Fusobacterium nucleatum modulates serum binding to Porphyromonas gingivalis biofilm

Jeomil Choi, Soo-Jin Kim, Sung-Jo Kim

Department of Periodontology, School of Dentistry, Pusan National University, Pusan, Korea

I. ABSTRACT

Anti-P. gingivalis immune sera were obtained from mice immunized with either *P. gingivalis* alone , or *F. nucleatum* followed by *P. gingivalis*. Two groups of immune sera were examined for binding capacity to *P. gingivalis* biofilm by confocal laser scanning microscope. Antibody avidity index was also determined for each immune sera. The results indicated that prior immunization of mice with *F. nucleatum* impaired *P. gingivalis*-specific immune sera in binding capacity to biofilm and antibody avidity to *P. gingivalis*.

Elevated antibody responses in patients with destructive periodontal disease has often been related to suboptimal level of protective antibody (opsonophagocytosis)[1-3], while post-immune sera obtained with experimental animals using a single periodontal pathogen demonstrated satisfactory levels of protective function against the homologous bacterial challenge[4, 5].The reason is unclear why elevated IgG responses in periodontal patients to periodontal pathogens do not necessarily reflect their protective function. Such an immune deviation might be derived from the fact that destructive periodontal disease is a cumulative result of immunopathologic processes responding to an array of different colonizing microorganisms sequentially infecting in the subgingival environmental niche.

Fusobacterium nucleatum is one of the key pathogens in gingivitis, in the transitional phase of conversion of gingivitis into destructive periodontitis, and in adult periodontitis[6-8]. It also plays a central role in coaggregation with other important microbial species in subgingival area[6, 9, 10] as well as in biofilm[11], especially with *Porphyromonas gingivalis* in synergism of virulence in human periodontal disease or in animal models[12-14]. This organism has also been reported to have immune modulating activity for secondary immune response to *Actinobacillus actinomycetemcomitans*[15]. It is presumed that sequential colonization and intermicrobial coaggregation between intermediate and late colonizers could potentially modulate the immune responses

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교신 저자 : 최점일, 부산시 서구 아미동 1-10 부산대학교치과대학 치주과학교실, 우편번호 : 602-739

and development of specific T cell phenotypes in periodontal lesions. We have recently demonstrated the skewed polarization of *P. gingivalis*-specific helper T cell clones in mice immunized with *F. nucleatum* followed by *P. gingivalis* [16].

Consequently, *F. nucleatum* may initially prime the immune cells and modify their responses to the successive organism, *P. gingivalis*. This could explain why one frequently observes non-protective serum antibodies to *P. gingivalis* in periodontal patients in contrast with those obtained from animals that were immunized with *P. gingivalis* alone [17].

The present study was performed to investigate the immune modulating effect of *F. nucleatum* on serum binding to experimental biofilms and the avidity of anti-*P. gingivalis* antibody.

II. Materials and Methods

1. Immunization of mouse with bacterial cells

F. nucleatum ATCC 10953 (American Type Culture Collection, Manassas, VA) and *P. gingivalis* 381 (kindly provided by Dr. Schifferle, SUNY at Buffalo, Buffalo, NY), grown and maintained anaerobically, were harvested and used for immunization of mice according to the methods described previously[16]. Briefly, bacterial cells were harvested, washed in phosphate-buffered saline and finally resuspended in pre-reduced half-strength Ringer solution to maintain viability. 10 BALB/c mice were immunized twice with of live *P. gingivalis* alone (5×10^8 cells) at 2-week intervals (Group 1). 10 BALB/c mice were immunized twice with live *F. nucleatum* (5×10^8 cells) at 2-week intervals via intraperitoneal injection followed by two subsequent injections of live *P. gingivalis* (5×10^8 cells) at 2-week intervals (Group 2). Mouse serum samples were collected by tail bleeding prior to the first immunization and 1 week after the final immunization, and stored at -20℃ until used.

2. Measurement of anti-P. gingivalis IgG titer

Mouse serum IgG titers were determined by a slight modification of the method previously reported[16-18]. Microtiter plates were coated in triplicate with 100 μl of whole cell antigens (10 μg/ml) of *P. gingivalis* diluted in phosphate buffer. After overnight incubation at 4 ℃, the plates were washed three times with PBS containing 0.05% Tween 20 (PBS/Tween). A total of 50 μl of mouse serum samples serially diluted in PBS/Tween was added to each well and incubated for 2 hours at room temperature. The plates were washed three times with PBS/Tween, and then 100 μl of peroxidase-conjugated rabbit anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA). After being incubated for 2 hours at room temperature, the plates were washed three times with PBS/Tween. 100 μl of tetramethylbenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, MD) were added to each well and incubated for 15 minutes at room temperature followed by adding 100 μl of 0.18 M H_2SO_4 to stop the reaction. Optical densities read at 450 nm of wavelength were plotted as a function of serum dilution factor, and regression analysis was performed. One of the pre-immune control sera was assigned an ELISA unit of 100 and serum IgG and IgG subclass titers of other control and test sera were calculated. For a statistical comparison of antibody levels between groups, a paired Student's *t*-test was performed.

3. Measurement of antibody binding on the biofilm

Porphyromonas gingivalis were cultured as biofilms on the slide glasses for 24 hours in anaerobic chamber by inoculating the media on the glass

slides placed in tilted position. The slide glasses were gently washed by phosphate-buffered saline to remove nonadherent layer. The biofilm was fixed by 4 % paraformaldehyde for 30min at room temperature and washed three times by phosphate buffer (40 mM, pH 7.0). Then biofilms were incubated with two different sources (sera from Groups 1 or 2, respectively) of mouse anti-sera against whole cells of *P. gingivalis*.(1:100), respectively, washed again by the same buffer, and then incubated in FITC-conjugated goat affinity-purified antibody to mouse IgG (1:100) overnight at 4℃. After washing three times, the microorganism in the artificial biofilms were stained with propidium iodide (PI, 5 micrograms/ml) for 30min. Fields of equal PI fluorescence intensity were selected for matched comparison of FITC intensity between Group 1 and Group 2. For a qualitative comparison of red (PI), blue (FITC) and yellow color (indicating overlapping areas of PI and FITC colors), fields were randomly selected for sequential monitoring of fluorescence at variable penetration depth levels. The fluorescence intensity of FITC and propidium iodide was determined by the confocal laser scanning microscope.

4. Avidity of immune sera

Relative antibody avidity was determined by the method described previously[19]. Polystyrene microtiter plates (Immulon II, Dynatech) were coated with bacteria followed by replicate dilutions of mouse sera (1:100 in PBS) applied. The serum was incubated for 1 hour at 37°C and washed with PBS/Tween. The plates were incubated with 100 microliters of increasing concentrations of NH_4SCN (0-5 M diluted in sterile H_2O) for 30 minutes at 37°C on a rotator. The application of NH_4SCN was repeated once in the same manner. The plates were washed and the bound antibody was detected by adding 100 microliters of HRP-conjugated rabbit anti-mouse IgG (1:3,000, Zymed, San Francisco, CA). The color reaction was developed by adding tetramethlybenzidine (1 mg/ml, Kierkegaard & Perry Laboratories, Gaithersburg, MD) for 20 minutes at room temperature. Optical densities were read at 45 nm. An avidity index was calculated as the molar concentration of NH_4SCN required to elute 50% of the bound antibody[20]. For a statistical comparison of antibody levels between groups, a paired Student's *t*-test was performed.

III. Results

Post-immune IgG titer against *P. gingivalis* in Groups 1 and 2 were 2532.5 ± 138.1 and 2488.2 ± 292.4, respectively (Table 1). All the post-immune IgG titers were significantly higher than pre-immune level ($p<0.01$), however there was no significant difference between the two groups ($p>0.05$).

At the preliminary experiments, PI intensities measured at variable biofilm depth were consistently comparable between the different slides that

Table 1. Pre-immune and post-immune serum IgG titer against *P. gingivalis* 381 (ELISA units ± s.d.)

	IgG titer to *P. gingivalis* 381	
	pre-immune	post-immune
Group 1 (N=10)	101.9 ± 18.2	2532.5 ± 138.1*
Group 2 (N=10)	105.3 ± 10.0	2488.2 ± 292.4*

* significant higher than pre-immune serum IgG titer ($p < 0.01$) or control group, and no statistical difference between Groups 2 and 3 ($p > 0.05$) by Student's t-test

Table 2. Mean fluorescence intensity of FITC was determined by the confocal laser scanning microscope (±s.d.)

	Group 1	Group 2	p value
Mean fluorescence intensity (± s.d.)	38355.8 ± 22806.6	12960.2 ± 12330.9	< 0.01

Table 3. Antibody avidity index to *P. gingivalis* of antisera obtained from Groups 1 and 2 (± s.d.)

	Group 1 (N=10)	Group 2 (N=10)	p value
Avidity index (± s.d.)	5.62 ± 1.14	4.68 ± 0.79	< 0.05

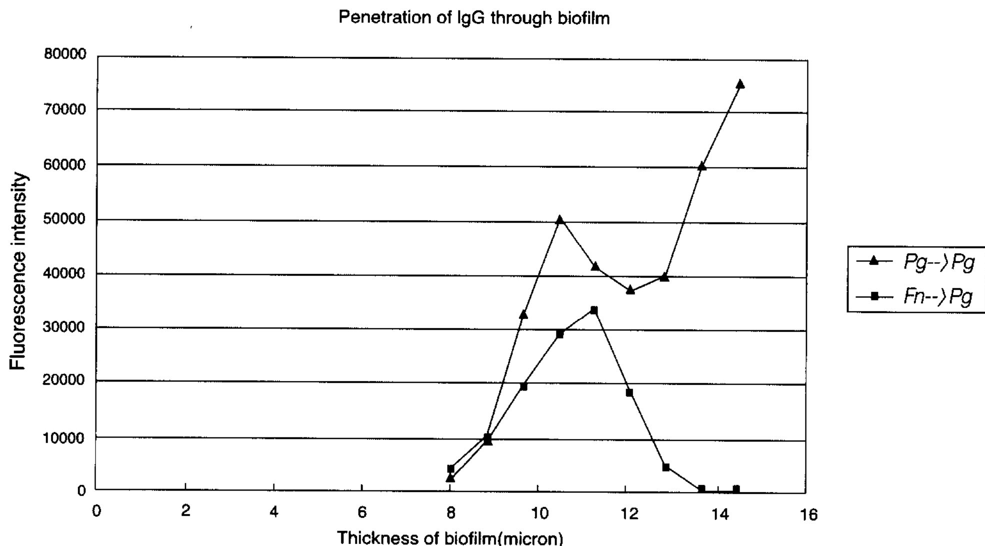

Figure 1. FITC intensity measured by confocal scanning laser microscope at variable depth levels of *P. gingivalis* biofilm. The intensity is consistently higher in Group 1 (*Pg-> Pg*) than in Group 2 (*Fn-> Pg*).

enabled the matched comparison between the experimental groups. Mean fluorescence intensity obtained from 9 different depth levels of biofilm mass was 38355.8 ± 22806.6 in Group 1 and 12960.2 ± 12330.9 in Group 2 (Figure 1). The mean FITC intensity of Group 1 was significantly higher than Group 2 ($p<0.01$, Table 2). For a sequential qualitative comparison, one field from each group was randomly selected and fluorescence color photographs were taken at variable depths of biofilm mass (Figure 2). The FITC intensity was very high in Group 1 when compared to Group 2, and areas stained with yellow color indicating the overlapping structure of PI (bacteria) and FITC (sera) were apparently wider in Group 1.

Relative avidity indices of sera from Groups 1 and 2 were 5.62±1.14 and 4.68±0.79, respectively (Table 3). The avidity index of sera from Group 1 was significantly higher than that of Group 2 ($p<0.05$).

IV. Discussion

The fact that an elevated level of antibacterial antibody does not necessarily reflect the enhanced

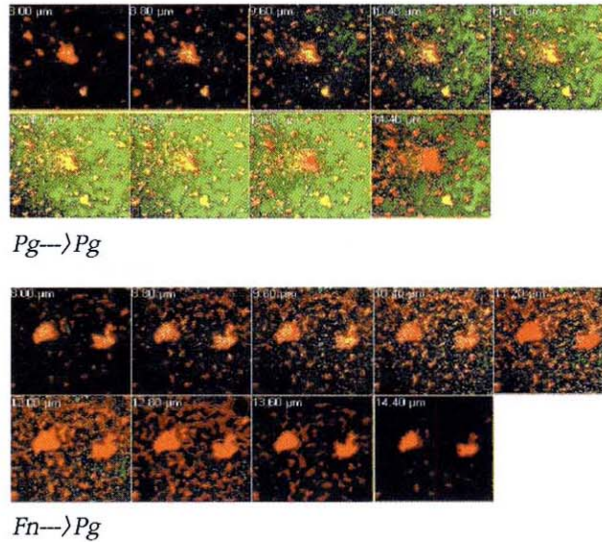

Figure 2. Color photomicrograph of PI and FITC fluorescence observed by the confocal laser scanning microscope indicating the serum (FITC-labeled) binding to bacteria (PI-labeled) at variable depths of biofilm on the slide glass (Upper panel: Group 1 (*Pg*-〉 *Pg*), Lower panel: Group 2 (*Fn*-〉 *Pg*). Yellow color indicates the overlapping zone of the red (PI-stained) and the green fluorescence (FITC-stained). Intensity of green fluorescence and yellow color is apparently higher in Group 1.

protective function against the periodontopathic bacteria and subsequent disease progression. One of the explanation may be that the heavy or light chain repertoire of human immunoglobulin structure following repeated vaccinations[21]. Other possibility may exist in that IgG2 subclass level is extremely high that demonstrate the suboptimal level of phagocytic killing. In the early-onset periodontitis, the production of IgG2 was under the immunogenetic control[22] and antibody molecule demonstrated low avidity against *P. gingivalis*. In the present study, the unsatisfactory level of antibody binding to bacterial biofilm and avidity in Group 2 may be explained by the fact that the immune anti-*P. gingivalis* sera was obtained from mice which has been pre-immunized by a second bacteria. In our previous reports[16, 17], we have addressed that the T cell polarization was skewed and the opsonophagocytosis level was impaired when the mice were sequentially immunized with *F. nucleatum* followed by *P. gingivalis*. The IgG2 class was significantly higher in the group than the mouse group that were immunized *P. gingivalis* alone.

In periodontitis patients, the elevated anti-*P. gingivalis* antibody level may reflect the similar problem that the antibody is also elevated to multiple bacteria which had previously been introduced into subgingival niche during gingivitis phase or early phase of destructive periodontitis.

Recently, Zhu, et al.[23] have shown that anti-*S. mutans* antibody can equally bind to biofilms formed either in the presence or absence of polysaccharide. The anti- *Streptococcus mutans* antibody used in the study was one which was obtained from mouse immunized with *Streptococcus mutans* alone. Our study also indicated that sera from mouse immunized *P. gingivalis* alone demonstrated

higher level of binding to biofilm. The problem lies in that, in periodontitis patients, the quality of anti-*P. gingivalis* antibody is not of such one that is comparable to antibody molecule obtained from mono-immunized animals, because of its multiplicity nature. This concept is very important in understanding why elevated antibody levels in the advanced periodontitis patients.

V. References

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-국문초록-

Porphyromonas gingivalis biofilm에 대한 면역혈청의 침투력에 대한 Fusobacterium nucleatum의 조절효과

최점일 · 김수진 · 김성조

부산대학교 치과대학 치주과학교실

P. gingivalis를 단독면역하거나 또는 Fusobacterium nucleatum 선면역 후 P. gingivalis를 면역하여 P. gingivalis 항혈청을 각각 얻어냈다. 두 종류의 항혈청이 P. gingivalis biofilm을 침투해 들어가는 능력을 confocal laser scanning microscope를 이용하여 비교 감증하였다. 항혈청의 P. gingivalis에 대한 avidity index도 측정하였다. 결과적으로 F. nucleatum 의 선면역은 P. gingivalis-특이 항혈청에 대해 세균성 biofilm의 침투능력을 저하시키고, 동일한 세균에 대한 avidity도 감소시켰다.

주요어 : *Porphyromonas gingivalis*, , *Fusobacterium nucleatum*, biofilm, serum binding, avidity