T lymphocyte response to periodontal complex bacterial biofilm

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I. Introduction

Bacterial biofilm is covered by protective surface glycoproteins with convection, metabolic, oxygen, and nutrient flow circulating that renders resistance to antimicrobial agents or phagocytes[1]. Periodontal disease is primarily an inflammatory disease initiated by infection of mixed bacterial co-aggregates composed of complex biofilm mass[2,3]. Among complex oral microbial biofilms, *P. gingivalis* is a major pathogenic organism that causes destructive periodontal diseases[4]. However, majority of microbiological and immunological studies on this organism have been made on the bacteria particularly grown as a planktonic culture[4-6]. Only a few approaches have been performed on *P. gingivalis* biofilm with respect to growth characteristics and antimicrobial resistance[3,7]. For more comprehensive understanding of virulence mechanisms and survival strategies that *P. gingivalis* biofilm utilizes, tools for bacterial genomics, proteomics and immunology may have to be constitutively practiced. While scarcity for identifying differentially expressed gene(s) or protein(s) in *P. gingivalis* biofilm may warranty the focused research in the area, elucidating the mechanisms by which *P. gingivalis* biofilm evade and perturb the host response could also be of potential value in understanding antimicrobial resistance and consequently designing the effective chemotherapeutic agents. Characterizing the host immune responses to *P. gingivalis* biofilm grown not only as single organism but also as mixed biofilm will also provide valuable information in understanding unique behavioral mechanisms of *P. gingivalis* biofilm in subgingival complex microbial biofilms. *Fusobacterium nucleatum* is one of the key pathogens in periodontal disease[8,9], playing a central role in intermicrobial coaggregation in subgingival area[2,8,10] and formation of mixed biofilm[3,7]. The organism also works synergistic with *P. gingivalis* in virulence[11] and immunologically modulates *Actinobacillus actinomycetemcomitans*[12]. Though the exact mechanism has not been clearly understood, we have recently demonstrated that *F. nucleatum* modulated anti-*P. gingivalis* anti-

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body function and cellular[13] or humoral immune responses[14] to *P. gingivalis*, when mice were immunized *F. nucleatum* followed by *P. gingivalis* grown as a planktonic culture.

Host responses to oral microbial biofilms have not been extensively studied. Using *P. gingivalis* biofilm (single or mixed) as a model, we are interested in seeing how outer membranes are differentially expressed/repressed in microbial biofilms. It is also critical to evaluate the pattern how these proteins are recognized by human sear or immune animal sera. We are also interested in evaluating how T- and B-cells are responding when biofilm-grown bacterial cells are immunized in animal. We will perform *in vitro* evaluation of immune serum function and *in vivo* protection assay.

The overall aim of this application is to evaluate how the cellular and humoral immune response is perturbed by *Porphyromonas gingivalis* biofilm that was immunized in animal.

II. Materials and Methods

1. Growth condition of P. gingivalis biofilm

Growth of *P. gingivalis* biofilm- pure or mixed. *P. gingivalis* 381 and *F. nucleatum* 10953 will be grown respectively in Tryptic soy broth (Difco, MI) supplemented with 5 micrograms/ml hemin and 0.5 micrograms/ml Vitamin K in anaerobic chamber (COY, MI). Overnight-grown bacteria will be inoculated onto three different kinds of solid tissue culture plates (cell culture dish, 6-well cell culture plate, membrane filter) coated with either polystyrene or mixed cellulose esters, respectively. Two different kinds of biofilms will be induced; *P. gingivalis* alone (pure) or mixed *P. gingivalis* plus *Fusobacterium nucleatum*. Outer membrane proteins will be isolated from each pure *P. gingivalis* biofilm grown on different plates, and subject to SDS-PAGE for a comparison of electrophoretic profiles. If the pattern is similar between different plates, we will select 6-well tissue culture plate for inducing biofilms. *P. gingivalis* or *F. nucleatum* grown respectively as planktonic culture will be used as the controls for immunization of animals.

2. Immunization of mice with P. gingivalis biofilm

Immunization of mice with *P. gingivalis* biofilm-pure or mixed. 10 BALB/c mice per each group were immunized three times respectively with 1 x 10^7 cells of 1) planktonic-cultured *P. gingivalis* 381 (*Pg*-planktonic group), 2) biofilm-cultured *P. gingivalis* 381 (pure *Pg*-biofilm group), 3) biofilm-cultured *P. gingivalis* 381+*F. nucleatum* 10953 (mixed *Pg*+*Fn*-biofilm group), and 4) planktonic-cultured *F. nucleatum* 10953 (*Fn*-planktonic group). The bacteria will be washed and resuspended in pre-reduced half-strength Ringer solution according to the method previously reported[12]. Mice will be immunized by three subcutaneous inoculations of bacteria at 2-week interval. 10 BALB/c mice sham-immunized with PBS served as the negative control group.

3. Measurement of anti-P. gingivalis or anti-F. nucleatum antibody titer

Peripheral blood will be collected by cardiac puncture to obtain immune sera. To determine anti-*P. gingivalis* IgG antibody titers in all groups and anti-*F. nucleatum* IgG titer in groups 3) and 4) by ELISA, microtiter plates will be coated respectively with aliquots of whole bacterial cells of *P. gingivalis* or *F. nucleatum* and incubated for overnight[13,14]. After washing plates, mouse serum samples serially diluted will be added and incubated. The plates will

be washed followed by adding peroxidase-conjugated rabbit anti-mouse IgG. After washing the plates, aliquots of tetramethylbenzidine (Kierkegaard and Perry Laboratories, Gaithersburg, MD) will be added followed by addition of 0.18 M H_2SO_4 to stop the reaction. Optical densities will be plotted as a function of serum dilution factor to calculate the titer. For the comparison of antibody levels between groups, a Student's *t*-test will be performed. Similarly, anti-*P. gingivalis* IgG subclass antibody titer will also be determined in groups 1-3.

4. Establishment of P. gingivalis-specific T cell lines

7 days after the final immunization, mouse splenic T cells in all groups will purified using mouse T cell enrichment columns according to the our method described previously[12]. Purified T cells will then be incubated with 1 x 10^8 *P. gingivalis* cells. Spleen cells treated with mitomycin C will be added as the antigen presenting cells. After 2 weeks of incubation, T cells were washed and allowed to rest without adding APC's or bacteria for 1 week. After the resting period, APC's and bacteria will be added to induce T cell proliferation according to the method described previously[13,14].

5. Phenotype characterization of T cell clones

T cells from T cell lines established from each group will be stained with FITC-conjugated rabbit anti-mouse CD4 or CD8 monoclonal antibody (PharMingen, San Diego, CA). Phenotypic expression of each T cell clone will be screened by flow-assisted cytometric screening using Coulter Epics Elite XL Flow Cytometry (Coulter, Hialeah, FL).

6. Determination of cytokine concentrations.

After 72 hours incubation of T cells (5 x 10^6 cells/well) from each clone with mitomycin C-treated APCs (1 x 10^7 cells/well) and 1 x 10^8 cells of either *P. gingivalis* or *F. nucleatum*, culture supernatants will be harvested for determination of cytokine levels by sandwich ELISA according to the method reported previously[13]. Briefly, 96-well plates (Corning, Coming, WY) will coated respectively with rat anti-mouse INF-gamma, IL-4, IL-6, IL-10, or IL-12. After washing the plates, each supernatant sample and standard recombinant mouse INF-gamma, IL-4, IL-6, IL-10, IL-12 will be added, respectively, and incubated. The plates will be washed and biotinylated rat anti-mouse INF-gamma, IL-4, IL-6, IL-10, IL-12 will be added respectively. After incubation for 1 hour, plates will be washed. Hydroperoxidase-conjugated streptavidin will be added and then will be washed followed by adding of o-phenylenediamine and incubation. To stop the color reaction, 4 N H_2SO_4 will be added and optical densities will be read at wavelength of 490 nm. Optical densities of standard cytokines will be plotted against the dilution factors and cytokine concentration of each sample was determined.

Table 1. Anti-*P. gingivalis* IgG titer of immune sera obtained from each group (mean±s.d.).

	IgG titer
Pg-planktonic group	400.3±83.6
pure *Pg*-biofilm group	403.2±91.4
mixed *Pg*+*Fn* biofilm group	414.5±38.9
Fn-planktonic group	409.3±13.0
Control group	100.4±8.9

Table 2. Cytokine concentrations (ng/ml) of culture supernatants from *P. gingivalis*-specific T cell lines established from each group (mean±s.d.)

	INF-gamma	IL-4	IL-10
Pg-planktonic group	0.29±0.03	-	-
pure *Pg*-biofilm group	0.11±0.04	-	-
mixed *Pg*+*Fn* biofilm group	0.12±0.12	-	-
Fn-planktonic group	0.11±0.10	-	-

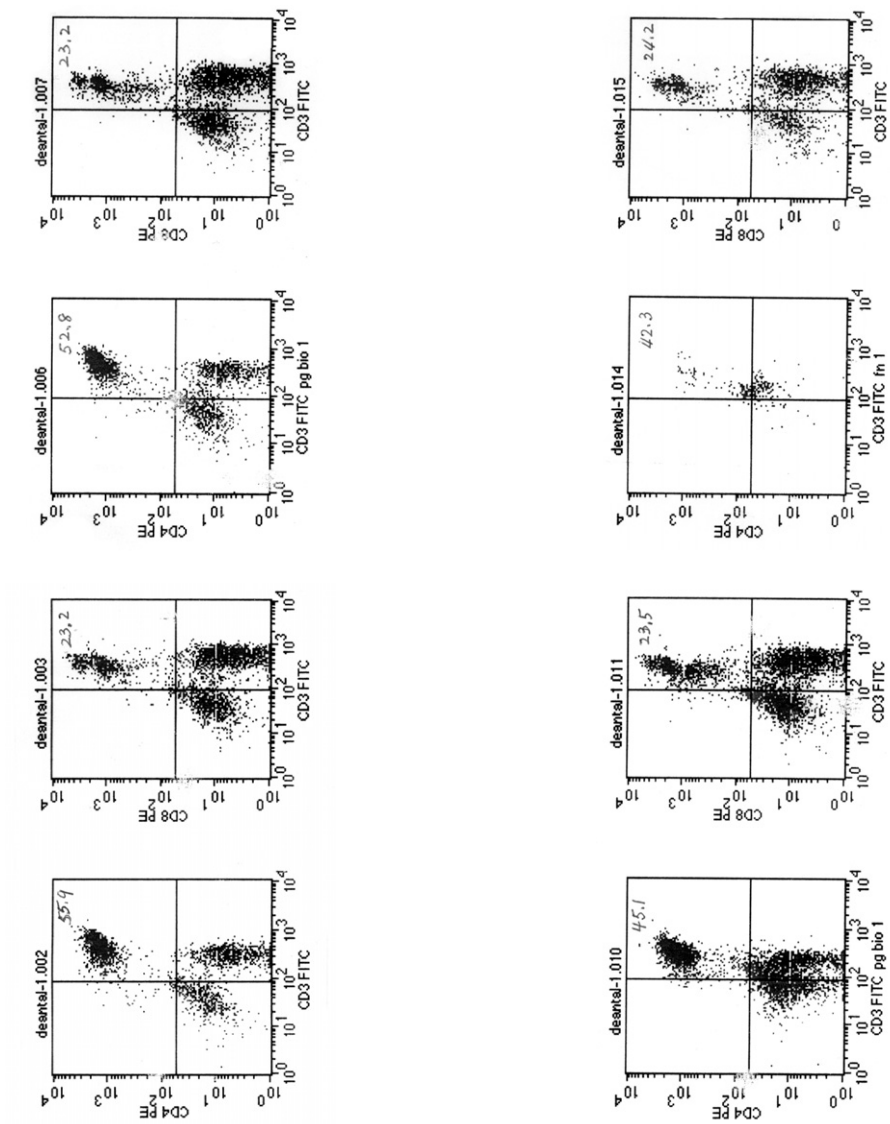

Figure 1. Flow cytometry profile of CD3+/CD4+ or CD3+/CD8+ T cells from representative T cell line established from each group, consisting of planktonic-cultured *P. gingivalis* (upper left), pure biofilm-cultured *P. gingivalis* biofilm (upper right), mixed *P. gingivalis* + *F. nucleatum* biofilm (lower left), and planktonic-cultured *F. nucleatum (lower right)*.

III. Results

1. Serum anti-P. gingivalis IgG titers

Anti-*P. gingivalis* IgG titers of each group were similar in magnitude (Table 1) ranging from 400.3 to 414.5. Mean IgG titers were all higher than that of control group.

2. Establishment of P. gingivalis-specific T cell lines

Splenic T cells were also isolated from groups 1)-3), respectively, and stimulated with *P. gingivalis* to establish *P. gingivalis*-specific T cell lines. Cytokine profiles and phehotype of T cells in established *P. gingivalis*-specific T cell lines were also examined. INF-gamma levels produced by T cell lines were also significantly lower in T cell lines established from biofilm groups (Table 2).

3. T cell phenotype profiles

All the T cell lines established from each group demonstrated a mixture of CD3+/CD4+ and CD3+/CD8+ T cells Figure 1). CD4+ cells ranged from 42.3%-55.9% and CD8+ cells ranged from 23.2%-24.2%, respectively.

IV. Discussion

All the immne mice had a elevated anti-*P. gingivalis* IgG titers compared with nonimmunized control. It wa sinteresting to see the similar IgG titer in mice group immunized with planktonic-cultured *F. nucleatum*. This phenomenon was observed in our previous study[13] indicating antigenic cross-reactivities between *P. gingivalis* and *F. nucleatum*. The biofilm-cultured *P. gingivalis*, pure or mixed, eradicated T cell cytokine, IFN-gamma, thus leading to the deviating of helper T cell polarization into Th2 subset. The implication of this unique polarization in protective capacity of host remains to be clarified further after T cell adoptive transfer. it is presumed that a different polarization of helper T cell by *P. gingivalis* may be one of their strategy to survive against host defense mechanisms and perturb host responses.

We will forward into the following new areas; 1) *In vivo* protection assay after adoptive transfer of T cell clones into SCID mice, *in vitro* testing of immune serum function as well as biofilm penetrating power of immune sera observed by confocal laser scanning microscope, and identification of differentially expressed/recognized outer membrane proteins of *P. gingivalis* biofilm by larger numbers of immune animal sera and patients sera.

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-국문초록-

치주복합세균성 바이오필름에 대한 T 임파구 반응

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본 연구는 *P. gingivalis* biofilm을 쥐에 면역했을 경우 숙주의 면역체계중에서 T 임파구가 어떻게 반응하는가를 비교연구한 것으로 *P. gingivalis* biofilm은 단일 세균으로 구성된 것과, *F. nucleatum*과 복합된 것 공히 T 임파구가 유리하는 cytokine 중에서 IFN-gamma의 현저한 감소를 초래하는 것으로 판명되어, 향후 숙주 면역 방어체계에 미치는 영향을 연구하는 중요한 지침자료를 제공해 주었다.

주요어 : biofilm, T lymphocyte, Porphyromonas gingivalis (바이오필름, T 임파구, Porphyromonas gingivalis)