Original Article

EFFECTS OF A MIXED INFECTION WITH *Porphyromonas gingivalis* AND *Treponema denticola* ON ABSCESS FORMATION AND IMMUNE RESPONSES IN MICE

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Abstract

Porphyromonas gingivalis and *Treponema denticola* have been found together in lesions of human periodontitis. We examined the ability of a mixed infection by both bacteria to synergistically form abscesses and disturb immune responses in mice. Absorbance of an invasive *P. gingivalis* 16-1 strain grown in tryptic soy broth and *T. denticola* ATCC 33520 strain grown in TYGVS medium were adjusted. BALB/c mice were injected with 200 μ l of the cell suspension at a site on the lateral dorsal area. The sizes of the subsequent subcutaneous abscesses were measured with a caliper gauge, and the area was expressed in square mm. Mixed infections by *P. gingivalis* and *T. denticola* produced larger abscesses than those formed after mono-infections by either *P. gingivalis* or *T. denticola*. The abscesses caused by mixed infection reached their maxima on the 6th day and maintained that size for the subsequent 5 days. The delayed type hypersensitivities against extracted antigens of *P. gingivalis* in mixed infection mice were significantly lower than those in the mono-infected mice. However, the IgG response to sonicated antigen of *P. gingivalis* did not differ between the two groups. The sizes of the abscesses caused by mixed infections in mice immunized with whole cells of *P. gingivalis* 16-1 were compared to those caused in sham-immunized mice. The average size of the abscess caused by mixed infection in immunized mice did not differ from that in sham-immunized mice, but many of the abscesses in immunized mice ruptured on the 4th or 5th day, followed by recovery in two weeks. These results suggest that mixed infection with *P. gingivalis* and *T. denticola* attenuates protective immune responses.

Key words: *Porphyromonas gingivalis* — *Treponema denticola* — Mixed infection

INTRODUCTION

Subgingival plaque contains various kinds

of bacterial species, mainly anaerobic rods and spirochetes^{16-18,22,23}. Many research groups have shown that the microbial complexity of

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subgingival plaque can contribute to the pathogenesis and be responsible for human periodontal disease^{27,28,30)}. It has been repeatedly reported that the combination of *Porphyromonas gingivalis* and *Treponema denticola* is closely associated with adult periodontitis^{8,13,15,24,29)}. We have also reported that the coaggregation between *P. gingivalis* and *T. denticola* *in vitro* might correlate with their synergistic virulent factors^{15,21)}.

A cysteine protease, gingipain, of *P. gingivalis* possesses various pathogenic factors such as adherence activity, degradation of host tissues and immunoglobulins, and suppression of leukocyte functions^{4,31)}. Another protease, dentilisin, of *T. denticola* also has various virulence factors such as degradation of host tissues and alteration of host defense mechanisms^{9,11)}. In addition, both microorganisms are known to produce endotoxin and endotoxin-like substances^{11,12)}. In patients with periodontitis, complicated immune responses against these microorganisms have been also reported^{9,12,25)}. Humoral and cellular immune responses have been implicated to play critical roles in the immunopathogenesis of periodontal disease^{5,6)}. In the process, mixed infections in periodontal lesions may influence the onset and progression of human periodontal disease.

The present study was performed to examine abscess formation and immune responses caused by mixed infections of *P. gingivalis* and *T. denticola* in mice.

MATERIALS AND METHODS

1. Bacterial strains and growth conditions

An invasive strain of *P. gingivalis* 16-1 and *T. denticola* ATCC 33520 were used in this study. Strains of *P. gingivalis* were maintained on tryptic soy agar (Becton Dickinson, Cockeysville, MD) supplemented with hemin (5 µg/ml), menadione (0.5 µg/ml) and 10% defibrinated horse blood. *T. denticola* strain was maintained on TYGVS medium as reported by Ohta *et al.*¹⁹⁾ Cells grown in tryptic soy broth medium (Becton Dickinson) supplemented

with hemin and menadione and TYGVS in an anaerobic chamber containing with 80% N₂, 10% CO₂, and 10% H₂ at 37°C were harvested and washed once with phosphate buffered saline (PBS, pH7.4).

2. Experimental mice

Animal experiments were conducted according to the guidelines for the treatment of experimental animals at Tokyo Dental College. A total of 70 BALB/c male mice, 6 to 7 weeks old, were used. In an experimental protocol, 30 mice were divided into 3 groups, 10 mice were injected with *P. gingivalis* alone, 10 mice were injected with *T. denticola*, alone and the last 10 mice were injected with a mixture of *P. gingivalis* and *T. denticola*. The rest of the mice were used to examine the effects of immunization with whole cells of *P. gingivalis* 16-1 on the mono-infection by *P. gingivalis* and the mixed infection by *P. gingivalis* with *T. denticola* ATCC 33520.

3. Immunization with whole cells of *P. gingivalis* 16-1

The cultured cells of *P. gingivalis* 16-1 that had been killed with 0.5% formalin for 24 hours and washed twice with PBS were used for immunization. Harvested cells were suspended in PBS at a concentration of 40 mg wet weight cells per 1 ml. Ten mice were immunized with whole cells of *P. gingivalis* 16-1, and another 10 mice were sham-immunized with PBS.

Ten mice were immunized with 100 µl of prepared *P. gingivalis* antigen into both bilateral inguina. One week after the first immunization, a booster immunization with 100 µl of the antigen was administered to the same sites.

4. Inoculation and abscess examination in mouse skin model

To examine abscess-forming pathogenicity after injection of these microorganisms, the murine abscess model described by Kesavalu *et al.*¹⁴⁾ was used in this study. Cells grown in broth media were harvested by centrifugation, suspended in PBS, and syringe-passaged

3 times to form an equal suspension. Absorbance of each suspension was measured at 600 nm, and their cell concentrations were adjusted to about 10^{11} cells/ml according to protocols in previous manuscripts^{32,33}. Mice were injected at sites on the dorsal area with 200 μ l (about 2×10^{10} cells) of each suspension or 200 μ l of a mixed suspension of *P. gingivalis* 16-1 (100 μ l; about 1×10^{10} cells) and *T. denticola* ATCC 33520 (100 μ l; about 1×10^{10} cells). Sizes of subcutaneous abscess lesions were measured with a caliper gauge, and their areas were determined and expressed in square mm.

5. Measurement of serum IgG

Two weeks after the injections, a blood sample was collected by a capillary tube from the eye-ground. After clotting, the serum was separated by centrifugation. Serum IgG levels against whole cells of *P. gingivalis* 16-1 were measured by an enzyme-linked immunosorbent assay (ELISA). Sonicated antigen of *P. gingivalis* in 50 μ l of 50 mM carbonate buffer (pH 9.6) was used to coat each well of a 96-well plate at 4°C overnight. Wells were blocked with 200 μ l of 3% bovine serum albumin in PBS at 37°C for 60 min. Aliquots of 50 μ l of 100 times diluted serum was applied to each well and incubated at 37°C for 60 min. After washing with PBS, the secondary antibody, peroxidase-labeled goat anti-mouse immunoglobulin (Dako A/S, Glostrup, Denmark) diluted at 1:2,000 was added, and then the wells were incubated at 37°C for 60 min. After development, absorbance at 490 nm was measured with a microplate reader (Bio-Rad Laboratories, Hercules, Calif.). The IgG levels were expressed as the absorbance at 490 nm.

6. Sensitization of

delayed type hypersensitivity (DTH)

To prepare the antigen for DTH sensitization, a total of 200 mg wet weight cells of washed *P. gingivalis* 16-1 were suspended in 2 ml PBS as described by Okuda and Takazoe²⁰. After incubation of the suspension at 60°C for 2 hours, the suspension was centrifuged at $12,000 \times g$ for 15 min. The supernatant was

used for the antigen to determine DTH. After 2 weeks of infection, 25 μ l of the extracted antigen was injected into the right hind footpad, and the same volume of PBS was injected into the other footpad. The thickness of each footpad was measured 24 hours later with a microradial thickness gauge (Ozaki Co., Tokyo). The differences between the thickness of the antigen injected site and the PBS injected site were used for calculations.

7. Statistical analysis

Statistical analysis was performed by multiple comparison by Scheffe's test using SAS version 8.02. Student *t*-test was used to compare the levels of DTH in each group.

RESULTS

1. Subcutaneous abscess lesions

We compared the abscess sizes in mice among the mono-infections with *P. gingivalis* 16-1 or *T. denticola* ATCC 33520 and the mixed infection with both bacterial species. Average sizes of the abscess after 5 to 11 days are shown in Fig. 1. In the mono-infection experiments, we injected 200 μ l of viable cells of *P. gingivalis* 16-1 or *T. denticola* ATCC 33520. In the mixed infection group, we injected 200 μ l of mixed suspension with 100 μ l *T. denticola* and 100 μ l *P. gingivalis*. The average abscess sizes in mice challenged by mixed cells were significantly larger than those by mono-infection with *P. gingivalis* 16-1 on days 5, 6, 8, and 10 after infection ($p < 0.01$). The average sizes of the in the mixed infection group was bigger than that after mono-infection with *T. denticola* ATCC 33520 on days 5, 6, 7, 8, 10, and 11 after infection ($p < 0.01$). Viable cells of *P. gingivalis* were detected in pus samples obtained from the abscess of 3 mice in the mixed infected group 7 days after the challenge.

2. Serum IgG response

We determined the serum IgG responses against whole cells of *P. gingivalis* 16-1 in mice challenged by mono-infection or by mixed infection with *P. gingivalis* 16-1 and *T. denticola*

Fig. 1 Average areas of abscesses after mono-infection by *P. gingivalis* 16-1, mono-infection by *T. denticola* ATCC 33520, or mixed infection with both strains. Mice were infected with 200 μ l of viable cell suspension of *P. gingivalis* 16-1, with 200 μ l viable cell suspension of *T. denticola* ATCC 33520, or with 200 μ l mixture of *P. gingivalis* (100 μ l) and *T. denticola* (100 μ l). Multiple comparison by Scheffe's test was performed using SAS ver. 8.02. *: $p < 0.05$

ATCC 33520. The mean ELISA IgG levels at OD490 of 100 times diluted serum against whole cells of *P. gingivalis* 16-1 from mixed infected group and those from *P. gingivalis* mono-infected group were 0.24 ± 0.019 and 0.24 ± 0.03 , respectively. These were increased from the normal serum level (0.10) but were not statistically different.

3. DTH responses in challenged mice

To evaluate the effect of mixed infection on DTH response, footpad swelling was evaluated 24 hours after injection of antigen extracted from cells of *P. gingivalis* 16-1. The results are summarized in Fig. 2. The average DTH responses in mice doubly infected with *P. gingivalis* and *T. denticola* were significantly lower than those after mono-infection by *P. gingivalis* ($p < 0.05$).

4. Effect of immunization on the abscess lesion

We examined the abscess lesions induced

Fig. 2 Comparison of DTH responses against extracted antigen in *P. gingivalis* mono-infected mice of with those in mice infected with a mixture of *P. gingivalis* and *T. denticola*. The DTH response in mixed infection mice was lower than that in mono-infected mice as calculated by Student *t*-test. *: $p < 0.05$

by the mixed infection of *P. gingivalis* and *T. denticola* in mice previously immunized with whole cells of *P. gingivalis*.

The time course of these abscess lesions induced by mixed infection with *P. gingivalis* 16-1 and *T. denticola* ATCC 33520 is shown in Fig. 3. Unexpectedly, the average size of the abscess lesions induced by mixed infection of *P. gingivalis* and *T. denticola* in sham-immunized mice was not statistically different from that in mice immunized with whole cells. On the 3rd, 4th, 7th, 9th and 11th day after mixed infection, the average abscess sizes in immunized mice were smaller than those in sham-immunized mice, but the difference was not statistically significant. The mixed infection in immunized mice reached its maximum on the 3rd or 4th day. Many of the abscesses ruptured on the 4th or 5th day, and the lesions then recovered in 2 weeks.

Fig. 3 Comparison of areas of abscesses caused by mixed infection with *P. gingivalis* 16-1 and *T. denticola* ATCC 33520 in mice immunized with whole cells of *P. gingivalis* 16-1 to the areas in sham-immunized mice.

DISCUSSION

Synergistic effects of periodontal pathogens in periodontal lesions are thought to play important roles in periodontal disease^{7,15,26}. In addition, immune responses against periodontopathic bacteria are thought to affect the process of periodontitis. In this study, we examined the synergistic effects of a mixed infection by *P. gingivalis* and *T. denticola*. Mixed infection by *P. gingivalis* 16-1 and *T. denticola* ATCC 33520 produced larger abscesses in mice than those formed after mono-infection by *P. gingivalis* alone. The effects of mixed infections with *T. denticola* and *P. gingivalis* in the murine subcutaneous abscess model were earlier reported by Kesavalu *et al.*¹⁴ They demonstrated that, at low *P. gingivalis* challenge doses, *T. denticola* significantly enhanced the virulence of *P. gingivalis* and concluded that the enzymatic activity of *T. denticola* had a potential role in the enhanced virulence in *P. gingivalis*. The hemolytic activity produced by *T. denticola* was also reported to be involved in the pathogenesis of the microorganisms^{2,3}. It is possible that the enzymatic activity and hemolytic activity of *T. denticola* may have enhanced the synergistic pathogenesis of the mixed infection in this study.

Immune suppressing activity of *T. denticola*

has been reported by several groups^{9,25}. The present study showed that DTH response against *P. gingivalis* in mixed infected mice was lower than those of the mono-infected mice. We previously indicated that the abscess forming activity is strongly attenuated by mutants lacking dentilisin, which is a major surface protease of the microorganisms¹⁰. Beaujourn *et al.*¹¹ reported that a protease from *T. denticola* caused a reaction from pro-interleukin 1 to interleukin 1. Recently, Miyamoto *et al.* in our laboratory demonstrated that cells and extracted dentilisin of *T. denticola* degraded interleukin 2, suggesting that dentilisin affects the cytokine network and suppresses the immune responses (*J Dent Res* **82**, Special Issue B 361, Abstract). It is possible that the dentilisin activity of *T. denticola* in the mixed infection mice is linked to the IgG responses. After mixed infection with *P. gingivalis* and *T. denticola*, the average abscess size did not significantly differ from that in sham-immunized mice. This result suggests that the addition of *T. denticola* attenuated the protective immune response against *P. gingivalis* infection. It is possible that the cytokine degradation activity attenuated the protective immune responses. We found that many of the abscesses that were formed by mixed infections in immunized mice with

whole cells of *P. gingivalis* 16-1 strain ruptured during the 4th or 5th day after the challenge. After rupturing, most of the lesions recovered in 2 weeks after the challenge. It is possible that cellular responses such as DTH in mice immunized with whole cells of *P. gingivalis* were involved in the rupture and early recovery of the abscess.

The present study suggested the mixed infection of *T. denticola* and *P. gingivalis* enhances virulence of both strains and helps explain the high detection rates of the combination of both microorganisms in adult periodontitis lesions^{11,26,28}.

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