口腔バイオフィルムの性質

1．バイオフィルムとは

バイオフィルムは菌体外多糖体によって取り囲まれた細菌のコミュニティーと考えられている．単一菌種でもバイオフィルムを形成するものがあるが，口腔内のバイオフィルムは多種多様な細菌種が主に歯面を足場として構築した細菌の集団社会である（図1～4）．そのなかでは，環境に適応するため，あるいは宿主の防御機構を回避するために，細菌がお互いに情報伝達を行いながら共同生活をしている．コミュニティーを形成した細菌は，単独行動をとる浮遊菌（planktonic cell）に比べて，宿主内での生存率を格段に上昇させている．

2．バイオフィルムの形成と構成細菌

口腔内プラークバイオフィルムは次のような過程を経て形成される（図1）．

①歯表面への conditioning film（ペリクル*5）の形成

②浮遊細菌のペリクルへの付着

③付着した細菌の増殖とそれに伴う菌体外多糖の産生

④多様な菌種の付着とマイクロコロニーの形成

⑤コミュニティーの形成とバイオフィルムの成熟

⑥バイオフィルムの剝離・バイオフィルムからの細胞遊離による細菌伝播

バイオフィルムはマイクロコロニーから成熟した細菌の凝集体とそれを取り巻く菌体外マトリックス（菌体外多糖）からなる基本構造をとる（図2）．典型的なバイオフィルムはキノコ状の構造をとるが，口腔内では環境的な要因，すなわち，ポケットの深さ（酸素分圧）や唾液の性状，あるいは栄養物濃度が影響し，その構造や細菌叢が変化する（図3）．バイオフィルムは成熟すると，融合する場合があるが，その間には栄養源を取り込んだり老廃物を排出するための水路（water channel あるいは fluid channel）を残す（図2）．栄養環境の良い場合にはこのような水路のない密なバイオフィルム（dense biofilm）となることもある（図3）．また，周辺の流体力学的剪断力が強い場合にはバイオフィルムは細長い形態をとるようになる（図1）．

口腔内バイオフィルムがどのような細菌種で構成されているのかを模式化して示す（図4）．バイオフィルム形成の初期過程には streptococci が主体となり，その後に *F. nucleatum*，そして *P. gingivalis* や *T. denticola*，*T. forsythia* といった偏性嫌気性細菌が構成菌となる．主要な歯周病原細菌である *P. gingivalis* と *T. denticola* は共凝集してバイオフィルム形成に関与する．バイオフィルム中の歯周病原細菌の割合が高くなれば，その病原性も高くなると考えられる．

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*5 ペリクル：唾液や歯肉溝滲出液中の糖タンパク質が歯面に吸着して形成される1 μm ほどの薄膜．細菌付着の足場となるが，酸などから歯を守る役目ももつ．

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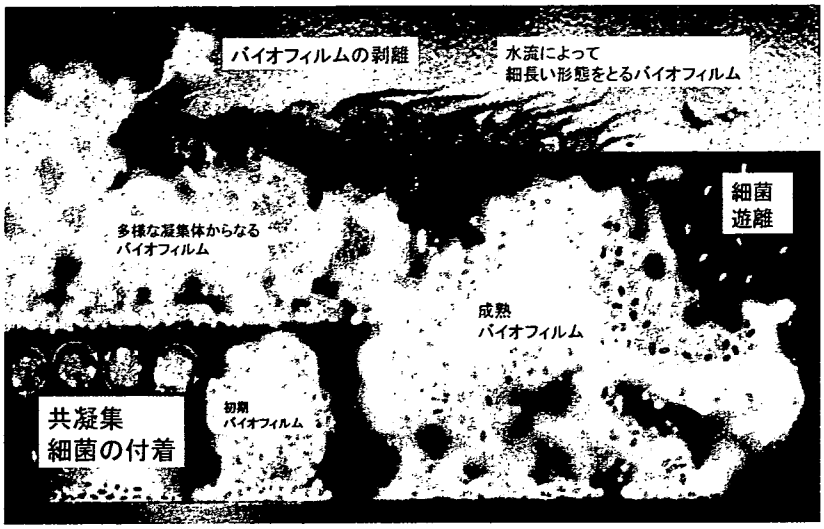

図1　バイオフィルムの形成過程と形態
(Fux CA, et al: *Expert Rev Anti Infect Ther*, 2003[4] より引用改変)

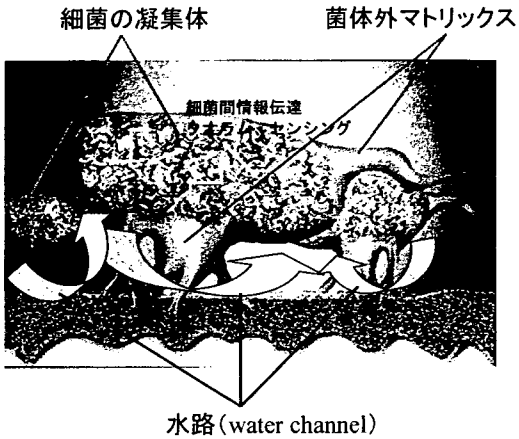

図2　バイオフィルムの基本構造(Overman PR: *Journal of Contemporary Dental Practice*, 2000[6] より引用改変)
典型的なキノコ状の構造をとるバイオフィルムの模式図．バイオフィルムは細菌の凝集体とそれを取り巻く菌体外多糖物質で構成される．凝集体は時に融合するが，凝集体の間には栄養源を取り込むための水路（water channel）が残る．細菌間ではクオラム・センシング機構を介した情報伝達と遺伝子発現調節が行われている．

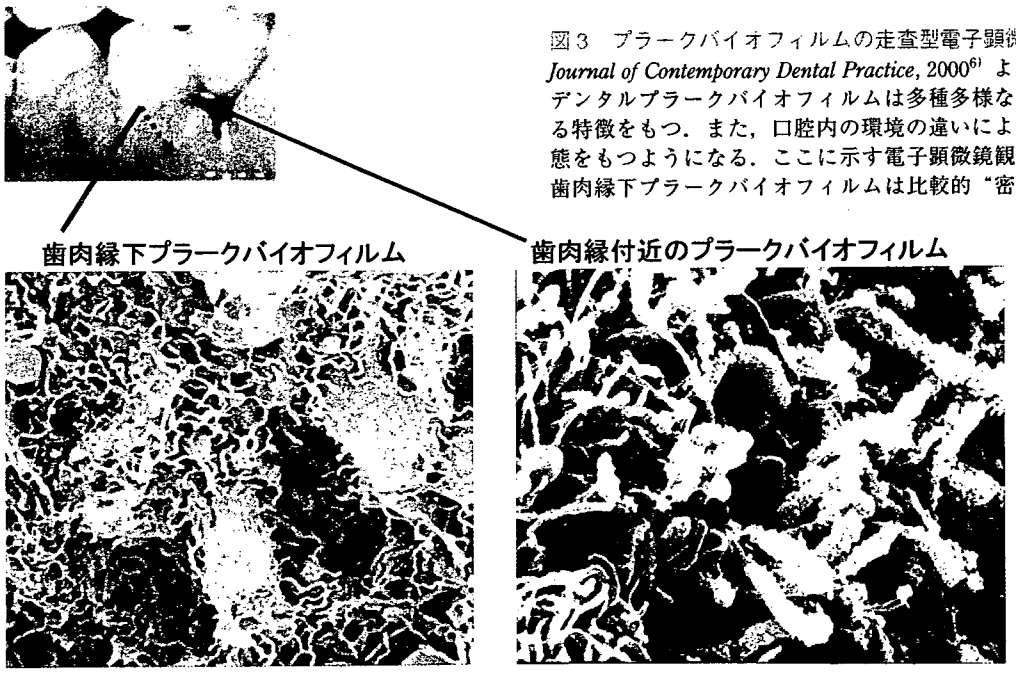

図3　プラークバイオフィルムの走査型電子顕微鏡観察像(Overman PR: *Journal of Contemporary Dental Practice*, 2000[6] より引用)
デンタルプラークバイオフィルムは多種多様な細菌種によって構成される特徴をもつ．また，口腔内の環境の違いによってさまざまな生態と形態をもつようになる．ここに示す電子顕微鏡観察像はその一例である．歯肉縁下プラークバイオフィルムは比較的“密”な形態をとっているものを示した．深い歯周ポケット内のバイオフィルムであり，スピロヘータが構成細菌の主体となっている．縁上に近い部位のバイオフィルムはcorncob（トウモロコシの軸）様の形態をとっているものを例として示した．

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3．バイオフィルムの生態と病原性

◌病原性を高める菌体外マトリックス

バイオフィルムの立体構造は菌体外マトリックスによって維持されている（図2）．菌体外マトリックスの本態は多糖体であり，菌体外多糖物質（extracellular polymeric substance: EPS）とよばれる．EPSは細菌の宿主定着の足場として機能するだけではなく，メカニカルなバリアとして細菌細胞を消毒薬や抗菌薬から，あるいは乾燥から保護する役目をもっている．さらに，抗原性の低い多糖物質に対して生体は有効な免疫応答を起こすことができない．これらのことが，バイオフィルム感染症の慢性化や治療抵抗性の原因になる．

◌バイオフィルム細菌の性状──菌と菌のインターラクション

バイオフィルム中の細菌は単体で存在する浮遊菌とは異なる性質をもつ．すなわち，バイオフィルム中では異なった菌種間においても菌と菌とのインターラクションがあり，お互いがコミュニケーションをとって特定の遺伝子発現を制御している．この細菌間の情報伝達システムはバイオフィルム中の細菌細胞密度を感知することで制御されることが多く，クオラム・センシング機構（quorum-sensing system）とよばれる．クオラム・センシング機構によって細胞密度や病原性因子の発現が調節され，バイオフィルム中の細菌は統率のとれた多細胞生物のように活動する．

微生物間情報伝達物質は自己誘導体（autoinducer）とよばれ，グラム陰性菌の同種間情報伝達分子となるアシル化ホモセリンラクトン（図5）やグラム陽性菌のペプチドフェロモンあるいはグラム陽性・陰性両者にみられるautoinducer-2が知られている．歯周病原細菌においても伝達物質を介したクオラム・センシング機構によって*P. gingivalis*の線毛[1]やプロテアーゼ分子[2]あるいは*A. actinomycetemcomitans*の白血球毒素[3]の発現が調節されていると報告されている．

クオラム・センシング機構を介した性状の変化以外にも，バイオフィルム細菌は病原性に関連した以下の特徴をもつ．

①細菌密度の高いバイオフィルム内ではプラスミド伝達あるいはトランスポゾン*6伝達を介した薬剤耐性遺伝子や病原遺伝子などの遺伝情報の交換が容易となる．

②増殖速度の遅いバイオフィルム内の細菌は分裂の際に効果を発揮するタイプの抗生物質（ペニシリン，セフェム系など）に対して高い薬剤耐性を示す．

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*6 トランスポゾン：いわゆる“動く遺伝子”．薬剤耐性遺伝子や病原遺伝子など複数の遺伝子から構成されている場合が多い．遺伝子間を伝播するために必要なトランスポーセースの遺伝子も含んでいる．

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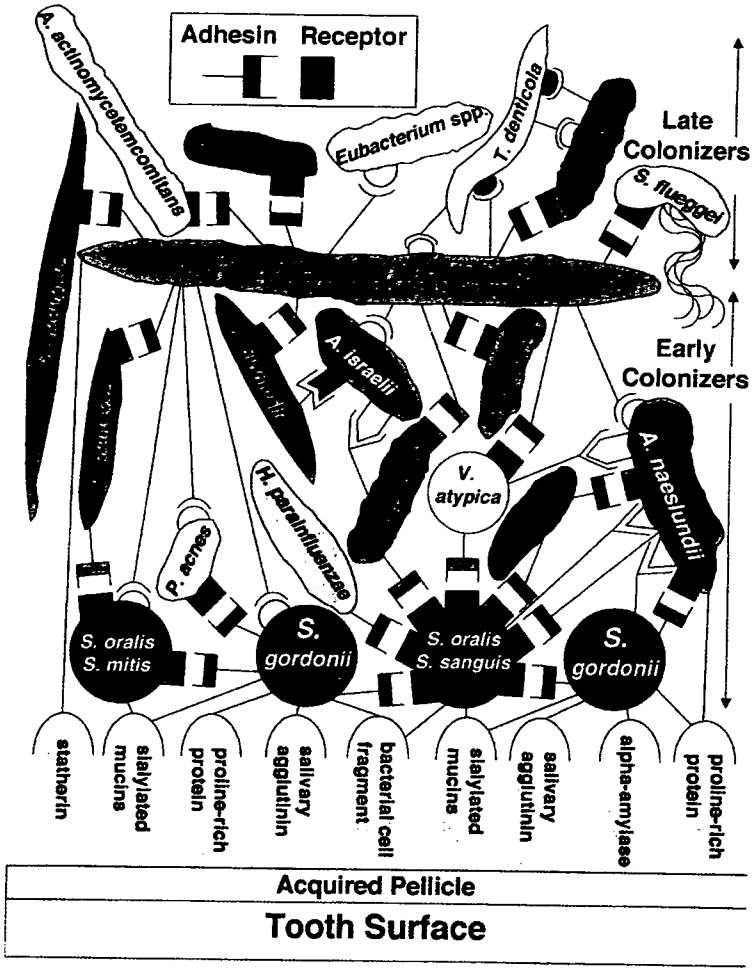

図4　バイオフィルムの構成細菌（Kolenbrander PE, et al: *Microbiol Mol Biol Rev*, 2002[5] より引用）
プラークバイオフィルム中ではさまざまな細菌種が凝集し，情報伝達が行われている．プラークの初期形成には streptococci が主体となり（early colonizers），その後 *Fusobacterium*，そして *P. gingivalis* や *T. denticola* などの偏性嫌気性菌がバイオフィルム形成に関与する（late colonizers）．*Fusobacterium* の菌体は大きく，他の細菌と共凝集することでプラーク形成の中心的役割を担っていると考えられている．

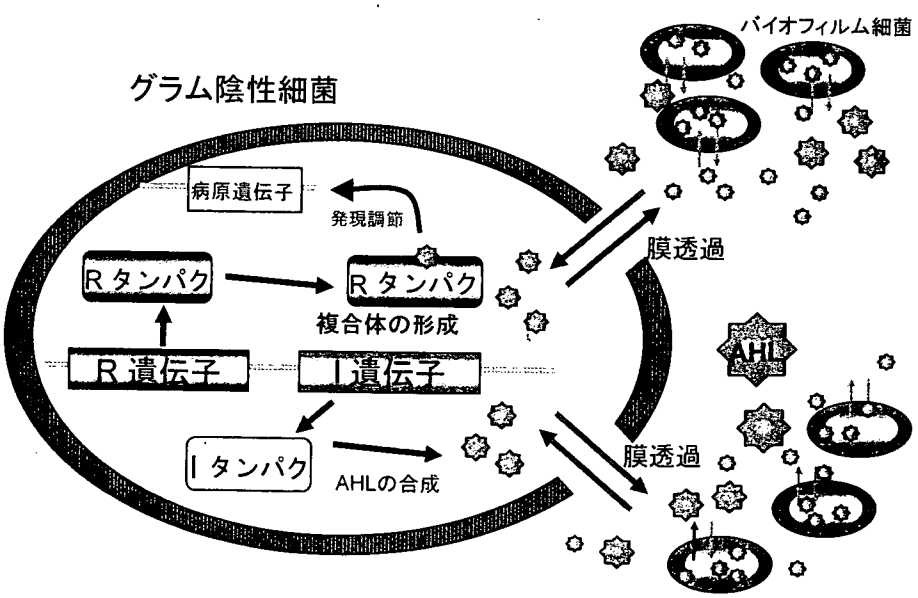

図5　グラム陰性細菌のクオラム・センシング機構
グラム陰性菌は自己誘導体（autoinducer）であるアシル化ホモセリンラクトン（AHL）を介するクオラム・センシング機構によって細胞間での情報伝達を行う．グラム陰性細菌のクオラム・センシング機構には 3 つの共通基本因子が存在する．すなわち，高い膜透過性をもつ AHL と AHL の合成に関与する I タンパク，そして AHL と複合体を形成して病原因子などのさまざまな遺伝子の発現調節を行う R タンパクである．

4．バイオフィルムに対する今後の展開

クオラム・センシング機構を介した細菌間の情報伝達によって歯周病原細菌はバイオフィルムを形成し，免疫応答や抗菌薬に対する抵抗性を獲得する．バイオフィルム内では，構成細菌種の生存に有利なようにお互いの活動が調整されており，バリアを獲得した細菌は病原因子の発現量を増加させ，宿主に挑んでくる．これによって慢性的に歯周局所に感染巣が存在することになり，歯周組織の破壊を引き起こす．さらに，感染症が慢性化することによって，全身に歯周病原細菌が移行するリスクが高くなり，心・血管疾患や誤嚥性肺炎などの全身疾患を誘発することになる．このようなバイオフィルム感染症である歯周病に対し，現段階ではスケーリングやルートプレーニングなどの機械的除去療法が治療の中心となっている．抗菌薬としてはテトラサイクリン系抗菌薬の歯周ポケット内局所投与が有効とされ，広く活用されている．また，マクロライド系抗菌薬に抗バイオフィルム作用のあることがわかり，歯周炎治療への応用が期待されている．さらに今後，バイオフィルム形成の分子メカニズムやクオラム・センシング機構の解明から，初期付着の阻害や細菌のコミュニケーションネットワークを阻害する新しい口腔バイオフィルムへの戦略が期待されている．

トピックス―指先からの自己採血による歯周病原細菌感染のリスク診断

バイオフィルム感染症である歯周病の診断や治療評価には細菌学的な検査が不可欠である．バイオフィルムを量的・質的に評価するためには，大きく2とおりの方法がある．1つは細菌培養法やPCR法などによって，口腔内から直接細菌種を検出するやり方である．もう1つは各菌種に対して産生されたIgGあるいはIgA抗体量を測定する方法である．特異抗体を検出・定量することは，感染細菌種を特定するだけでなく，歯周病原細菌に対する生体反応を評価し，歯周炎の活動度を診断することにつながる．歯周病原細菌のなかでは，*P. gingivalis* に対する抗体価が歯周病の病状をよく表していることが最近の研究で明らかになってきた（図6）．

従来，歯周病原細菌に対する抗体測定検査には，静脈血を利用するのが一般的であった．採血から血清分離，そして抗体価測定に至る一連の検査操作には特別な技術や測定機器が必要である．このため，歯周病の抗体測定検査は大学病院などの施設に限られて実施されている．このような現状では有用な検査であっても，広く一般に普及させて臨床に活用することは困難である．そこで現在，全国の大学が協力して，指先の毛細血管からの採血で実施可能な検査システムが開発されようとしている（図7）．これまでの研究によって，指先からの毛細血管血を用いた抗体測定検査が，静脈血と同様の検査結果を示すことが明らかとなった．この検査システムが活用できるようになれば，歯周病の血清学

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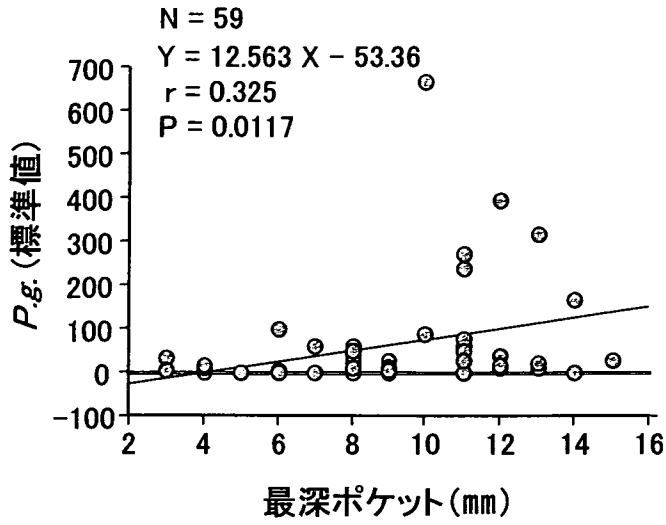

$$\text{標準値} = \frac{\text{患者血清抗体価} - \text{健常者平均抗体価}}{2 \times \text{標準偏差（健常者）}}$$

図6　*P. gingivalis*(*P. g.*)に対するIgG抗体価と歯周病の病状との関係
*P.g.*に対するIgG抗体量は，健常者の抗体量と比較しやすいように標準値として表した（計算式をグラフ下に示す）．歯周病患者の病状（最深のポケット深さ）が悪いほど，*P.g.*に対する抗体量は増加傾向を示す．

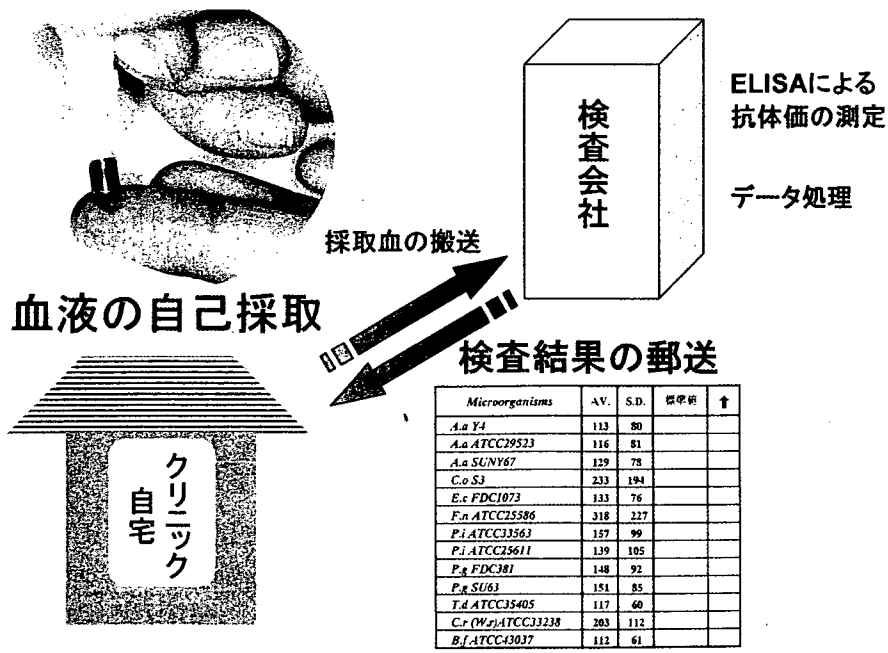

図7　指先からの自己採血による新しい歯周病の検査システム
指先から自己採血し，採血サンプルを検査会社に搬送するためのキットを作製する．このキットを用いることで一般開業医や自宅での歯周病診断が可能となる．

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的診断を広く，一般の歯科医院に普及できると考える．さらに，検査キットを市販することによって，歯周病の自己診断が可能となり，歯周病の潜在患者が自主的に歯科医院を受診することへとつながり，国民の健康増進に寄与できるだろう．

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ORIGINAL RESEARCH ARTICLE

Role of salivary tumour necrosis factor α in HIV-positive patients with oral manifestations

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Summary: In HIV-1-infected patients, oral manifestations such as recurrent apthous ulcers are often seen. A total of 29 HIV-infected patients were examined to determine salivary tumour necrosis factor α (TNFα) concentrations by enzyme-linked immunosorbent assay, the amount of HIV-1 RNA copy by Amplicor HIV-1 Monitor test, number of CD4 cells by flow cytometry and oral manifestations by oral examination. TNFα concentration was significantly correlated with the amount of HIV-1 RNA, however, not with the number of CD4 cells in HIV-1-infected patients. Further, patients with oral manifestations showed significantly higher concentrations of TNFα in saliva and HIV-1 RNA copies in serum than those without oral manifestations. Following recovery from oral ulcers, TNFα concentration was decreased by half to 20 times lower than the level of that during ulcer incidence. Our results suggest that salivary TNFα is a good indicator for oral manifestations and HIV RNA amounts in HIV-1-infected patients.

Keywords: TNFα, HIV, oral manifestations, HAART, saliva

INTRODUCTION

Between 1991 and 2002, the number of AIDS patients in Japan increased more than seven-fold, from 38 to 304,[1] and at present, there are more than 10,000 HIV-infected patients in Japan. In the oral cavities of AIDS- and HIV-infected patients, various oral manifestations, including candidosis, recurrent apthous ulcers, hairy leukoplakia, Kaposi sarcoma and other types of ulcers, have been observed.[2,3] Most of those are thought to be caused by an opportunistic infection and influenced by the immune state of the host.

The concentrations of various cytokines are increased in the blood of HIV-infected patients, including tumour necrosis factor α (TNFα), which is a key proinflammatory cytokine produced by a number of cells, including macrophages, muscle neutrophils, endothelial cells and vascular smooth muscle cells (VSMCs). This protein acts locally at tissue sites with vessel wall damage and has many biological activities, including control of cell differentiation, tissue renewal and restructuring, defence against microorganism infection, growth/differentiation of lymphocytes and induction of apoptosis of cancer cells, as well as some functions with normal cells, such as fibroblasts, epidermal cells and endothelial cells.[4-11] TNFα induces apoptosis signalling by binding specifically to its receptor in the cell membrane[12-14] and is abundant in all rheumatoid arthritis tissues.[15] Further, the expression of TNFα in intestinal mucosa from Crohn's disease patients is markedly enhanced.[16] Some oral manifestations are inflammatory reactions, so it is suspected that those in HIV-infected patients are associated with TNFα.

Various substances in saliva are useful as biochemical markers of oral diseases with regard to diagnosis and prognosis, as well as to evaluate therapy effects.[17,18] For the diagnosis of HIV-positive patients, a quick test using oral fluid has been used recently instead of blood,[19] with the results found helpful to educate those patients regarding window periods and elucidate false routine confirmatory testing in clinics. In an attempt to develop a quick system for diagnosis of oral manifestations, we analysed the relationships between oral manifestations and salivary TNFα concentrations in HIV-1-infected patients.

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MATERIALS AND METHODS

Subjects

The subjects were 29 HIV-1-infected patients who had consulted the Oral Surgery Section of Tokyo Medical

University (age 39.8 ± 12.3 years; 19 men, 10 women), while 32 persons who did not have systemic disease and were not infected with HIV-1 (age 41.3 ± 21.4 years; 19 men, 13 women) served as the control group. Nineteen of the patients had received highly active antiretroviral therapy (HAART), which was defined as treatment with three or more antiretroviral agents, including at least one protease inhibitor or at least one non-nucleoside reverse-transcriptase inhibitor.

Oral examination

Oral manifestations were observed by two dentists, who were experts in oral surgery. The distribution of patients by oral manifestation is shown in Table 1. Patients who had received HAART were examined both before and after the therapy.

Laboratory methods and data collection

Non-stimulated whole saliva samples were collected for 5 minutes from each patient prior to a dental health check up. Serum samples were isolated from 5 mL of peripheral blood taken from each patient. Measurements of the various parameters were performed by SRL (Tokyo, Japan). Spontaneously flowing whole saliva samples were collected in sterilized bottles and subjected to a centrifuge at 16,000 revolutions per minute (rpm). The supernatants obtained were used for the detection of TNFα with an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine® HS, R&D Systems, Inc., Minneapolis, MN, USA). Plasma HIV-1 RNA was assayed with an Amplicor HIV-1 Monitor test (Roche Diagnostic Systems, Branchburg, NJ, USA). CD4 T-cell measurements were made by flow cytometry using thawed whole-blood samples that had been cryopreserved in 10% dimethylsulphoxide with directly conjugated monoclonal antibodies (Becton Dickinson Immunocytometry, San Jose, CA, USA) at one month after the TNFα detection assay.

Table 1 Distribution of oral manifestation

Oral manifestation	Number of subjects
Aphtha	5
Candidosis	4
Ulcer	4
Oral hairy leukoplakia	3
Linear gingival erythrosis	2
Necrotizing ulcerative gingivitis	1
Herpes simplex	1
Herpes zoster	1
Kaposi sarcoma	1
Cytomegalovirus (CMV) stomatitis	1

Four subjects have more than two oral manifestations.

Statistical methods

Values below the quantification limit of 50 copies/mL were assigned a value of 50 copies before analysis. Comparisons of continuous variables between two groups were made using the Mann-Whitney *U* test. The χ^2 test was used to assess the statistical significance for bivariate comparison when the variables were nominal. Differences at the 0.05 level were considered statistically significant. SPSS for Windows (version 10.0) was used for all statistical analyses.

RESULTS

Figure 1 shows the relationship between TNFα concentration and the amount of HIV-1 RNA in serum, as well as the number of CD4 cells in the HIV-1-infected patients. The concentration of TNFα was significantly correlated with the amount of HIV-1 RNA, however, not with CD4 cell number.

Table 2 shows the relationships between TNFα concentrations and oral manifestations. We compared TNFα concentrations among the control group, HIV-1-infected patients without oral manifestations (HPON group) and HIV-1-infected patients with oral manifestations (HPOP group). None of the control subjects had oral manifestations. The HPOP group showed significantly higher concentrations of TNFα than the other groups (HPON $P = 0.040$, control $P = 0.000$). TNFα concentration in the HPON group was higher than in the control, though it was not significant ($P = 0.075$). In the HPOP group, patients with non-infected oral manifestations (NIOMs) showed significantly higher TNFα concentrations than those in the

Table 2 Tumour necrosis factor α (TNFα) concentration and HIV-1 RNA level

	TNFα (pg/mL)			Log10 HIV-1 RNA copies/mL		
	Median	25th, 75th percentiles	P	Median	25th, 75th percentiles	P
Control (32)	1.5	0.5, 2.5		–	–	
HPON (10)	3.1	1.3, 5.4	*	50	50, 10,037.5	
HPOP (19)	5.8	3.2, 26.9		35,000	1000, 90,000	**

Comparisons were by Mann-Whitney *U* test.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
Bracket: Control vs. HIV-1 infected patients without oral manifestations (HPON group) or HIV-1 infected patients with oral manifestations (HPOP group) in TNFα assay, and HPON vs. HPOP in HIV-1 RNA assay.

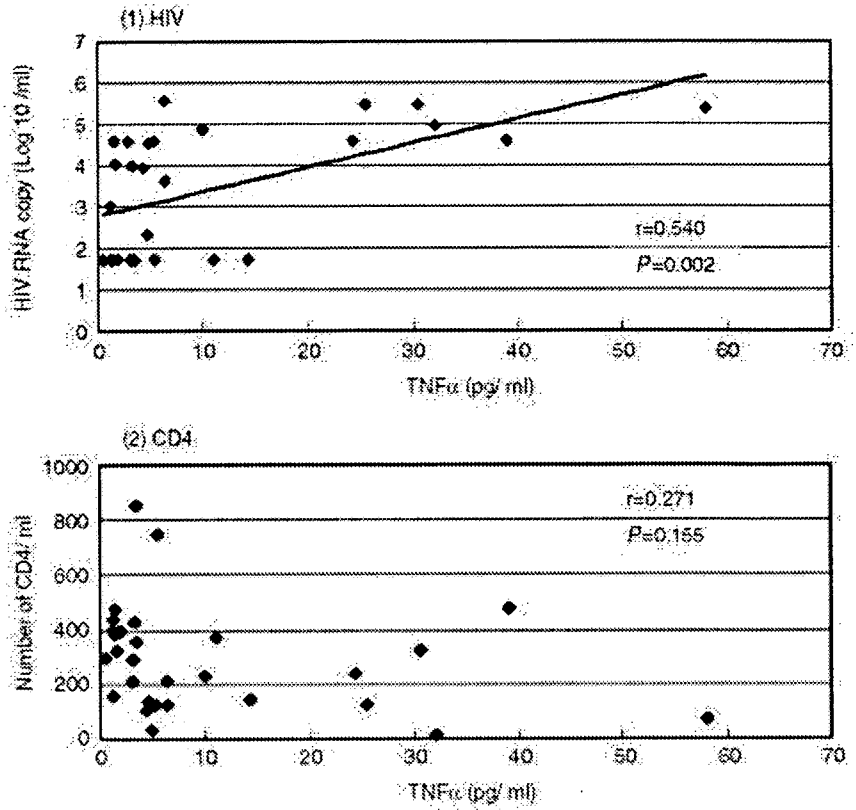

Figure 1 Scatter plot of tumour necrosis factor α (TNFα) concentrations in saliva, amounts of HIV-1 RNA in serum and number of CD4 cells among peripheral blood mononuclear cells. TNFα concentration was strongly correlated with the amount of HIV-1 RNA (1) ($r=0.540$, $P=0.002$), but not CD4 number (2) ($r=0.271$, $P=0.156$)

HPON and control groups (data not shown; HPON $P=0.044$, control $P=0.000$). Further, the TNFα concentration in patients with infected oral manifestations (IOMs) was significantly higher than that in the control group, whereas it was not significantly higher than that in the HPON group (data not shown; HPON $P=0.193$, control $P=0.005$). In addition, the HIV-1 RNA level in the HPOP group was significantly higher than that in the HPON group ($P=0.007$).

In the HPOP group, patients with ulcers showed a high concentration of TNFα (with ulcers, 35.5 pg/mL; entire HPOP group, 14.2 pg/mL; average values). Further, the concentrations of TNFα in saliva in four of the HIV-1-infected patients who had ulcers were determined after recovery from the ulcers and compared with those in the same patients before recovery (Figure 2). Following recovery from oral ulcers, TNFα concentrations were decreased by half to 20 times less than the level during ulcer incidence.

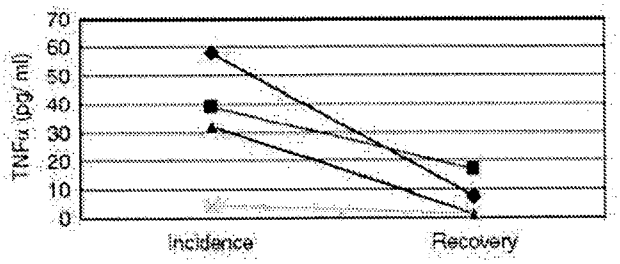

Figure 2 Tumour necrosis factor α (TNFα) levels in saliva from patients with ulcers in the oral cavity. Comparisons of TNFα concentrations between periods of incidence and recovery were performed using whole saliva samples from four patients with ulcers

DISCUSSION

Elevated levels of salivary TNFα have been found in HIV-infected macrophages, monocytes, trophoblastic cells and lymphocytes of HIV-infected patients,[20–23] and a statistically significant correlation between HIV-1 RNA and TNFα in plasma was also reported.[24] In our study, the concentration of TNFα in saliva, in the same way as that in blood, had a significant relationship with the amount of HIV-1 RNA in serum. This correlation is thought to be partly explained by the following. TNFα is excessively produced to suppress opportunistic infections that are caused by immunodeficiency in HIV-infected patients.[25] We considered that the excessive salivary TNFα production seen in our subjects may have been from the activities of the defence mechanism against opportunistic microorganism infection. Patients who showed a high concentration of

TNFα in whole saliva did not show significant production of TNFα in parotid saliva (data not shown). Therefore, it was considered that the TNFα was produced by mucosal and local responses to microorganisms in the oral cavity. Another is that TNFα induces HIV expression via stimulation of the HIV promoter.[26] Tat protein was reported to transactivate TNFα gene expression in HIV-1-infected and in tat-transfected T-lymphocytic and monocytic cell lines.[27] It is reported that HIV-1 infected salivary gland epithelial cell lines.[28] These reports induce a speculation that TNFα gene expression was transactivated by HIV-1 gene products in HIV-1-infected salivary cells of patients. It is known that extracellular tax induces the production and release of TNFα.[29] HIV-1 might also express viral factors that exert extracellular induction of the production and release of TNFα as HTLV1. It was reported that induction of TNFα expression in the genital tract is likely to be involved in producing the adverse effects. Cervical lavage in genital tract from patients with bacterial vaginosis induces TNFα expressions and toll-like receptor (TLR)-2 and 4 expressions in the peripheral blood mononuclear cells and THPP-1 monocyte cell line.[30] However, in patients with HIV-1 RNA below the quantitation limit, various concentrations (0.5-14.2 pg/mL) of TNFα were seen (Figure 1). Therefore, the extent of TNFα production is considered to be directed by not only HIV-1 RNA concentration, as there is a possibility that TNFα production is induced by HIV-1 infection via some other mechanisms.

Eleven patients with HIV-1 RNA below the quantitation limit had received HAART and only three (27.2%) had oral manifestations. The rate of oral manifestations in patients who underwent HAART (52.6%) was significantly lower than that (90%) in those who did not. Based on our medical examinations and the treatment records, it was found that five patients in the HPON group with HIV-1 RNA below the quantitation limit had some oral manifestations before HAART. The extensive decrease of HIV-1 RNA following HAART may be related to a low level of TNFα and disappearance of oral manifestations.

TNFα concentration in the HPOP group was significantly higher than that in the HPON group. TNFα is an inflammatory cytokine that induces inflammatory reactions in injured tissues.[31] An excess production of TNFα and then stabilization of TNFα mRNA cause a disruption of cells, which leads to various chronic inflammatory diseases, such as chronic rheumatoid arthritis and Crohn's disease.[32-34] Cell disruption by TNFα is also considered to cause inflammatory lesions in the oral cavity, as well as other organs. Some of the oral manifestations observed in the present patients were considered to be caused by that mechanism.

Ulcers result from a tissue state in which cells in the oral cavity become disrupted. In the present patients, TNFα concentrations decreased drastically following recovery from ulcers, which suggests that salivary TNFα is associated with ulcer induction in the mucosal membrane of the oral cavity. As for other non-infective oral manifestations, some relationship with TNF, which might be involved with a decrease in immunity or another mechanism, is suspected. In infective oral manifestations, TNFα is considered to be induced and disrupt infected cells or invaded microorganisms, such as candida. The reason for not finding a significant difference in TNFα concentration between the present IOM and HPON groups may have been because of the small sample size.

This is the first known study to show that salivary TNFα is correlated with oral manifestations and the amount of HIV-1 RNA in HIV-1-infected patients, in whom salivary TNFα was present in higher concentrations than in uninfected individuals. Excess TNFα is considered to be associated with oral manifestations in various ways. Thus, improvement in oral health is considered necessary for increasing the quality of life of HIV-1-infected patients. Our findings will provide useful and important information for elucidation of the effects of salivary TNFα on the incidence of oral manifestations, as well as regarding oral care for HIV-1-infected patients. In addition, the correlation between salivary TNFα concentration and amount of HIV-1 RNA seen suggests that measuring salivary TNFα concentration is useful for determining the amount of HIV-1 RNA and diagnosing oral manifestations in these patients.

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Glycosylation of the OMP85 homolog of *Porphyromonas gingivalis* and its involvement in biofilm formation

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Abstract

OMP85 is a highly conserved outer membrane protein in all Gram-negative bacteria. We studied an uncharacterized OMP85 homolog of *Porphyromonas gingivalis*, a primary periodontal pathogen forming subgingival plaque biofilms. Using an outer-loop peptide antibody specific for the OMP85 of *P. gingivalis*, loop-3 Ab, we found a difference in the mobility of OMP85 on SDS–PAGE gel between the *P. gingivalis* wild-type and the isogenic *galE* mutant, a deglycosylated strain, suggesting that OMP85 naturally exists in a glycosylated form. This was also supported by a shift in OMP85 PAGE mobility after chemical deglycosylation treatment. Further, loop-3 Ab cross-reacted with the *galE* mutant stronger than the wild-type strain; and could inhibit biofilm formation in the *galE* mutant more than in the wild-type strain. In conclusion, this is the first report providing the evidence of OMP85 glycosylation and the involvement of OMP85 in biofilm formation.

Keywords: *Porphyromonas gingivalis*; Gram-negative bacteria; Periodontal disease; OMP85; *galE*; Glycoprotein; Biofilms; Glycosylation; Outer membrane protein

Periodontitis is one of the most common infectious diseases and is caused by bacteria or groups of bacteria found in subgingival pockets. Among the bacterial population, *Porphyromonas gingivalis*, a Gram-negative anaerobe, is a primary component forming subgingival plaque biofilms in periodontitis [1]. Data suggest *P. gingivalis* is also associated with systemic diseases and complications including cardiovascular diseases, atherosclerosis, preterm births, and low-birth-weight babies [1]. Hence, it is important to delineate the molecular basis for biofilm formation by this bacterium.

OMP85 is a highly conserved outer membrane protein (OMP) in all Gram-negative bacteria, for which complete sequences are available. The OMP85 from both *Neisseria meningitidis* and *Escherichia coli* are essential for cell viability and are involved in the OMP assembly [2,3]. Recently, *E. coli* OMP85 was shown to form hetero-oligomeric complexes with four different lipoproteins [4,5] and to recognize sorting signals that can be detected at the C-termini in the vast majority of bacterial OMPs [6]. Additionally, OMP85 homologs from some bacterial species are immmunogenic where immunization of animals using a vaccine preparation based on recombinant proteins of each homolog confers protection against the bacterial challenge [7,8]. This is yet to be shown for the OMP85 homolog of *P. gingivalis*.

Protein glycosylation in bacteria is studied because most glycoproteins found in bacterial pathogens are localized at the bacterial surface; and appear to influence

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host–bacterial interaction [9]. For Gram-negative bacteria, although only a few glycoproteins are known, most of the glycoproteins play critical roles in virulence: e.g., the type IV pili of *N. meningitidis* [10] and *Pseudomonas aeruginosa* [11], the flagella of *Campylobacter jejuni* [12], three adhesins of *E. coli* [13–15], and the gingipains of *P. gingivalis* [16].

Here, we focused on the OMP85 of *P. gingivalis* and tried to characterize the molecule in the light of the glycosylation biology. We generated an outer-loop peptide antibody, loop-3 Ab, specific for OMP85 and examined whether *P. gingivalis* OMP85 is modified with glycan or not, using Western blot analysis with loop-3 Ab. We also examined whether glycosylation of OMP85 is associated with biofilm formation of *P. gingivalis*. Here we present new information concerning the roll of OMP85 glycosylation in biofilm formation.

Materials and methods

Bacterial strains, plasmids, and oligonucleotides. E. coli and *P. gingivalis* strains were cultured as described previously [17]. The bacterial strains and plasmids used in this study are shown in Table 1. The oligonucleotides used in this study are shown in Supplementary Table 1.

Sequencing. The entire *omp85* gene (2.7 kb) from *P. gingivalis* strain 33277 and 381 was cloned into pMD20-T using the DNA ligation kit Mighty Mix (Takara Bio, Shiga, Japan). The resultant plasmids were DNA sequenced (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, CA). The sequence of the *omp85* gene from strain W83 was obtained from the GenBank database. The amino acid sequences corresponding to the *omp85* genes among strains 33277, 381, and W83 were aligned using Genetyx 7.0.3 software (Genetyx Co., Tokyo, Japan). The cleavage site of the N-terminal signal peptide of the OMP85 and the molecular mass after the cleavage were predicted by Genetyx 7.0.3.

Topological prediction of OMP85. PROFtmb program [18], a fully automated Hidden Markov Model-based method for predicting the topology of bacterial outer membrane proteins directly from amino acid sequences, was used to predict the topology of *P. gingivalis* OMP85, according to the manufacture's instructions.

Peptide synthesis and generation of peptide antisera. Peptides were synthesized using a stepwise solid-phase procedure at Asahi Techno Glass Co. (Tokyo, Japan). The peptides were conjugated to keyhole limpet hemocyanin and used as the immunogen to produce rabbit antiserum. Antisera were produced in Japanese White rabbits at Asahi Techno Glass Co. using conventional immunization protocol. The specificity and cross-reactivity of antibody were examined using antigen-specific ELISA and whole-cell ELISA as described previously [19,20].

Over-expression and purification of GST-OMP85 fusion protein. A PCR fragment corresponding to the open reading frame of OMP85 was ligated to the expression vector pGEX-6P-1 (GE Healthcare Biosciences, Piscataway, NJ) according to manufacture's instructions. The GST-OMP85 fusion protein was over-expressed in *E. coli* harboring the constructed expression vector. As most of the GST-OMP85 protein was found to be in the form of inclusion bodies, the GST-OMP85 inclusion bodies were solubilized using 50 mM Tris–HCl buffer (pH 8.0) containing 0.5% SDS, 1 mM EDTA, and 0.1 M NaCl. Then, the solubilized samples were fractionated with a SDS–PAGE-based strategy using PREPPHORESIS-S and a fraction collector (ATTO Co., Tokyo, Japan); and the recombinant GST-OMP85 protein was purified without detectable contaminating bands.

Outer membrane extraction. The differential detergent method was used to extract the outer membrane (OM) of *P. gingivalis* as described previously [21] with some modifications. Briefly, bacterial cells were washed and suspended in 10 mM HEPES–NaOH (pH 7.4) buffer; and were disrupted using sonication at 8 W for 1 min on ice in the presence of protease inhibitors, RNaseA, and DNaseI. The remaining intact bacterial cells were removed using centrifugation at 1000*g* for 10 min at 4 °C. The supernatant was ultracentrifuged at 100,000*g* for 1 h at 4 °C. The cell envelope fraction recovered in the pellet was re-suspended using 1% Triton X-100 in Hepes–NaOH buffer with 20 mM $MgCl_2$ for 30 min at 20 °C. Finally, the OM fraction was recovered as a pellet after ultracentrifugation (100,000*g* for 1 h at 4 °C). To prepare the OM fraction for the glycoprotein staining and mass analysis, the OM extracts were passed twice through a Detoxigel (Pierce, Rockford, IL) column to remove LPS according to manufacture's instructions. The resultant OM extracts did not contain detectable LPS using Limulus Amebocyte Lysate assay (Seikagaku Co., Tokyo, Japan); but contained major OMPs using a mass spectrometry (data not shown) indicating the OMP was resolved.

SDS–PAGE and Western blot. SDS–PAGE was performed in a 7.5% gel where 10-μg samples were used. The Gelcode Blue Stain Reagent (Pierce) was used for Coomassie brilliant blue (CBB) staining. The Gelcode Glyco-

Table 1
Bacterial strains and plasmids used in this study

Bacterial strain/plasmid	Usage, relevant phenotypes, or selective marker[a]	Source and/or description
Escherichia coli		
DH5α	Usage for cloning	Takara Bio
BL21	Usage for protein expression	GE Healthcare Biosciences
Porphyromonas gingivalis		
ATCC 33277	Wild-type	American Type Culture Collection
ATCC 33277 *galE*	*galE* mutant by insertion of *ermF-ermAM* cassette into the Bse RI site of the corresponding gene, Erm^r	[17]
ATCC 33277 *galE-c2*	*galE* complementary strain, Tet^r, Erm^s	This study[b]
Plasmid		
pMD-20T	Cloning vector, Amp^r	Takara Bio
pGEX6P-1	Expression vector, Amp^r	GE Healthcare Biosciences
pUC19Q	Contains *tetQ* cassette between SmaI and BamHI sites in pUC 19, Amp^r, Tet^r	[17]
pQG2	Contains a region of 0.8 kb downstream of *galE* between Bam HI and XbaI sites of pUC19Q, Amp^r, Tet^r	This study[b]
pGQG2	Contains 0.3-kb upstream and whole sequence of *galE* between KpnI and SmaI sites of pQG2, Amp^r, Tet^r	This study[b]

[a] Amp^r, ampicilin resistant; Erm^r, erythromycin resistant; Erm^s, erythromycin sensitive; Tet^r, tetracycline resistant.
[b] The strategy used to construct *galE* -c2 strain is shown in Supplementary Fig. S2.

protein Staining Kit (Pierce) was used for carbohydrate staining. For Western blot, the materials in the SDS–PAGE gel were transferred onto PVDF membrane and probed with loop-3 Ab diluted 1:10. Horse radish peroxidase (HRP)-labeled anti-rabbit Ig antibody (GE Healthcare Bio-Sciences) diluted 1:200,000 was used as the second antibody. Chemiluminescence was detected using ECLplus (GE Healthcare Bio-Sciences).

Chemical deglycosylation assay. Chemical deglycosylation of the OM extract was performed using anhydrous trifluoromethanesulfonic acid (TFMS) with 10% anisole, as described previously [22]. Briefly, 150 μl of 10% anisole in TFMS was added to 1 mg of lyophilized OM extract and incubated at 4 °C for 1 or 3 h and the solution was neutralized with 60% pyridine solution. The neutralized solution was purified by dialysis using a Slide A Lyzer Dialysis Cassette (MWCO 10 kDa, Pierce) with 20 mM Tris–Cl (pH 8.0), 10 mM EDTA, and 0.1% SDS, then concentrated to a 100-μl volume by ultra-filtration with a MWCO 30-kD filter (ATTOPREP UF-30, ATTO Co.).

Biofilm inhibition assay using loop-3 Ab. Loop-3 Ab or pre-immune serum was diluted with PBS to various ratios (50%, 20%, 10%, and 5%) and mixed with 2×10^7 CFU of *P. gingivalis* cells in a volume ratio of one to one. After a 1-h pre-incubation at 37 °C in an anaerobic chamber, the bacterial cells were harvested, washed with PBS twice, and used in the biofilm formation assays as described previously [17].

MALDI-TOF mass spectrometry and peptide mass fingerprinting. After SDS–PAGE, CBB-stained protein bands were excised from the gels, and in-gel digestion with trypsin was performed as described previously [23]. MALDI-TOF mass spectra were acquired with a Bruker Ultraflex mass spectrometer (Bruker Daltonics Inc., Billerica, MA). Peptide masses were compared to the MSDB database using the

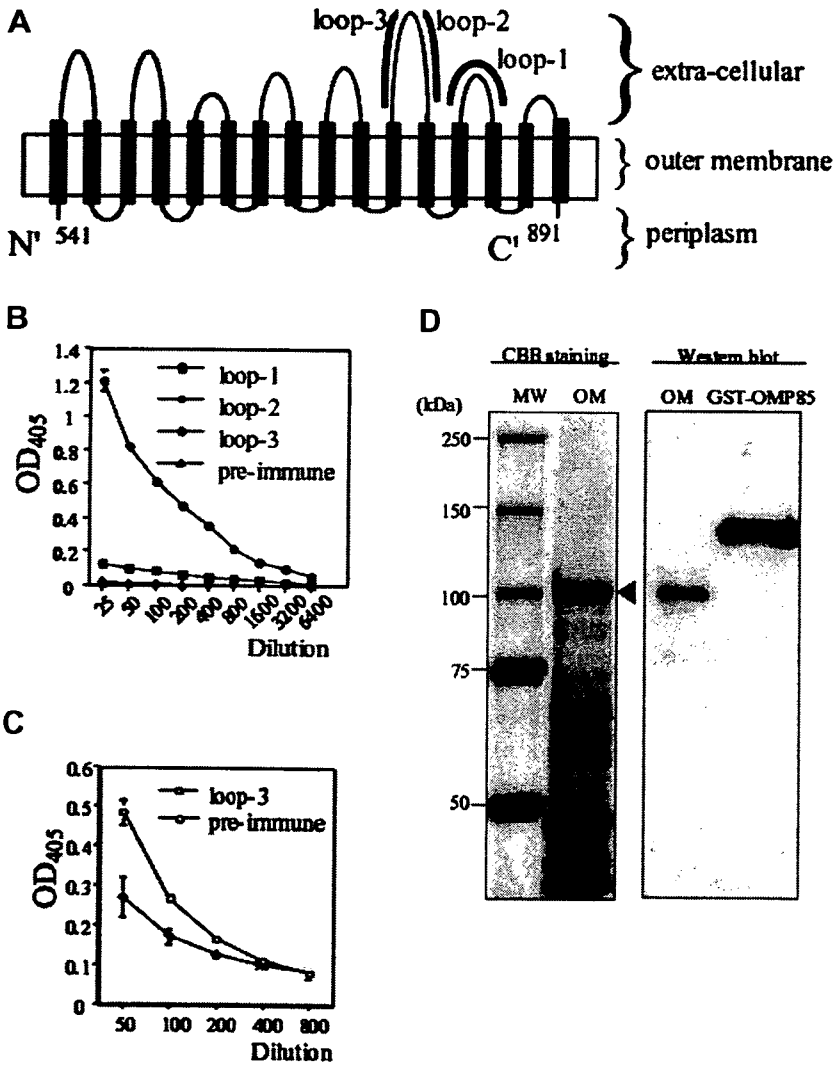

Fig. 1. Topology of OMP85 and cross-reactivity of OMP85 peptide Ab. (A) The transmembrane β-barrel domain of OMP85 and designed peptides. The OMP85 topology of *P. gingivalis* strain W83 was predicted to consist of two domains, the N-terminal periplasmic domain up to residue 541; and the rest, the C-terminal β-barrel transmembrane domain. Three peptides corresponding to the outer loops of OMP85, loop-1, -2, and -3, were designed. The amino acid sequences are shown in Supplementary Fig. S1. (B) Cross-reactivity of the three loop Ab to GST-OMP85. ELISA on plates coated with GST-OMP85 was performed using Ab from the three loop peptides and pre-immune serum. The results are expressed as the means ± SD of triplicate assays. (C) Cross-reactivity of the loop-3 Ab to whole cells of *P. gingivalis*. ELISA on plates coated with whole cells of *P. gingivalis* wild-type strain was performed using loop-3 Ab and pre-immune serum. The results are expressed as the means ± SD of triplicate assays. (D) Ten micrograms of sample of OM extract (lane: OM) from the wild-type strain was applied to SDS–PAGE using a 7.5% polyacrylamide gel. The CBB staining and Western blot with loop-3 Ab are shown in the left and right figures, respectively. Purified GST-OMP85 (lane: GST-OMP85) was also tested as a positive control. The molecular mass of the GST portion of the fusion protein is approximate 26 kDa. MW: molecular weight marker. Approximately 100-kDa band in CBB staining is shown by an arrowhead and identified as the RagA protein using TOF/MS analysis.

Mascot search engine (http://www.matrixscience.com/cgi/search_form.pl? FORMVER=2&SEARCH =PMF). Search parameters were defined with a mass accuracy of ±0.5 Da considering one missing cleavage by trypsin, carbamidomethylation of cysteine, and oxidation of methionine.

Nucleotide sequence accession numbers. The nucleotide sequence of the *omp85* homologous gene, PG0191, in *P. gingivalis* strain W83 is listed under GenBank Accession No. AE015924.

Statistical analysis. Statistical analysis was performed using the Mann–Whitney's *U*-test. *P*-values of less than 0.05 were considered to indicate statistical significance.

Results and discussion

Characterization of P. gingivalis OMP85

Using a BLAST search, a putative outer membrane protein of *P. gingivalis*, PG0191, was found as an OMP85 homolog in the whole protein database of *P. gingivalis* strain W83. The OMP85 homolog of *P. gingivalis* shares 25% and 21% identity (43% and 38% similarity) with the OMP85 of *N. meningitidis* and the YaeT (OMP85) of *E. coli*, respectively, while the deduced amino acid sequences of OMP85 among the three different strains of *P. gingivalis* were highly conserved (Supplementary Fig. S1). The molecular mass of the *P. gingivalis* OMP85 after the cleavage of the signal peptide was estimated to be 100.1 kDa whose size is relatively larger than the other OMP85 homologs. Further, a topological prediction program specific for bacterial OMP predicted that *P. gingivalis* OMP85 contains a C-terminal β-barrel transmembrane domain that consists of 16-strand β-sheets connected by short periplasmic turns and long irregular external loops in the outer membrane (Fig. 1A). Based on this information, three peptides, loop-1, -2, and -3, were designed and synthesized. The peptides were conserved among the three strains of *P. gingivalis* (Fig. S1) and corresponded to loops exposed to the outer environment (Fig. 1A). As a result, antibody against loop-3 peptide, loop-3 Ab, cross-reacted with the recombinant GST-OMP85 fusion protein (Fig. 1B) and with whole cells of *P. gingivalis* (Fig. 1C). The purity and specificity of loop-3 Ab to OMP85 was verified using Western blot with an OM extract of *P. gingivalis* (Fig. 1D). As expected, a strong band of GST-OMP85 was observed at approximate 126 kDa (Fig. 1D).

Evidence of OMP85 glycosylation

As some bacterial glycoproteins have been shown to be deglycosylated by mutation of the *galE* gene [24,25], we hypothesized that OMP85 from *P. gingivalis galE* mutant is also deglycosylated if OMP85 naturally exists in a glycosylated form. We compared the OMP85 migrations on the SDS–PAGE gel between the wild-type strain and the *galE* mutant. OM extracts from the wild-type, the *galE*, and the complementary strain, *galE*-c2, were applied to SDS–PAGE, followed by CBB staining or Western blot using the loop-3 Ab. We found the OMP85 band in the OM extract from the *galE* strain had electrophoresed more rapidly (Fig. 2, lane 2) than the wild-type strain (lane 1). The mobility of OMP85 in the *galE*-c2 (lane 3) was the same as that of the wild-type. This suggests the *galE* gene is involved in the modification of OMP85 and OMP85 is naturally modified, in all likelihood, by glycans. To confirm whether the difference in OMP85 mobility depends on the glycosylation of OMP85, chemical deglycosylation assay of the OM extract from the wild-type strain was performed using anhydrous TFMS and anisole where this deglycosylation procedure is effective in both preserving the core protein and removing glycan from the protein. After deglycosylation of the OM extract, the OMP85 band in the Western blot was shifted to a lower molecular weight (Fig. 3). This demonstrated that the native OMP85 is glycosylated.

Approximately 100-kDa bands shown with arrowheads in Fig. 1D and Fig. 3 were found in CBB-stained

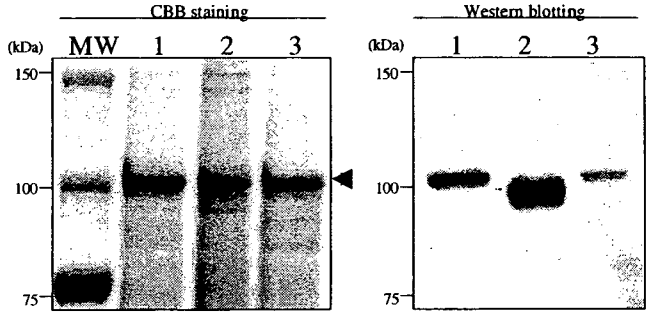

Fig 2. Western blot of OM extracts from wild-type, *galE*, and *galE*-c2 strains using OMP85 antibody. Ten micrograms of samples of OM extracts from the wild-type (lane 1), *galE* (lane 2), and *galE*-c2 (lane 3) strains were applied to SDS–PAGE; followed by CBB staining or Western blot with loop-3 Ab. MW: molecular weight marker. Approximately 100-kDa bands in CBB staining are shown by an arrowhead and identified as the RagA protein using TOF/MS analysis.

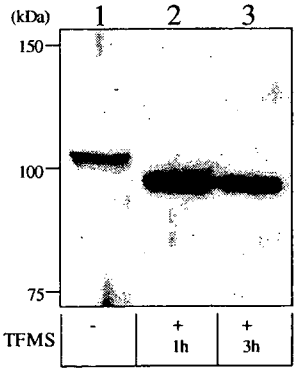

Fig. 3. Western blot of non-treated and deglycosylated OM extracts from the wild-type strain. The OM extract from the wild-type strain was treated with TFMS and anisole at 4 °C for 1 h (lane 2) or 3 h (lane 3) and were applied to SDS–PAGE followed by Western blot using loop-3 Ab. Lane 1: untreated OM extract from the wild-type strain.

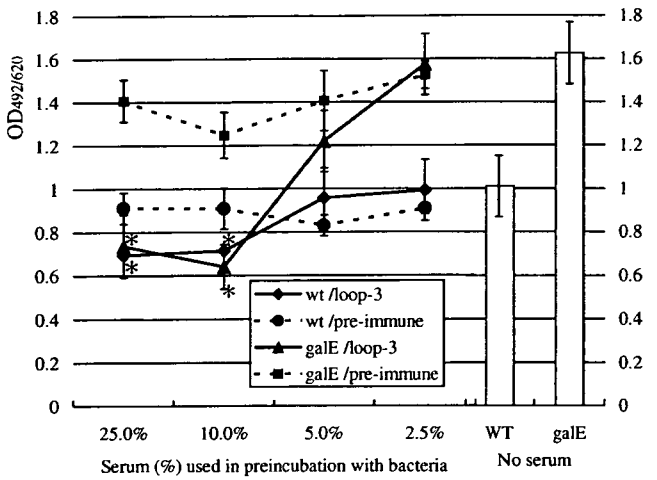

Fig. 4. Inhibitory effect of OMP85 antibody on biofilm formation using strain 33277 and its *galE* mutant. Loop-3 Ab or pre-immune serum at various concentrations (25%, 10%, 5%, or 2.5%) was pre-incubated with cells of the wild-type or *galE* strain for 1 h in an anaerobic chamber. Then the bacterial cells were washed twice and applied to the biofilm formation assay. Safranin-stained biofilms were measured by $OD_{492/620}$ absorbance. The biofilm mass of both strains in the absence of serum is shown in the right bar graph. Data shown are representative of three independent experiments. The results are expressed as the means ± SD of triplicate assays. Asterisks denote a significant decrease in biofilm formation after pre-incubation with loop-3 antiserum (versus pre-immune serum, $P < 0.05$).

gels of OM extracts from all the three strains of *P. gingivalis*. In mass spectrometry analysis a major protein in the bands was identified as not OMP85 but as RagA (PG0185) (data not shown), a well-known major OMP of *P. gingivalis* [26]. In addition, the positive band shown at the corresponding molecular mass was invisible in the glycoprotein-stained gel (data not shown). This data suggest the OMP85 of *P. gingivalis* appears to be a minor OMP consistent with previous OMP85 studies from other bacteria [10,15].

Effects of loop-3 Ab on biofilm formation

We investigated whether the loop-3 Ab has an effect on biofilm formation of *P. gingivalis* and further examined the association between OMP85 glycosylation and biofilm formation. The biofilm inhibition assay using the loop-3 Ab was performed in the wild-type strain or in the *galE* mutant whose biofilms were found to be enriched compared to those of the wild-type strain [17]. We found the loop-3 Ab inhibited biofilm formation of both strains of *P. gingivalis* after the pre-incubation, in a dose-dependent manner (Fig. 4). This indicated OMP85 is involved in biofilm formation in *P. gingivalis*. Similar results were found in *P. aeruginosa* where an Ab specific for the OMP85 homolog of *P. aeruginosa* also inhibited biofilm formation (unpublished data).

As shown in Fig. 4, the inhibitory effect of the loop-3 Ab in biofilm formation with the *galE* mutant was stronger than the wild-type strain. To determine why the biofilm formation of *galE* mutant was more susceptible to the treatment of the loop-3 Ab, we further investigated the cross-reactivities of the loop-3 Ab to both strains using triplicate ELISA assays. We found the cross-reactivity of the loop-3 Ab to the *galE* mutant was significantly stronger than the wild-type strain (0.610 ± 0.010 and 0.335 ± 0.083, respectively; expressed as means ± SD of OD_{405} nm at 30 min.). The result suggests that it is possible to uncover or improve access of the loop-3 Ab to the loop-3 region by removing glycosylation in the *galE* mutant, followed by the increased inhibition level of attachment and biofilm formation of *P. gingivalis*. Although the identification of the glycosylation site(s) and carbohydrate components in loop-3 region has to await further investigation, we found three candidates of the sites for *O*-glycosylation; Ser-752, Ser-756, and Thr-760 in the loop-3 region (Fig. S1).

Reports show glycosylation may serve to modulate the fine tuning in cell-cell recognition and signaling [9]. Although this post-translational modification system has generally been considered to be restricted to eukaryotes, there is an increased awareness that prokaryotic glycoproteins may serve this same function. At present, only a few glycoproteins have been found in Gram-negative bacteria; however in many instances, the glycoproteins are shown to be involved in bacterial attachment and colonization [27–30]. Here we provide compelling evidence to show OMP85 glycosylation and a possible involvement of glycosylation of OMP85 in biofilm formation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.11.035.

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ORIGINAL

Effects of *Enterococcus faecium* on *Streptococcus mutans* Biofilm Formation Using Flow Cell System

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Key words : *Streptococcus mutans*／*Enterococcus faecium*／*Lactobacillus casei*／biofilm／flow cell system

Abstract : *Enterococcus faecium*, a lactic acid bacterium, is a normal bowel commensal inhabitant that is rarely found in the oral cavity. We investigated whether *E. faecium* functions as a probiotic strain inhibiting biofilm formation by *Streptococcus mutans*, which is an etiological agent for dental caries, using a flow cell system. Cell suspensions were cultured in flow cell systems coated with salivary components in tryptic soy broth including 0.25% sucrose without dextrose. The resultant biofilm formation was stained using a LIVE/DEAD® *Bac*Light™ Viability Kit, and examined using confocal laser scanning microscopy. *E. faecium* showed cell density-dependent inhibition of biofilm formation in dual species culture with *S. mutans* in flow cell cultures at ratios of 100 : 1 and 10 : 1. Biofilm formation with increased numbers of voids and hollows was observed at the base of the culture using a confocal microscope. In contrast, increasing the ratio of *L. casei* or *L. salivarius*, other lactic acid bacteria, to *S. mutans* did not affect biofilm formation. In addition, a sonic extract sample of *E. faecium* was sub-purified by salting out and gel filtration, and its inhibitory effects on *S. mutans* biofilm were similarly observed in the same assays. Together, our results suggest that *E. faecium* possesses an inhibitory substance and functions as a probiotic bacterial inhibitor of streptococcal biofilm formation. Further, more they provide important information regarding bacterial communication and diversity, as well as for potential therapies and materials for the prevention of biofilm development in the oral cavity.

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Introduction

Oral lactic acid bacteria have been reported to occur at high numbers in both superficial and deep caries[1], and are known to play secondary or opportunistic roles in caries development by producing lactic acid and extracellular polysaccharides[2]. Recently, lactic acid bacteria have been utilized as probiotics in dietary supplementation and medicine, as they are known to be useful for the enhancement of immunological activities[3] and adjustment of serum cholesterol[4], as well as for their anti-allergy effects[5]. Their potential mechanisms include the formation of an enhanced barrier to the translocation of bacteria and bacterial products across the mucosa[6,7], and the competitive exclusion of potential pathogens[8], along with modification of host responses to microbial products[9,10] that inhibit the growth of pathogens[11,12], such as *Klebsiella pneumoniae*[13], *Escherichia coli*[14], and *Candida albicans*[12]. In the field of dentistry, it was reported that a water-soluble extract of *Lactobacillus fermentum* completely inhibited the growth of *S. mutans*[15], while another report showed that the clinical strain S11 of *L. fermentum* and its culture supernatant significantly inhibited insoluble glucan formation by *S. mutans*[16]. However, it is not clear whether lactic acid bacteria possess activities to regulate oral biofilm formation.

Streptococcus mutans, the predominant etiologic agent of human dental caries[17] has been shown to be able to adhere to and form a biofilm on tooth surfaces, catabolize carbohydrates and generate acids, and survive at a low pH and under other environmental stress conditions, which are characteristics involved in its cariogenicity[2]. *S. mutans* interacts with other organisms including gram-positive streptococci and bacteria such as *Actinomyces, Neisseria*, and *Vellonela* in biofilm development[2,18,19]. Therefore, it is likely that cooperative and competitive interactions between *S. mutans* and other organisms play important roles in the development of dental biofilm and caries in the oral cavity[20].

Enterococci are gram-positive cocci that form a part of the normal gastrointestinal tract flora in animals and humans[21], function as lactic acid bacteria, and are generally considered to be normal bowel commensals, though they are also recognized as opportunistic pathogens[22]. Enterococci have long been implicated in persistent root canal and dentin infections[23–25], as well as endocarditis and urinary tract infections[24,26]. In addition, enterococci occur in natural foods and are used as probiotics in dairy products[27]. The genus *Enterococcus* consists of at least 23 species, 2 of which, *Enterococcus faecalis* and *E. faecium*, account for greater than 95% of the clinically important isolates.

Lactic acid bacteria such as *E. faecalis* and *E. faecium* are rarely found on oral surfaces. However, they are able to survive under conditions of root and dentin caries. Therefore, they may be associated with the development of microbiological communities in difficult-to-access areas of the oral cavity, and have unique effects on oral biofilm formation with streptococci. In the present study, we observed biofilm formation of *S. mutans* in mixed cultures with lactic acid bacteria and non-biofilm bacteria such as *E. faecium*, *Lactobacillus casei*, and *L. salivarius in vitro* using a flow cell system[28]. Our results further clarify the role of lactic acid bacteria in oral biofilm formation and provide useful information for the development of preventive medicines for oral diseases.

Materials and Methods

1. Bacterial strains and culture conditions

S. mutans MT8148, *E. faecium* 129 BIO 3B (provided by Biofermin Phamaceutical Co., Kobe, Japan), *L. casei* ATCC 393, and *L. salivarius* JCM1231 were used in this study. A strain of *E. faecium*, 129 BIO 3B (classified previously as *Streptococcus faecalis* 129 BIO 3B), reportedly does not produce various toxins, such as bacteriocin and hemolysin, and was proposed as a beneficial probiotic strain for intestinal flora conditions[29]. *S. mutans* and *E. faecium* were grown in Brain Heart Infusion medium (BHI ; Difco Laboratory, Detroit, MI, USA), while *L. casei* and *L. salivarius* were grown in Lactobacilli MRS medium (Difco).