David LL. Isolation and identification of *Prevotella tannerae* from endodontic infections. Oral Microbiol Immunol 2000: **15**: 273–275.
51. Ximenez-Fyvie LA, Haffajee AD, Socransky SS. Microbial composition of supra- and subgingival plaque in subjects with adult periodontitis. J Clin Periodontol 2000: **27**: 722–732.
52. Zambon JJ. *Actinobacillus actinomycetemcomitans* in human periodontal disease. J Clin Periodontol 1985: **12**: 1–20.
53. Zambon JJ, Christersson LA, Slots J. *Actinobacillus actinomycetemcomitans* in human periodontal disease. Prevalence in patient groups and distribution of biotypes and serotypes within families. J Periodontol 1983: **54**: 707–711.

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Expression of type IV collagen and laminin at the interface between epithelial cells and fibroblasts from human periodontal ligament

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The present study was undertaken to examine whether synthesis of type IV collagen and laminin around the epithelial rests of Malassez (ERM) requires direct contact between cells from ERM and periodontal ligament fibroblasts. Human periodontal ligament (HPDL) explants produced outgrowths containing both ERM cells and fibroblasts when cultured in a modified serum-free medium. The interface between ERM cells and fibroblasts was examined using phase-contrast microscopy (PCM) and scanning electron microscopy (SEM). Expression of type IV collagen and laminin was studied by immunohistochemistry and *in situ* hybridization. It was observed that ERM cells grew underneath fibroblasts or attached to them. At the interface, type IV collagen and laminin and their respective mRNAs were abundant in both ERM cells and fibroblasts, while these proteins and mRNAs showed little if any staining in cells further away from the interface. Hence, these findings indicate that synthesis of type IV collagen and laminin is induced by direct interaction between ERM cells and periodontal ligament fibroblasts.

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The epithelial rests of Malassez (ERM) are normal consistent constituents of the periodontal ligament (PDL) and persist throughout the life of a tooth as a network of cell clusters connected to each other and possibly also to the junctional epithelium of gingiva (1, 2). Although their functions are still unknown, it has been suggested that they play a role in maintaining the PDL space (3), and they are perhaps also associated with cementum formation and cementum repair (4–7). Increased proliferation of the epithelial-rest cells has been reported to occur after surgical trauma or infection (8, 9).

In general, epithelial cells are separated from the underlying connective tissue by a basement membrane, which is composed of a mixture of matrix components mainly including type IV collagen (10), laminin (11, 12), and heparin sulfate proteoglycan (10, 13). It has been shown that extracellular matrix molecules are essential for cell adhesion, migration, differentiation, and growth, and that production of the extracellular matrix is involved in the interaction between the epithelial and connective tissue cells of the periodontium (14–19). Similarly, the ERMs are surrounded by a continuous basement membrane (20, 21). In an *in vitro* ultrastructural study, BRUNETTE *et al.* (22) found material reminiscent of a basal lamina, apparently synthesized by epithelial cells cultured from monkey periodontal ligament, in an area where the epithelial cells were in contact with collagen-like material that may have been produced by fibroblast-like cells. HOU *et al.* (23) have suggested that human PDL fibroblasts adjacent to the mouse epithelial root sheath cells may exhibit enhanced intracellular fluorescence for collagen I and fibronectin.

The aim of the present study was to determine whether a basement membrane could develop *in vitro* under conditions that allowed epithelial cells and fibroblasts derived from human PDL to interact directly with each other under chemically defined conditions. Since extracellular matrix components are essential for the formation of a basement membrane between two cell types, we evaluated the synthesis extracellular matrix proteins and their corresponding mRNA to elucidate the effect of the interaction between epithelial cells and fibroblasts from human PDL.

Material and methods

Cell culture

Freshly extracted third molars from 34 patients between 17 yr and 28 yr of age were obtained from the Department

of Oral Surgery, Tohoku University Graduate School of Dentistry. Informed consent was obtained from the patients before extractions. After washing the teeth several times with alpha-minimum essential medium (α-MEM; Cosmo Bio, Tokyo, Japan) supplemented with 10% fetal bovine serum and antibiotics (60 $\mu g\ ml^{-1}$ kanamycin, 20 units ml^{-1} penicillin G, 10 $\mu g\ ml^{-1}$ fungison, Sigma, St Louis, MO, USA), PDL explants were carefully removed from the root with a scalpel. They were then plated in 35-mm culture dishes in α-MEM. The explants produced outgrowths that were primarily composed of fibroblasts. After 1 wk, α-MEM was replaced by a modified serum-free medium (3 : 1 (v/v) MCDB153 medium; Sigma) supplemented with 5 $\mu g\ ml^{-1}$ insulin (Sigma), 0.5 $\mu g\ ml^{-1}$ hydrocortisone (Sigma), 10 $\mu g\ ml^{-1}$ transferrin (Sigma), 14.1 $\mu g\ ml^{-1}$ phosphorylethanolamine (Sigma), 10 $ng\ ml^{-1}$ epidermal growth factor (EGF; Sigma) and antibiotics (24–26): α-MEM) including 40 $\mu g\ ml^{-1}$ bovine pituitary extract (Kyokuto, Tokyo, Japan) (27–29). This resulted in outgrowths of epithelial cells next to fibroblast-like cells. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Differential adhesion to the surface in the presence of 0.25% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) solution was used to produce cultures that were predominantly of one cell type (i.e. either fibroblasts (5–10 min) or epithelial cells (15–20 min)) (30).

Gingival tissues were removed from clinically healthy patients at the time of the third molar extraction. After mincing they were cultured as described above. Cultures of PDL epithelial cells only or PDL fibroblasts only were used as controls.

Growth curves

Epithelial cells from PDLs and gingival tissues (second subculture) were seeded at 2×10^4 cells cm^{-1} in 12-well culture plates (Falcon; Nippon Becton Dickinson, Tokyo, Japan). The number of cells per dish on successive days was determined using a hemocytometer after trypsinizing the cells from the dish.

Statistical analysis

Statistical analysis of data was performed using Student's *t*-test ($P < 0.05$).

Scanning electron microscopy

Outgrowths of both epithelial cells and fibroblasts from human PDL cultured on the dishes were fixed with 2.5% glutaraldehyde in PBS at 4°C for 1 h and processed according to the tannic acid technique of KATSUMOTO *et al.* (31). Specimens were dried in a critical point dryer (HCP-2; Hitachi, Tokyo, Japan), ion-sputtered with Au–Pd in a coating unit (Hitachi 01–101), and examined with a scanning electron microscope (Hitachi S-510).

Immunohistochemistry

Cells were fixed with 4% paraformaldehyde at room temperature for 10 min. Alternatively, cells cultured on plastic discs (Sumilon; Sumitomo Bakelite, Tokyo, Japan) were dehydrated using a graded series of ethanol treatments, and then embedded in paraffin. Serial sections of 10-μm thickness were cut on a microtome. Both the cells on the dish and the vertical sections were processed for immunohistochemical examination. To inhibit endogenous peroxidase, 3% hydrogen peroxide was added. The cells and the vertical sections were then incubated with 10% normal goat serum for 30 min to block non-specific binding. They were then treated at room temperature for 2 h with a primary antibody to monoclonal mouse antihuman cytokeratin AE1/AE3 (1 : 50) (Dako, Carpinteria, CA, USA) to detect epithelial cells. The cells and vertical sections were then incubated overnight at 4°C with primary antiserum to rabbit antiporcine 25 kDa amelogenin (0.1 $\mu g\ ml^{-1}$) (courtesy of Dr T. Uchida, Second Department of Oral Anatomy, Hiroshima University School of Dentistry, Japan), which recognizes both human and rat amelogenin (6, 32), and with primary antibodies to monoclonal mouse antihuman type IV collagen (1 : 500) (Sigma) or polyclonal rabbit antihuman laminin (1 : 50) (Rockland, Gilbertsville, PA, USA). After rinsing in PBS, the cells were incubated with biotinylated immunoglobulin at room temperature for 30 min and stained by the avidin-biotinylated peroxidase complex (ABC) method, using an ExtrAvidin peroxidase staining kit (Sigma) and an AEC (3-amino-9-ethylcarbazole) chromogen kit (Sigma).

Proliferating, DNA-synthesizing cells in co-cultures were identified by nuclear incorporation of 100 $\mu mol\ l^{-1}$ 5-bromo-2′-deoxy-uridine (BrdU; Boehringer Mannheim Biochemica, Germany) for 24 h. Labeled nuclei were detected with monoclonal mouse anti-BrdU antibodies for 30 min at 37°C. After washing three times in PBS, the cells were incubated with HRP-conjugated goat antimouse immunoglobulin (Chemicon International, Temecula, CA, USA) for 30 min at 37°C and stained with 3,3-diaminobenzidine (DAB) and H_2O_2. Mayer's hematoxylin solution was applied for counterstaining. For control staining, PBS was used instead of the primary antibody.

In situ hybridization

The oligonucleotide probes used for *in situ* hybridization were synthesized by Nihon Gene Research Laboratories, Sendai, Japan. The sequences were as follows:

Probe 1: 5′-TCC AGG GTA GCC CCT CTC TCC TTT TTC TCC CAA AGG TCC TGT GCC-3′ for type IV collagen α-1 mRNA (33);
Probe 2: 5′-GCC ACC AGG TGG TGT CGG CCT GGT TGT TGT AGT CGG TCA GGA AGG-3′ for laminin γ-1 mRNA (34).

A biotin label was added at the 3′ end.

A computer-assisted search (GenBank) using the above antisense sequences, as well as the corresponding sense sequences, revealed no significant homology with any known sequences, other than that of the type IV collagen α-1 chain and the laminin γ-1 chain, respectively.

In situ hybridization was carried out using the *In Situ* Hybridization Detection Kit for Biotin Labeled Probes (Sigma). Briefly, the cells were fixed with 4% paraformaldehyde at room temperature for 10 min. They were then immersed in PBS including RNase inhibitor and then digested with proteinase K (0.1 $\mu g\ ml^{-1}$) at room temperature. To inhibit endogenous peroxidase, 3% hydrogen peroxide was added. The specimens were hybridized with biotin-labeled probes in the hybridization solution in a humid incubation chamber overnight at 37°C. After washing

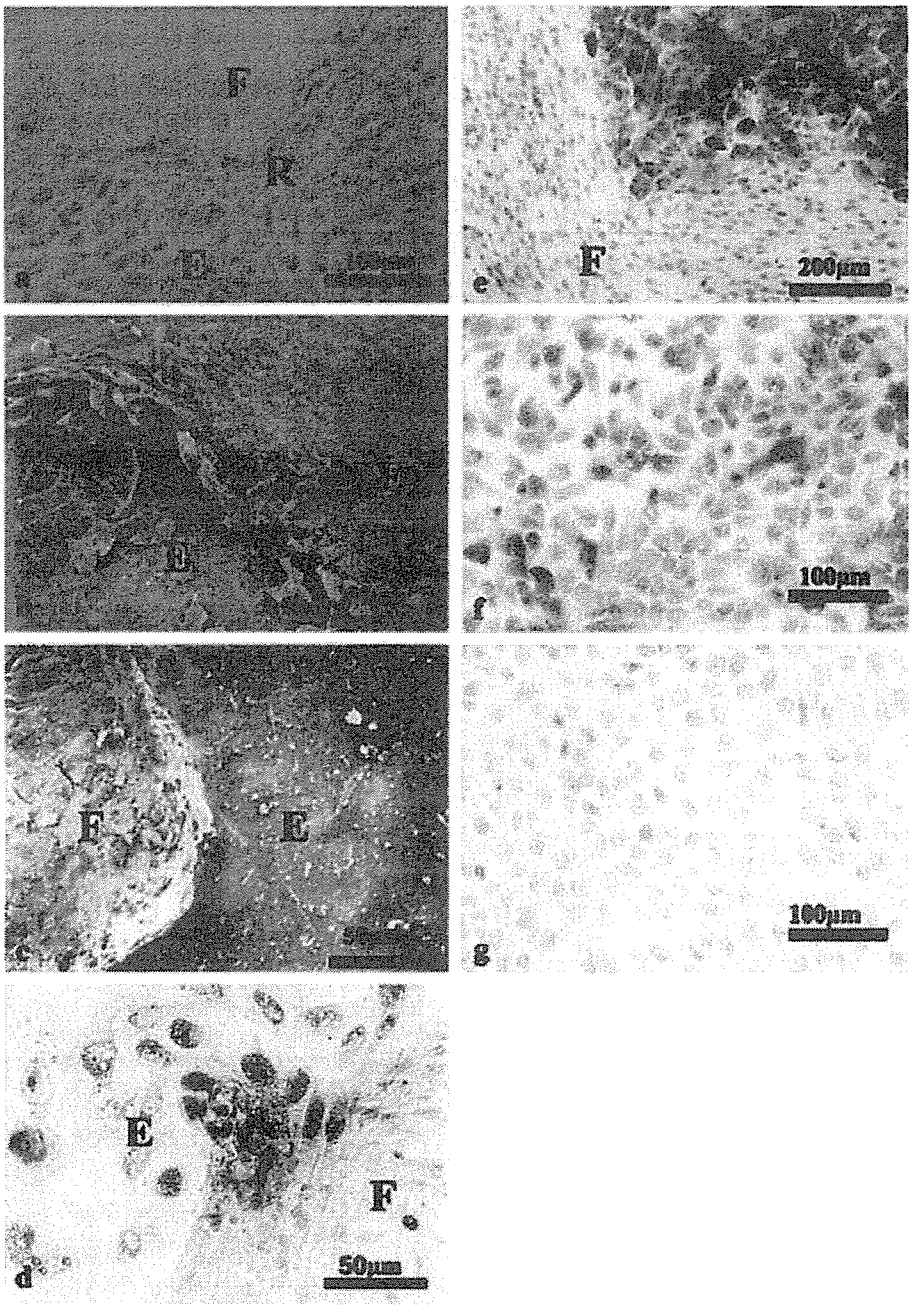

Fig. 1. (a) Phase-contrast photomicrograph of a primary coculture from human periodontal ligament. A sheet of epithelial cells (E) is covered by a ribbon (R) of fibroblasts (F). (b,c) Scanning electron micrographs of a primary cultures: (b) in the mixed multilayer, the epithelial cells (E) are seen to move under the fibroblasts (F); (c) epithelial cells (E) tightly attach to each other and push out the fibroblasts (F). (d) Photomicrograph showing assessment of proliferation by BrdU-labeling in epithelial cells (E) and fibroblasts (F) from human periodontal ligament. (e) Photomicrograph showing intense immunoreactivity for cytokeratin AE1/AE3 in epithelial cells (E) from human periodontal ligament. Immunostaining for cytokeratin in fibroblasts (F) is not seen. (f) Photomicrograph showing intense immunoreactivity for amelogenin in epithelial cells from human periodontal ligament; (g) immunostaining for amelogenin is not seen in epithelial cells from human gingival tissue.

in PBS, the specimens were reacted with blocking solution (5% BSA, 500 $\mu g\ ml^{-1}$ normal sheep IgG, 100 $\mu g\ ml^{-1}$ salmon testicular DNA, and 100 $\mu g\ ml^{-1}$ yeast tRNA in PBS) at room temperature for 15 min. They were then incubated with ExtrAvidin peroxidase solution (Sigma) at 37°C for 20 min, reacted with biotin-conjugated anti-avidin antibody (Sigma) in a humid chamber at 37°C for 30 min, and re-reacted with ExtrAvidin peroxidase solution. Following this, they were washed three times in PBS and the peroxidase sites were visualized using a solution containing

DAB and H_2O_2, with counterstaining using Mayer's hematoxylin solution.

Results

Characterization of cells in mixed cultures

Explant cultures from human PDL showed initial outgrowth of fibroblasts from the explant edges when maintained in α-MEM for 1 week. After replacing α-MEM with the modified serum-free medium, outgrowth of epithelial cells was induced in 50 out of 225 explant cultures (22.2%), and multilayers of both epithelial cells and fibroblasts were seen within 2–3 wk. All the co-cultures showed similar epithelial–mesenchymal interaction phenomena. When both epithelial cells and fibroblasts were cultured together in the modified serum-free medium, they were distinguishable from each other morphologically (Fig. 1a). Furthermore, scanning electron microscopy revealed that the epithelial colonies grew under fibroblasts (Fig. 1b), or attached adjacent to them (Fig. 1c).

Figure 2 shows typical growth curves of epithelial cells from PDLs and gingival tissues. The PDL epithelial cells had a lower growth rate than gingival epithelial cells under our culture conditions. In addition, PDL epithelial cells incorporated BrdU more extensively than PDL fibroblasts derived from the same PDL explant, indicating that PDL epithelial cells have a higher proliferation rate than PDL fibroblasts (Fig. 1d).

Immunohistochemistry

Human PDL epithelial cells in mixed cultures stained positive for broad-spectrum antibodies to cytokeratins (AE1/AE3), indicating their epithelial origin, while fibroblasts in the same culture did not show cytokeratin expression (Fig. 1e). Moreover, immunoreactivity for amelogenin was observed in human PDL epithelial cells, indicating their odontogenic epithelial origin, while human gingival epithelial cells did not show amelogenin expression (Fig. 1f,g).

In co-cultures (Fig. 3a) and vertical sections (Fig. 3c,d), the intensity of immunostaining for type IV collagen in human PDL fibroblasts immediately adjacent to human PDL epithelial cells was markedly high. Similarly, the intensity of immunostaining for laminin was increased at the interface (Fig. 3b). Immunostaining for both proteins showed little if any staining in areas further away from this interface. Control epithelial cells cultured alone showed only weak staining for type IV collagen (Fig. 3e) and laminin (data not shown). Control fibroblasts cultured alone showed little if any staining for type IV collagen (Fig. 3f) and laminin (data not shown).

In situ hybridization

To localize mRNA expression of type IV collagen α-1 chain and laminin γ-1 chain, the cells were hybridized *in situ* with biotin-labeled antisense oligo-DNA probes. The PDL fibroblasts immediately adjacent to PDL epithelial cells showed higher positive signals for both type IV collagen α-1 mRNA and laminin γ-1 mRNA (Fig. 4a,b). These results were consistent with the immunohistochemical observations (Fig. 3a,b). In the epithelial cells or the fibroblasts cultured alone, signals for type IV collagen α-1 mRNA and laminin γ-1 mRNA (data not shown) were weak compared with the co-culture.

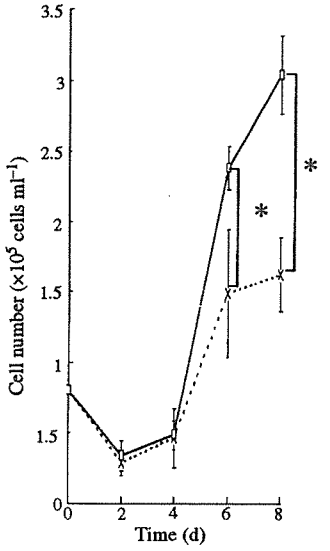

Fig. 2. Growth curves of epithelial cells from human periodontal ligament (dashed line with Xs) and gingival tissue (unbroken line with boxes). Values are the mean ± S.D. of five samples. $^*P < 0.05$.

Discussion

Both fibroblasts and epithelial cells were successfully cultured from human PDL tissue, using the explant technique in a modified serum-free medium. There were two steps in our co-culture system: in the first step, outgrowth of fibroblasts was induced from explants in α-MEM; and in the second step, outgrowth of epithelial cells was induced from the same explants, after replacing α-MEM with a modified serum-free medium. In this case, two different cell types were derived from the same tissue, but these cells did not mix, and instead formed fibroblasts as multilayers around epithelial cells. Keratin expression clearly distinguished epithelial cells from fibroblasts, since only the former cell type expressed this protein (Fig. 1e). In addition, amelogenin was expressed only in the cultured human PDL epithelial cells, and was not detectable in the human gingival epithelial cells (Fig. 1f,g). In a previous *in vitro* study, amelogenin synthesis has been reported in rat ameloblast-lineage cells (35), and these results suggest that our cultured human PDL epithelial cells were derived from the odontogenic epithelium, the epithelial cells of Malassez.

The morphological properties observed in the present study were similar to those found in an *in vitro* culture of monkey PDL fibroblasts and epithelial cells (22), and

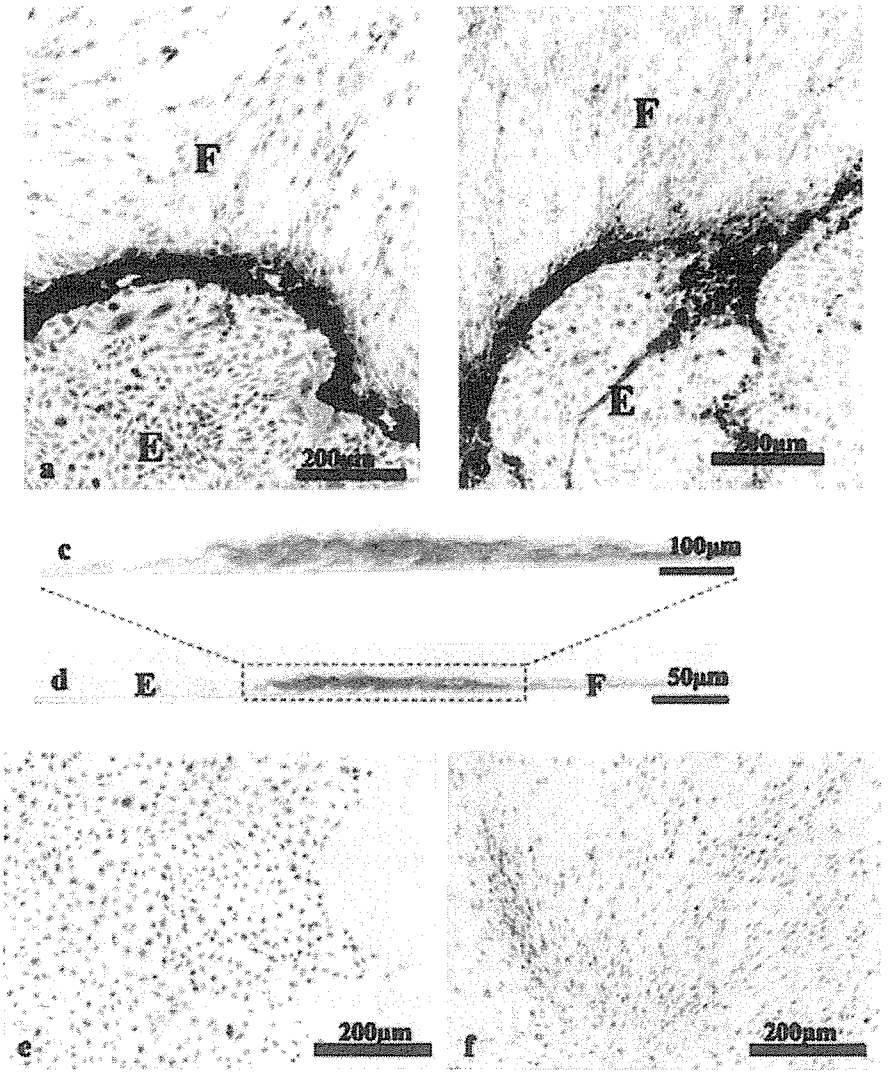

Fig. 3. (a,b) Photomicrographs showing intense immunoreactivity for type IV collagen (a) and laminin (b) between epithelial cells (E) and fibroblasts (F) from human periodontal ligament. (c,d) Photomicrographs showing intense immunoreactivity for type IV collagen at the interface between epithelial cells (E) and fibroblasts (F) from human periodontal ligament in vertical section. (e) Photomicrograph showing weak immunoreactivity for type IV collagen in control epithelial cells from human periodontal ligament. (f) Photomicrograph showing little immunoreactivity for type IV collagen in control fibroblasts from human periodontal ligament.

those of human PDL fibroblasts and mouse PDL epithelial cells (23). Ultrastructural changes of fibroblasts adjacent to the epithelial cells have also been reported for intracellular organelles, and have been related to protein synthesis, processing and transport (23). Additional features of interest in the ultrastructural studies were the presence of extracellular matrix between epithelial cells and fibroblasts under co-culture conditions (22, 23).

It is well established that extracellular matrix molecules regulate various physiological activities, such as cell growth, migration and differentiation (14–19). The basement membrane, a part of the extracellular matrix, separates the connective tissue from the epithelium and contains collagen (mainly type IV) and non-collagenous glycoproteins such as laminin. Under *in vitro* culture conditions, many cells secrete the components of the basement membrane (14, 15, 34, 36–41). In our study, enhanced expression and distribution of type IV collagen and laminin were found at the interface between epithelial cells and fibroblasts, while little staining for these proteins was apparent in areas further away from the interface of the mixed culture. These results suggest that interactions between epithelial cells and fibroblasts stimulate type IV collagen and laminin expression in epithelial cells *in vitro*. The modulation of the expression of extracellular molecules in fibroblasts by epithelial cells has been documented in previous cell culture studies (23), which showed that, under co-culture conditions, the synthesis of type I and III collagen and fibronectin was stimulated in PDL fibroblasts adjacent to PDL epithelial

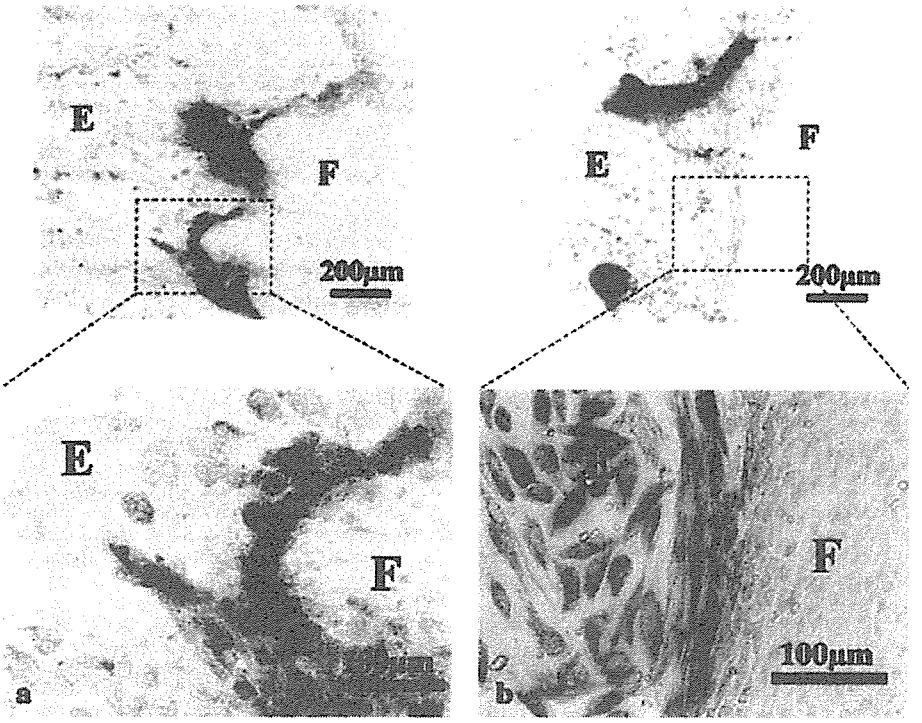

Fig. 4. Photomicrographs showing intense immunoreactivity for type IV collagen mRNA (a) and laminin mRNA (b) at the interface between epithelial cells (E) and fibroblasts (F) from human periodontal ligament.

cells. These observations are concordant with our observations, but the matrix proteins (type I and III collagen and fibronectin) detected are not components specific to the basement membrane. In another study in organotypic co-cultures of epithelial cells and fibroblasts derived from gingival tissue (41), type IV collagen and laminin were observed immunohistochemically underneath the basal epithelial cells. However, to our knowledge, our study is the first to show the synthesis of basement membrane constituents at the interface between epithelial cells and fibroblasts derived from PDL.

The effects of cell density and cell-cell contacts on protein syntheses appear very interesting. In our study, fibroblasts were clearly distinguishable from epithelial cells (Fig. 1a), and type IV collagen and laminin were strongly expressed only at the interface (Fig. 3a,b) where direct cell–cell contacts occurred, suggesting the involvement of such contacts in protein expression. During cell culture, an area of high cell density is sometimes produced, and in our study this area was observed in the culture of fibroblasts alone and epithelial cells alone (Fig. 3e,f), but significant protein expression was not observed in either of these cultures. Hence, these observations do not support the hypothesis that cell density itself affects protein expression. However, it is still unclear whether increased protein expression requires cell–cell contacts, synthesis of other proteins, or an appropriate cell density, and this remains to be elucidated.

The role of PDL epithelial cells in periodontal physiology is unclear. However, it is known that, when stimulated by endodontal infection, these cells can proliferate and give rise to periapical cyst formation (1, 42). It has been reported that epithelial cells derived from porcine rests of Malassez secrete prostaglandin, which possibly contributes to cyst growth (43). It has also been speculated that PDL epithelial cells may participate in periodontal diseases by proliferating during pocket formation and development of new junctional epithelium during postoperative gingival healing (2).

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References

1. TEN CATE AR. The epithelial cell rests of Malassez and the genesis of the dental cyst. *Oral Surg Oral Med Oral Pathol* 1972; **34**: 956–964.
2. SPOGE JD. Rests of Malassez and chronic marginal periodontal disease. *J Can Dent Assoc* 1980; **11**: 712–716.
3. MELCHER AH. Repair of wounds in the periodontium of the rat. Influence of periodontal ligament on osteogenesis. *Arch Oral Biol* 1970; **15**: 1183–1204.
4. SLAVKIN HC, BOYDE A. Cementum: an epithelial secretory product? *J Dent Res* 1975; **53**: 157.
5. SLAVKIN HC. Towards a cellular and molecular understanding of periodontics: cementogenesis revisited. *J Periodontol* 1976; **47**: 249–255.

6. HAMAMOTO Y, NAKAJIMA T, OZAWA H, UCHIDA T. Production of amelogenin by enamel epithelium of Hertwig's root sheath. *Oral Surg Oral Med Oral Pathol* 1996; **81**: 703–709.
7. HAMMARSTROM L. Enamel matrix, cementum development and regeneration. *J Clin Periodontol* 1997; **24**: 658–668.
8. GRUPE AE JR, TEN CATE AR, ZANDER HA. A histochemical and radiobiological study of *in vitro* and *in vivo* human epithelial cell rest proliferation. *Arch Oral Biol* 1967; **12**: 1321–1329.
9. JOHANSEN JR. Incorporation of tritiated thymidine by the epithelial rests of Malassez after attempted extraction of rat molars. *Acta Odont Scand* 1969; **19**: 463–470.
10. TIMPL R, DZIADEK M. Structure, development, and molecular pathology of basement membranes. *Int Rev Exp Pathol* 1986; **29**: 1–112.
11. TIMPL R, ROHDE H, ROBEY PG, RENNARD SI, FOIDART JM, MARTIN GR. Laminin – a glycoprotein from basement membranes. *J Biol Chem* 1979; **254**: 9933–9937.
12. ENGEL J, ODERMATT E, ENGEL A, MADRI JA, FURTHMAYR H, ROHDE H, TIMPL R. Shapes, domain organizations and flexibility of laminin and fibronectin, two multifunctional proteins of the extracellular matrix. *J Mol Biol* 1981; **150**: 97–120.
13. CARLIN BE, JAFFE R, BENDER B, CHUNG AE. Entactin, a novel basal-lamina associated sulfated glycoprotein. *J Biol Chem* 1981; **256**: 5209–5214.
14. UITTO VJ, LARJAVA H, PELTONEN J, BRUNETTE DM. Expression of fibronectin and integrins in cultured periodontal ligament epithelial cells. *J Dent Res* 1992; **71**: 1203–1211.
15. YAMADA KM. Cell surface interactions with extracellular materials. *Annu Rev Biochem* 1983; **52**: 76–795.
16. PITAL S, HEKMATI M, METZGER Z, SAVION N. Epithelial–connective tissue interaction on the tooth surface: an *in vitro* model. *J Periodont Res* 1991; **26**: 461–467.
17. TERRANOVA VP, MARTIN GR. Molecular factors determining gingival tissue interaction with tooth structure. *J Periodont Res* 1986; **57**: 311–317.
18. LIN CQ, BISSELL MJ. Multi-faceted regulation of cell differentiation by extracellular matrix. *FASEB J* 1993; **7**: 737–743.
19. PITAL S, HEKMATI M, METZGER Z, SAVION N. The effect of partial demineralization and fibronectin on migration and growth of gingival epithelial cells *in vitro*. *J Periodontol* 1989; **60**: 293–301.
20. HAMAMOTO Y, NAKAJIMA T, OZAWA H. Ultrastructure of epithelial rests of Malassez in human periodontal ligament. *Archs Oral Biol* 1989; **34**: 179–185.
21. HAMAMOTO Y, SUZUKI I, NAKAJIMA T, OZAWA H. Immunocytochemical localization of laminin in the epithelial rests of Malassez of immature rat molars. *Arch Oral Biol* 1991; **36**: 623–626.
22. BRUNETTE DM, KANOZA RJ, MARMARY Y, CHAN J, MELCHER AH. Interactions between epithelial and fibroblast-like cells in cultures derived from monkey periodontal ligament. *J Cell Sci* 1977; **27**: 127–140.
23. HOU LT, KOLLAR EJ, YAEGER JA. Epithelial cell–fibroblast interactions: modulation of extracellular matrix protein in cultured oral cells. *J Periodont Res*, 1993; **28**: 102–114.
24. RHEINWALD JG, GREEN H. Serial cultivation of strains of human epidermal keratinocytes. *Cell* 1975; **6**: 331–334.
25. RHEINWALD JG, GREEN H. Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. *Nature* 1977; **265**: 421–424.
26. BOYCE ST, HAM RG. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal cultures and serum-free serial cultures. *J Invest Dermatol* 1983; **81**: 33–40.
27. YAMANAKA T, SAKAMOTO A, TANAKA Y, ZHANG Y, HAYASHIDO Y, TORATANI S, AKAGAWA Y, OKAMOTO T. Isolation and serum-free culture of epithelial cells derived from epithelial rests of Malassez in human periodontal ligament. *In Vitro Cell Dev Biol* 2000; **36**: 548–553.
28. PITTELKOW MR, SCOTT RE. New techniques for the *in vitro* culture of human skin keratinocytes and perspectives on their use for grafting of patients with extensive burns. *Mayo Clin Proc* 1986; **61**: 771–777.
29. COFFEY RJ JR, DERYNCK R, WILCOX JN, BRINGMAN TS, GOUSTIN AS, MOSES HL, PITTELKOW MR. Production and auto-induction of transforming growth factor-α human keratinocytes. *Nature* 1987; **328**: 817–820.
30. BRUNETTE DM, MELCHER AH, MOE HK. Culture and origin of epithelium-like and fibroblast-like cells from porcine periodontal ligament explants and cell suspensions. *Arch Oral Biol* 1976; **21**: 393–400.
31. KATSUMOTO T, NAGURO T, IINO A, TAKAGI A. The effect of tannic acid on the preservation of tissue culture cells for scanning electron microscopy. *J Electron Microsc* 1981; **30**: 177–182.
32. UCHIDA T, TANABE T, FUKAE M. Immunocytochemical localization of amelogenins in the deciduous tooth germs of the human fetus. *Arch Histol Cytol* 1989; **52**: 543–552.
33. BRAZEL D, OBERBAUMER I, DIERINGER H, BABEL W, GLANVILLE RW, DEUTZMANN R, KUHN K. Completion of the amino acid sequence of the α1 chain of human basement membrane collagen (type IV) reveals 21 non-triplet interruptions located within the collagenous domain. *Eur J Biochem* 1987; **168**: 529–536.
34. HANN CR, SPRINGETT MJ, WANG X, JOHNSON DH. Ultrastructural localization of collagen IV, fibronectin, and laminin in the trabecular meshwork of normal and glaucomatous eyes. *Ophthalmic Res* 2001; **33**: 314–324.
35. TABATA MJ, MATSUMURA T, LIU J-G, WAKISAKA S, KURISU K. Expression of cytokeratin 14 in ameloblast-lineage cells of the developing tooth of rat, both *in vivo* and *in vitro*. *Arch Oral Biol* 1996; **41**: 1019–1027.
36. DE ARCANGELIS A, LEFEBVRE O, MECHINE-NEUVILLE A, ARNOLD C, KLEIN A, REMY L, KEDINGER M, SIMON-ASSMANN P. Overexpression of laminin α1 chain in colonic cancer cells induces an increase in tumor growth. *Int J Cancer* 2001; **94**: 44–53.
37. TOKIDA Y, ARATANI Y, MORITA A, KITAGAWA Y. Production of two variant laminin forms by endothelial cells and shift of their relative levels by angiostatic steroids. *J Biol Chem* 1990; **265**: 18123–18129.
38. ZHANG X-H, SUN H-M, YUAN J-Q. Extracellular matrix production of lens epithelial cells. *J Cataract Refract Surg* 2001; **27**: 1303–1309.
39. BAKER SE, DIPASQUALEIP AP, STOCK EL, QUARANTA V, FITCHMUN M, JONES JC. Morphogenetic effects of soluble laminin-5 on cultured epithelial cells and tissue explants. *Exp Cell Res* 1996; **228**: 262–270.
40. ODIOSO LL, DOYLE MJ, QUINN KW, BARTEL RL, ZIMBER MP, STEVENS-BURNS D. Development and characterization of an *in vitro* gingival epithelial model. *J Periodont Res* 1995; **30**: 210–219.
41. TOMAKIDI P, FUSENIG NE, KOHL A, KOMPOSCH G. Histomorphological and biochemical differentiation capacity in organotypic co-cultures gingival cells. *J Periodont Res* 1997; **32**: 388–400.
42. GAO Z, MACKENZIE IC, WILLIAMS DW, CRUCHLEY AW, LEIGH I, LANE B. Patterns of keratin-expression in rest of Malassez and periapical lesions. *J Oral Pathol* 1988; **17**: 178–185.
43. BRUNETTE DM, HEERSCHE JNM, PURDON AD, SODEK J, MOE HK, ASSURAS JN. *In vitro* cultural parameters and protein and prostaglandin secretion of epithelial cells derived from porcine rests of Malassez. *Archs Oral Biol* 1979; **24**: 199–203.

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Characteristic Intestinal Microflora of Specific Pathogen-Free Mice Bred in Two Different Colonies and their Influence on Postnatal Murine Immunocyte Profiles

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Abstract: *Cecal microflora of BALB/c mice originating from two different SPF-breeding colonies were compared. The analysis of cultivable bacteria in the ceca showed significantly higher numbers of total bacteria in BALB/cCrSlc (SLC mice) than in BALB/cA Jcl (JCL mice) ($p<0.05$), which were mainly based on higher numbers and occurrence of Peptococaceae. Bifidobacteria were detected only in SLC mice. Feeding an oligosaccharide, raffinose, to the mice also induced different shifts in the composition of cecal microflora and the concentration of cecal organic acids. In the second experiment, hysterectomy-derived (HD) SLC mice were fostered to SPF lactating SLC mothers, or SPF lactating JCL mice, together with the mother's own natural birth (NB) pups in each isolator. HD mice fostered to SLC-mothers showed significantly higher percentages of T-cell receptor αβ cells expressing a CD8α homodimer ($p<0.05$) and a CD8αβ heterodimer ($p<0.001$) in the intraepithelial lymphocytes (IEL) compared with HD mice fostered to JCL-mothers. IEL profiles of HD mice corresponded well to those of NB mice that were breast-fed by the same mothers. Differences in the ratio of B220$^+$cells to Thy1.2$^+$cells in the splenocytes were also observed as a trend between both HD mice fostered to SLC or JCL mothers ($p=0.06$). These results suggest that postnatal colonization of various characteristic intestinal microflora derived from SPF-breeding colonies results in differences in development of lymphocyte populations in the intestinal and systemic organs of mice.*

Key words: *immunity, intestinal microflora, intraepithelial lymphocytes, SPF mice*

Introduction

It is considered that postnatal formation of intestinal microflora of mammals is influenced by bacterial contamination from the mother during lactation [5, 8]. It has also been reported that the composition of intestinal microflora of adult mice was based on genetic factors in each mouse strain [7] including levels of major histocompatibility complex [11], and was not affected very much by bacterial contamination from

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the mothers [7]. Thus if mice, particularly inbred mice, are bred in barrier-sustained colonies, their intestinal microflora should be strongly affected by their mother's microflora because they are strictly isolated from the external environment. It is possible that the characteristic composition of microflora in each breeding colony might have different effects on characteristics of SPF mice. Microbes have various characteristics related to microbial metabolism, immunogens and pathogenicity in hosts. However, there are few reports showing differences in intestinal microflora of mice in SPF-breeding colonies, and also the influence of these differences on characteristics of SPF mice. In order to clarify the situation, we focused on whether different SPF-intestinal microflora affect development of immune organs because the immune system is susceptible to colonization of intestinal microbes. For example, germ-free animals are well known to show immunological underdevelopment of systemic and intestinal immune organs, such as the spleen, lymph nodes [4], Peyer's patches [10] and intraepithelial lymphocytes (IEL) [2, 14], compared with conventional animals.

In this study, we compared the cecal microflora of two BALB/c mice from different breeding colonies and also examined the composition of cecal microflora and the concentration of cecal organic acids after feeding indigestible oligosaccharide to both groups of mice. Lastly, we demonstrated that postnatal association with these 2 kinds of SPF-microflora in hysterectomy-derived mice induces development of different immunocyte profiles in the hosts.

Materials and Methods

Animals

SPF animals, female BALB/cCrSlc mice (SLC mice), female BALB/cAJcl mice (JCL mice), and pregnant mice, were purchased from Japan SLC (Hamamatsu, Japan) and CLEA Japan (Tokyo, Japan). The mice used for analysis of intestinal microflora were housed in plastic cages at 22 ± 2°C with a 12 h light-dark cycle under conventional conditions. The mice used for foster-nursing experiments were housed in vinyl isolators sterilized with 2% peracetic acid. Metal cages and water were sterilized by autoclaving at 121°C for 70 min. The isolators were placed in a room with a controlled 12 h light dark cycle at 24 ± 1°C with a relative humidity of 55 ± 5%. All mice were given sterilized diet and water *ad libitum*. All experiments were performed in accordance with the guidelines for the care and use of laboratory animals of The University of Tokyo.

Diets

The purified basal diet was composed of (g/kg) casein 200.0, maize starch 481.7, α,β-starch 90.0, sucrose 50.0, cellulose 50.0, soyabean oil 60.0, mineral mixture (AIN-76) 50.0, vitamin mixture (AIN-76) 13.0, choline chloride 2.3, and methionine 3.0 (1). The oligosaccharide diet was prepared by adding raffinose (Nippon Beet Sugar Mfg, Tokyo, Japan, purity > 995 g/kg DM) to the basal diet (50 g/kg diet) instead of maize starch. The diets were pelletized and vacuum-sealed in plastic bags by Funabashi Farm (Chiba, Japan), and were sterilized by γ-irradiation at 10 kGy.

Oligosaccharide feeding experiment

BALB/cCrSlc mice and BALB/cAJcl (6 weeks of age) were randomly allocated to two groups (n=5 per group, total 4 groups). Each group was fed the basal diet or the oligosaccharide diet for 2 weeks. After the mice were sacrificed by cervical dislocation, ceca removed from the mice were each subjected to analysis of intestinal microflora and organic acids.

Foster-nursing experiment: association with different SPF microflora

Ten pregnant BALB/cCrSlc mice were maintained under SPF conditions. After the pups were removed from the pregnant mice by hysterectomy, the pups were randomly fostered to BALB/cCrSlc lactating mothers (n=5) or BALB/cAJcl lactating mothers (n=5), together with the mother's own natural birth pups in each vinyl isolator. After both the hysterectomy-derived (HD) and natural birth (NB) pups were weaned at 4 weeks of age, they were kept in the same isolators until 6 weeks of age. Thereafter, HD and NB mice were sacrificed by cervical dislocation, and the small intestine, spleen and mesenteric lymph nodes (MLN) removed from these mice were each subjected to flow cytometric analysis.

Analysis of intestinal microflora

Bacteriological analysis of mice cecal contents was carried out according to the method of Mitsuoka [9]. Briefly, fresh samples were immediately diluted in 10-

fold steps with anaerobic phosphate buffer and 0.05 ml of each dilution was inoculated on two non-selective media (BL and TS agar) and three selective media (mLBS, DHL and TATAC agar). BL and mLBS agar plates were incubated at 37°C for 48 h in an anaerobic steel wool jar filled with oxygen-free CO_2, and TS, DHL and TATAC agars were incubated for 24–48 h aerobically. Bacterial groups were identified using colony and cell morphology, Gram staining, spore formation and aerobic growth.

Determination of cecal organic acids

Cecal contents were weighed and homogenized with a 20-fold volume of 0.2 N HCl. Pyroglutamic acid was used as the internal standard. Organic acids in the cecal contents were determined by high performance liquid chromatography using the postcolumn method with bromothymol blue as pH indicator (wave length for detection; 445 nm, column; RSpak KC-811, Showa Denko K.K., Tokyo, Japan).

Lymphocyte preparation

IEL were prepared as previously described [6]. In brief, contents of the small intestines of mice were thoroughly washed out with Hank's balanced salt solution (HBSS: Gibco BRL, Gaithersburg, MD). Each intestine was inverted, and individually transferred to a 50-ml conical tube containing 45 ml of HBSS supplemented with 5% (v/v) fetal calf serum (Sigma-Aldrich, Mo, USA). The tubes were shaken at 150 rpm in the horizontal position for 45 min at 37°C, and then were shaken several times by hand. Each resultant cell suspension was collected and filtered through a glass-wool column. Subsequently, the cells were suspended in 30% Percoll (Pharmacia Biotech, Uppsala, Sweden) solution and centrifuged at 400 × g for 20 min. Cells pelleted at the bottom of the tube were applied to Percoll discontinuous density gradient centrifugation. IEL were recovered at the 44 to 70% Percoll interface. Single-cell suspensions of splenocytes and MLN cells were prepared by mashing the organ with the end of a syringe, and passing each cell suspension through a polyester mesh.

Flow cytometry

Lymphocytes (5×10^5 cells) were incubated with fluorochrome-labeled or biotinylated monoclonal antibodies (mAb) for 20 min in ice water after blocking Fc receptors with anti CD16/32 mAb (2.4G2; PharMingen, San Diego, CA). If necessary, fluorochrome-labeled streptavidins were used as the second antibodies. After staining, the cells were washed with HBSS containing 1% (w/v) fetal calf serum and 0.01% (w/v) sodium azide. Cytofluorometric analysis was performed by flow cytometry (FACSort, Becton Dickinson, Franklin Lakes, NJ). The following mAb were used: biotin-anti-TCRβ mAb (H57-597; PharMingen), fluorescein isothiocyanate (FITC)-anti-TCRγ mAb (GL-3; Cedarlane, Hornby, Ontario, Canada), biotin-anti-TCRγ mAb (GL-3, Cedarlane), FITC-anti-CD4 mAb (H129.12; PharMingen), phycoerythrin (PE)-anti-CD8α mAb (53–6.7; Gibco BRL), FITC-anti-CD8β mAb (Y8.77; Seikagaku-Kogyo, Tokyo, Japan), PE-anti-B220 mAb (RA3–6B2, Gibco BRL), FITC-anti-Thy1.2 mAb (30-H12, PharMingen), FITC-streptavidin (Gibco BRL), PE-streptavidin (Gibco BRL), and RED 670-streptavidin (Gibco BRL); all were purchased from the sources indicated.

Statistical analysis

Results were expressed as mean values with standard deviations. Differences in bacterial counts and organic acid concentrations in the ceca of mice were analyzed using Scheffe's F post-hoc test, and differences in occurrences of microbes were analyzed using Fisher's exact probability test. Differences in lymphocyte profiles of mice were analyzed using Student's *t* test.

Results

Intestinal microflora of mice bred in different colonies

Differences in cecal microflora between SLC mice and JCL mice were compared in terms of cultivable bacteria (Table 1). SLC mice showed significantly higher numbers of total bacteria than JCL mice ($p<0.05$). In particular, higher numbers and significantly higher occurrences of Peptococaceae in SLC mice were observed compared with those in JCL mice ($p<0.05$). The percentage of Peptococaceae to total bacteria was 52 ± 16% in SLC mice, while that in JCL mice was below 4%. Bifidobacteria were detected only in SLC mice. There were no differences between groups in numbers of facultative aerobes such as Lactobacilli, Enterobacteriaceae and Streptococaceae. Feeding of the oligosaccharide diet

Table 1. Comparison of cecal microflora of BALB/cAJcl and BALBc/CrSlc fed basal diet or oligosaccharide diet

	Basal diet				Oligosaccharide diet			
	BALB/cAJcl		BALB/cCrSlc		BALB/cAJcl		BALB/cCrSlc	
Total bacteria	9.1 ± 0.2	(100)[a)]	9.6 ± 0.3*	(100)	9.5 ± 0.1	(100)	9.9 ± 0.3	(100)
Bacteroidaceae	8.8 ± 0.2	(100)	9.1 ± 0.2	(100)	9.4 ± 0.2#	(100)	9.3 ± 0.4	(100)
Bifidobacteria		(0)	7.2	(40)		(0)	7.8 ± 0.4	(100)
Eubacteria	7.9	(40)	7.9	(40)	8.3	(20)	8.6 ± 0.6	(80)
Peptococaceae	7.6	(20)	9.3 ± 0.4	(100)*	6.6	(20)	9.6 ± 0.2	(100)
Clostridia		(0)	7.2	(40)	8.1	(40)	7.8 ± 0.4	(100)
Fusiform bacteria		(0)		(0)	8.0 ± 0.2	(60)#		(0)
Curved rods	7.6	(20)		(0)	7.6	(40)		(0)
Lactobacilli	8.6 ± 0.4	(100)	8.5 ± 0.2	(100)	8.0 ± 0.6	(100)	8.3 ± 0.4	(100)
Enterobacteriaceae	5.4 ± 0.5	(100)	5.6 ± 0.4	(100)	4.9 ± 0.9	(100)	4.4 ± 0.2#	(100)
Streptococaceae	6.6 ± 0.3	(100)	6.5 ± 0.5	(100)	6.6 ± 0.0	(100)	6.5 ± 0.7	(100)

The mice were fed purified basal diet or oligosaccharide diet (containing raffinose at 50 g/kg) for 2 weeks. a) Values are expressed as mean ± S.D. of bacterial counts (log no./g cecal contents). Figures in parentheses refer to frequency of occurrence (%). Significant difference * ($p<0.05$) between BALB/cA and BALB/cCr fed basal diet, # ($p<0.05$) between basal diet and oligosaccharide diet within the same mouse strain.

Table 2. Cecal organic acids of BALB/cAJcl and BALB/cCRSlc fed basal diet or oligosaccharide diet

	Basal diet		Oligosaccharide diet	
	BALB/cAJcl	BALB/cCrSlc	BALB/cAJcl	BALB/cCrSlc
Acetic acid	25.4 ± 7.5	23.1 ± 7.8	25.5 ± 8.8	29.2 ± 6.4
Propionic acid	3.3 ± 0.7	3.7 ± 0.8	4.8 ± 2.0	8.0 ± 1.9#
n-Butyric acid	2.7 ± 0.7	3.1 ± 1.6	4.7 ± 3.2	9.2 ± 3.0#
Formic acid	0.1 ± 0.1	1.1 ± 0.4*	0.0 ± 0.0	0.2 ± 0.3#
Succinic acid	0.0 ± 0.0	0.1 ± 0.1	0.2 ± 0.4	0.2 ± 0.2
Lactic acid	0.3 ± 0.2	0.0 ± 0.0	0.2 ± 0.2	1.2 ± 1.1#

Values are expressed as mean ± S.D. of organic acids (μmol/g cecal contents) of mice. * and #; see footnotes of Table 1.

(50 g raffinose/kg diet) to JCL mice for 2 weeks significantly increased numbers of Bacteroidaceae ($p<0.05$) and occurrence of fusiform bacteria ($p<0.05$). In contrast, SLC mice fed oligosaccharide diet showed increasing tendencies in the numbers or occurrences of various strict anaerobes such as Bacteroidaceae, bifidobacteria, eubacteria, Peptococaceae and clostridia, and a significant decrease in the number of Enterobacteriaceae ($p<0.05$). On the other hand, the organic acid concentration in the ceca of mice fed a basal diet showed a significant difference only in formic acid concentration between SLC and JCL mice (Table 2). There were no significant differences in cecal organic acids of JCL-mice between mice fed the basal diet and the oligosaccharide diet. However, the oligosaccharide diet fed to SLC mice induced significant increases of cecal propionic, butyric and lactic acid concentrations compared with those fed the basal diet ($p<0.05$).

Effect of postnatal association with different SPF microflora on lymphocyte profiles of the mice

HD mice were fostered to SPF lactating SLC mothers or SPF lactating JCL mothers in each isolator together with NB pups. With this method, intestinal microflora of HD mice should have been transferred from foster mothers. There were marked differences in the IEL populations between SLC-NB mice and JCL-NB mice (Fig. 1). The SLC-NB mice showed higher percentages of TCR$\alpha\beta$ IEL and lower percentages of TCR$\gamma\delta$ IEL than the JCL-NB mice. The high percent-

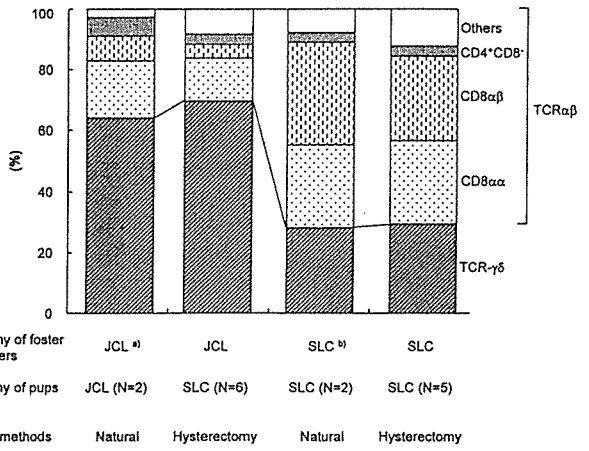

Fig. 1. Comparison of intraepithelial lymphocyte profiles among natural birth and hysterectomy-derived BALB/c mice fostered to lactating mothers from different colonies. The hysterectomy-derived (HD) SLC mice were fostered to SPF lactating mothers together with the mother's own natural birth (NB) pups until 4 weeks of age. Data show means of each population in intraepithelial lymphocytes of NB and HB mice at 6 weeks of age. a) JCL; BALB/cA Jcl. b) SLC; BALB/cCrSlc.

age of TCR$\alpha\beta$ IEL in the SLC-NB mice consisted of increased percentages of both CD8$\alpha\alpha^+$ cells and CD8$\alpha\beta^+$ cells. The percentages of CD8$\alpha\beta^+$ cells and CD8$\alpha\alpha^+$ cells in SLC-NB mice were 4-fold and 2-fold higher, respectively, than those in JCL-NB mice. There were few differences in the percentages of CD4$^+$ TCR$\alpha\beta$ IEL between SLC-NB mice and JCL-NB mice. On the other hand, IEL profiles of HD mice show good agreement with those of NB mice that were breast-fed by the same mothers. HD mice fostered to SLC-mothers showed significantly higher percentages of TCR$\alpha\beta$ IEL expressing CD8$\alpha\alpha$ ($p<0.05$) and CD8$\alpha\beta$ ($p<0.001$) compared with HD mice fostered to JCL-mothers. Therefore, the ratio of TCR$\alpha\beta$ IEL to TCR$\gamma\delta$ IEL was significantly higher in SLC-fostered HD mice compared with JCL-fostered HD mice ($p<0.05$: SLC-fostered HD mice, 2.4 ± 0.2; JCL-fostered HD mice, 0.4 ± 0.1). Moreover, the ratio of B220$^+$cells to Thy1.2$^+$cells in the spleen of SLC-fostered HD mice tended to be higher than that in JCL-fostered HD mice (Table 3).

Table 3. Ratio of B220$^+$ cells to Thy1.2$^+$ cells in the spleen and mesenteric lymph nodes of hysterectomy-derived mice fostered to lactating mothers for different colonies

	Foster mother		
	JCL[a)]	SLC[b)]	p
Spleen	1.88 ± 0.39	2.33 ± 0.06	0.06
Mesenteric lymph nodes	0.42 ± 0.09	0.52 ± 0.07	0.11

Ratio of B220$^+$ cells to Thy1.2$^+$ cells in the spleen and mesenteric lymph nodes of hysterectomy-derived mice shown in Fig. 1 (means ± SD, n=4) p values were calculated using Student's t test. a, b) see footnotes in Fig. 1.

Discussion

It is possible that the compositions of intestinal microflora in SPF experimental animals might differ among breeding colonies, and this difference might have some effect on development of immune organs in the animals. In order to prove this hypothesis, cecal microflora of different colonies of BALB/c mice, BALB/cCrSlc and BALB/cAJcl, were compared. An analysis of cultivable bacteria in the ceca showed higher numbers and occurrences of Peptococaceae in SLC mice than in JCL mice. Bifidobacteria were also detected only in SLC mice. These results show that there was a major difference in composition of cecal anaerobes between JCL and SLC BALB/c mice. Raffinose is known to be a fermentable indigestible oligosaccharide and growth factor of bifidobacteria *in vitro* [15] and in humans *in vivo* [3]. Feeding this sugar to mice also induced different changes of various bacterial counts between the two mice colonies. Analysis of changes in cecal organic acids by oligosaccharide feeding also gave different results: a significant increase of some organic acids in SLC mice but no changes in JCL mice. The difference in metabolism of orally fed oligosaccharide by indigenous microflora suggests that not only cultivable bacterial counts in the intestine but also essential microbes composing microflora differed in SLC and JCL mice.

In the second experiment, HD mice were fostered to SPF lactating SLC mothers or SPF lactating JCL mothers together with the mother's NB pups. This method resulted in association of HD and NB mice with the intestinal microflora of the foster mother in the isolator. HD and NB mice acquired identical intestinal microflora because the BALB/cCr and BALB/cA mice used in the present study have identical genetic backgrounds. SLC-NB mice and SLC-fostered HD mice showed significantly higher percentages of TCR$\alpha\beta$ cells

expressing CD8 molecules in IEL compared with those of JCL-NB and JCL-fostered HD mice. This result suggests that SLC-flora induce expansion of $CD8^+$ TCR$\alpha\beta$ IEL more strongly than JCL-flora. Moreover, the ratios of $B220^+$cells (B-cell marker) to $Thy1.2^+$cells (T-cell marker) in the splenocytes also differed between SLC-fostered HD mice and JCL-fostered HD mice.

It is known that the number of TCR$\alpha\beta$ IEL in germ-free mice is greatly reduced compared with conventional mice [2, 14]. The ratios of TCR$\alpha\beta$ cells to TCR$\gamma\delta$ cells in the IEL of the JCL-fostered HD mice in our results showed good agreement with those of germ-free mice reported previously (JCL-fostered HD mice, 0.4 ± 0.1; germ-free mice, 0.39 as reported in Reference No.13). These results suggest that JCL-flora might lack microbes able to induce expansion of $CD8^+$ TCR$\alpha\beta$ IEL. It has been reported that mono-association with segmented filamentous bacteria (SFB) in germ-free mice induced marked expansion of $CD8^+$ TCR$\alpha\beta$ IEL in the small intestine from germ-free levels to conventional levels [13]. In the present study, we were not able to clarify whether SFB colonized the intestinal lumens of JCL and SLC mice because SFB is a non-cultivable bacteria [12].

In conclusion, we showed that BALB/c mice bred in two different colonies have essentially different compositions of intestinal microflora in terms of not only bacterial counts but also metabolic responses to oligosaccharides. Moreover, postnatal association with each microflora in HD mice resulted in different development of lymphocyte populations in the intestinal and systemic immune systems. Specific indigenous microflora of each breeding colony might affect not only development of the immune system but also systemic metabolism and pathology. We consider that investigators should pay more attention to the possibility that results obtained from animal experiments might be influenced by characteristic microflora of each breeding colony.

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References

1. American Institute of Nutrition. 1979. Report of the American Institute of Nutrition ad hoc Committee on Standards for Nutritional Studies. *J. Nutr.* 107: 1340–1348.
2. Bandeira, A., Mota-Santos, T., Itohara, S., Degermann, S., Heusser, C., Tonegawa, S., and Coutinho, A. 1990. Localization of γ/δ T cells to the intestinal epithelium is independent of normal microbial colonization. *J. Exp. Med.* 172: 239–244.
3. Benno, Y., Endo, K., Shiragami, N., Sayama, K., and Mitsuoka, T. 1987. Effects of raffinose intake on human fecal microflora. *Bifidobacteria Microflora* 6: 59–63.
4. Berg, R.D. 1983. Host immune response to antigens of the indigenous intestinal flora. pp. 101–126. *In*: Human Intestinal Microflora in Health and Disease (Hentges, D. J. eds.), Academic Press, New York.
5. Davis, C.P., McAllister, J.S., and Savage, D.C. 1973. Microbial colonization of the intestinal epithelium in suckling mice. *Infect. Immun.* 7: 666–672.
6. Fujiura, Y., Kawaguchi, M., Kondo, Y., Obana, S., Yamamoto, H., Nanno, M., and Ishikawa, H. 1996. Development of $CD8\alpha\alpha^+$ intestinal intraepithelial T cells in β_2-microglobulin- and/or TAP1-deficient mice. *J. Immunol.* 156: 2710–2715.
7. Itoh, K., Oowada, T., and Mitsuoka, T. 1985. Characteristic faecal flora of NC mice. *Lab. Anim.* 19: 7–15.
8. Maejima, K., Maejima, F., Tajima, Y., and Okugi, M. 1967. Viable count of various bacteria in the digestive tract of mice. III. Changes of viable count of fecal bacteria with age. *Bull. Exp. Anim.* 16: 20–25.
9. Mitsuoka, T., Sega, T., and Yamamoto, S. 1965. Eine verbesserte methodik der qualitativen und quantitativen analyse der darmflora von menschen und tieren. *Zbl. Bakt. Hyg., I. Abt. Orig.* 195: 455–469 (in German).
10. Rothkotter, H. J. and Pabst, R. 1989. Lymphocyte subsets in jejunal and ileal Peyer's patches of normal and gnotobiotic minipigs. *Immunology* 67: 103–108.
11. Toivanen, P., Vaahtovuo, J., and Eerola, E. 2001. Influence of major histocompatibility complex on bacterial composition of fecal flora. *Infect. Immun.* 69: 2372–2377.
12. Umesaki, Y., Okada, Y., Matsumoto, S., Imaoka, A., and Setoyama, H. 1995. Segmented filamentous bacteria are indigenous intestinal bacteria that activate intraepithelial lymphocytes and induce MHC class II molecules and fucosyl asialo GM1 glycolipids on the small intestinal epithelial cells in the ex-germ-free mouse. *Microbiol. Immunol.* 39: 555–562.
13. Umesaki, Y., Setoyama, H., Matsumoto, S., Imaoka, A., and Itoh, K. 1999. Differential roles of segmented filamentous bacteria and clostridia in development of the intestinal immune system. *Infect. Immun.* 67: 3504–3511.
14. Umesaki, Y., Setoyama, H., Matsumoto, S., and Okada, Y. 1993. Expansion of $\alpha\beta$T-cell receptor-bearing intestinal intraepithelial lymphocytes after microbial colonization in germ-free mice and its independence from thymus. *Immunology* 79: 32–37.
15. Yazawa, K. and Tamura, Z. 1982. Search for sugar sources for selective increase of bifidobacteria. *Bifidobacteria Microflora* 1: 39–44.

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Effect of specific antigen stimulation on intraepithelial lymphocyte migration to small intestinal mucosa

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Summary

Migration of intraepithelial lymphocytes (IELs) into intestinal epithelium is not yet well understood. We established an IEL-cell line from ovalbumin (OVA) 23–3 transgenic (Tg) mice and investigated the effect of antigen stimulation on the dynamic process of IEL migration into small intestinal mucosa. The cell line was a T cell receptor (TCR) $\alpha\beta^+$ $CD4^+$ $CD8^-$ phenotype, expressing $\alpha E\beta 7$ integrin in 90% of cells. Under intravital microscopy, the lined IELs adhered selectively to the microvessels of the intestinal villus tip of the Tg mice. The accumulation of IELs was significantly inhibited by an antibody against β7-integrin and MAdCAM-1. When IELs were stimulated with OVA, the accumulation was attenuated compared to that of resting cells, with decreased expression of $\alpha E\beta 7$ integrin. In Tg mice fed with OVA, the number of IELs which migrated in the villus mucosa was significantly smaller than in the non-fed controls. The preferential migratory capacity of IELs to villus mucosa may be altered by specific antigen stimulations.

Keywords: adhesion molecules, cell trafficking, intraepithelial lymphocytes, ovalbumin, small intestine

Introduction

The intestinal epithelium is a region constantly exposed to intraluminal substances, such as intestinal flora and food antigens. It has been suggested that the functions of intraepithelial lymphocytes (IELs) are first-stage protection and maintenance of the epithelial layer. IELs consist of both αβ and γδ T cell receptor (TCR)-bearing cells with phenotypic and functional features distinct from those of cells in the peripheral lymphoid tissue [1]. Indeed, peripheral T cells and IELs have many different characteristics; for example, concanavalin A (ConA), which is mitogenic to peripheral T cells, cannot stimulate IELs [2]. These lymphocytes, mainly $CD8^+$ T cells, are largely unresponsive to proliferative signals mediated via conventional stimulation of the CD3–TCR complex [3]. Most IELs express characteristic integrin $\alpha E\beta 7$, which is hardly observed in peripheral T cells [4,5].

These T cells have diverse sources of origin: it is known that most peripheral T cells differentiate in the thymus, but a large population of IELs differentiate extrathymically. Some IELs may arise *in situ* in the gut epithelium [6]. Some may develop in the thymus, and yet others come from extraintestinal, extrathymic sources, and these lymphocytes reach the intestine. IEL precursors in the blood may gain access to the mucosal sites. It has been speculated that the γδ T cells present in the vaginal epithelium originate from the peripheral lymphoid organs [7]. There have been reports that injected peripheral T cells, under adequate conditions, can fill the epithelial compartments and acquire the characteristics of IELs [6,8]. However, there has been little information about the exact route and mechanisms by which IELs reach the microvessels of intestinal villi before they gain access to the epithelium.

There is evidence that the homing behaviour of lymphocytes can be altered profoundly by activation and differentiation. The migration properties of activated lymphocytes appear to be both more selective and more diverse than those of naive lymphocytes. Some migration properties of 'memory' lymphocytes resemble more closely those of activated lymphocytes [9–11]. Most memory and effector lymphocytes probably traffic through lymphoid organs, but unlike naive cells, they can also access and recirculate through extralymphoid immune effector sites such as the intestinal mucosa or inflamed skin and joints [11–13]. In humans, for example, CD4 cells that express both CLA and L-selectin preferentially accumulate in inflamed skin [14]. However, the effect of antigen-specific activation of IELs on their