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Gram-positive bacteria as an antigen topically applied into gingival sulcus of immunized rat accelerates periodontal destruction

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Background and objective: Periodontitis is generally accepted to relate to gram-negative bacteria, and the host defense system influences its onset and progression. However, little is known about the relation between gram-positive bacteria and periodontitis. In this study, we topically applied gram-positive and gram-negative bacterial suspensions to the gingival sulcus in rats after immunization, and then histopathologically examined their influence on periodontal destruction.

Materials and Methods: Rats previously immunized with heat-treated and sonicated *Staphylococcus aureus* or *Aggregatibacter actinomycetemcomitans* were used as immunized groups. The non-immunized group received only sterile phosphate-buffered saline. In each animal, *S. aureus* or *A. actinomycetemcomitans* suspension was applied topically to the palatal gingival sulcus of first molars every 24 h for 10 d. Blood samples were collected and the serum level of anti-*S. aureus* or anti-*A. actinomycetemcomitans* immunoglobulin G (IgG) antibodies was determined by enzyme-linked immunosorbent assay. The first molar regions were resected and observed histopathologically. Osteoclasts were stained with tartrate-resistant acid phosphatase (TRAP). The formation of immune complexes was confirmed by immunohistological staining of C1qB.

Results: Serum levels of anti-*S. aureus* and anti-*A. actinomycetemcomitans* IgG antibodies in the immunized groups were significantly higher than those in the non-immunized groups were. The loss of attachment, increase in apical migration of the junctional epithelium, and decreases in alveolar bone level and number of TRAP-positive multinuclear cells in each immunized group were significantly greater than in each non-immunized group. The presence of C1qB was observed in the junctional epithelium and adjacent connective tissue in the immunized groups.

Conclusions: Heat-treated and sonicated *S. aureus* and *A. actinomycetemcomitans* induced attachment loss in rats immunized with their suspensions. Our results suggest that not only gram-negative but also gram-positive bacteria are able to induce periodontal destruction.

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Key words: gram-positive bacteria; histopathological study; immunization; periodontal destruction

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Introduction

Periodontitis is a chronic inflammatory response resulting in loss of attachment and alveolar bone resorption. Both the effect of periodontal bacteria and response of the host defense system have been thought to influence its onset and progression (1,2). Kuramoto *et al.* (3) reported that the formation of immune complexes with lipopolysaccharide of *Escherichia coli* as an antigen and its specific antibody in gingival sulcus induces attachment loss and bone resorption in rats. This report also suggests that biological activity of antigens is important in periodontal destruction. Furthermore, Yoshinaga *et al.* (4) recently reported that topical application of lipopolysaccharide as an antigen induces periodontal destruction when the serum level of anti-lipopolysaccharide antibody is elevated in rats immunized with lipopolysaccharide. These experiments show that the combination of an antigen in the gingival sulcus and a specific antibody in gingival crevicular fluid (GCF) is involved in periodontal destruction. However, the lipopolysaccharide used as an antigen in these animal models of periodontitis is not derived from oral bacteria. Although the proportion of gram-negative bacteria increases markedly in gingival microflora with increasing severity of periodontitis (5,6), gram-positive bacteria are dominant in the gingival sulcus that is in a healthy condition. When gingivitis, an early stage of periodontitis, occurs, the number of gram-positive bacteria is still higher than the number of gram-negative bacteria (7,8). Therefore, it seemed important to investigate the influence of gram-positive bacteria on the onset of periodontitis. In this study, we used heat-treated and sonicated *Staphylococcus aureus* or *Aggregatibacter actinomycetemcomitans* as antigens. Although *S. aureus* is few in the oral cavity (9,10) among gram-positive bacteria, its biological activity of peptidoglycan and lipoteichoic acid is proved and it has been widely used in many experiments. *Aggregatibacter actinomycetemcomitans* is a gram-negative bacterium and well-known as a periodontopathic bacterium. We histopathologically examined their influences on periodontal tissue using a method similar to that in a previous report (4) and detected the existence of immune complexes by staining C1qB immunohistologically. As a result, it was observed that not only gram-negative but also gram-positive bacteria were able to induce loss of attachment and alveolar bone resorption in rats immunized with their suspensions.

Materials and methods

Rats

All rats were purchased from Charles River Japan (Tokyo, Japan) and maintained under specific pathogen-free conditions in the Biomedical Research Center, Center for Frontier Life Sciences (Nagasaki University, Nagasaki, Japan). Animal care and experimental procedures were carried out in accordance with Guidelines for Animal Experimentation of Nagasaki University and with approval from the Institutional Animal Care and Use Committee.

Bacteria

The bacteria used in this study were *S. aureus* strains ATCC21027 and *A. actinomycetemcomitans* Y4. After 24 h incubation on brain heart infusion (BD 211059) agar plates at 37°C, *S. aureus* was grown in brain heart

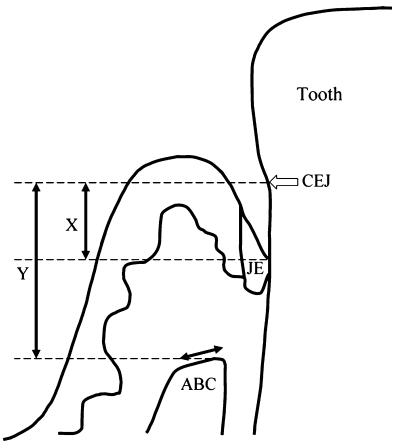

Fig. 1. Schema of rat periodontal tissue for histometrical analysis. Periodontal loss of attachment (X) was calculated by measurement of the distance between the CEJ and the coronal position of JE. A comparison of the alveolar bone resorption levels (Y) of the samples was performed by measurement of the distance between the CEJ and alveolar bone crest. Tartrate-resistant acid phosphatase-positive multinuclear cells in an area of 500 μm width on the surface of the alveolar bone were counted. CEJ, cemento-enamel junction; JE, junctional epithelium; ABC, alveolar bone crest; X, loss of attachment: distance between CEJ and the coronal portion of JE; Y, alveolar bone level: distance between CEJ and the level of ABC.

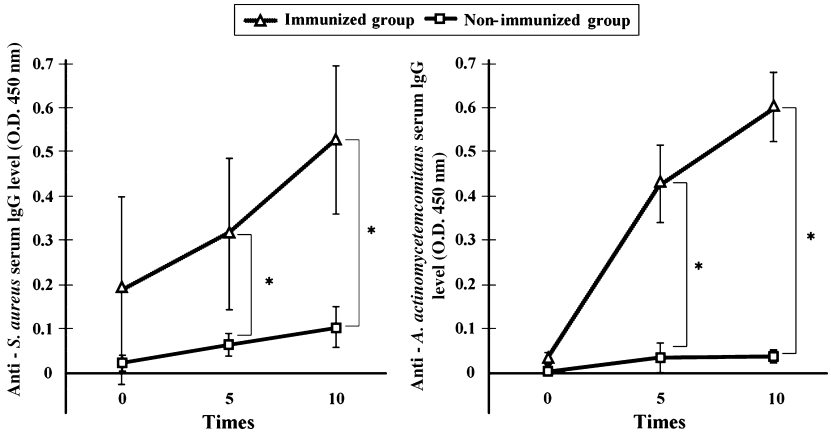

Fig. 2. Serum levels of anti-*S. aureus* and anti-*A. actinomycetemcomitans* IgG antibodies in the immunized and non-immunized groups. Both immunized groups show significantly higher levels than the non-immunized groups. Each bar represents the mean ± SD. *Significantly different from the non-immunized group. Mann–Whitney *U*-test, $P < 0.05$.

infusion broth (BD211059). *Aggregatibacter actinomycetemcomitans* was anaerobically incubated on Trypticase soy agar plates (BD 236950) with yeast extract (BD 212750) and defibrinated sheep blood at 37°C. After that, *A. actinomycetemcomitans* was grown anaerobically in Todd–Hewitt Broth supplemented with 0.3% yeast extract (BD 212750). Bacteria were harvested and washed three times with MilliQ water and lyophilized.

Bacterial suspension

Bacterial suspension was used for immunization and topical application. We mixed 0.5 mg of bacterial dry weight and 100 μL of sterile phosphate-buffered saline (PBS) for immunization. For topical application, the bacterial suspension was mixed at 25 μg/μL. All of these suspensions were fragmented with sonication for 1 min after heat treatment for 10 min.

Experimental design

Ten male 9-wk-old Lewis rats were divided into two groups, an *S. aureus*-immunized group and an *A. actinomycetemcomitans*-immunized group, consisting of five rats each. Another 10 rats were used in each bacterial non-immunized group. Animals in the immunized groups received intraperitoneal injections of a mixture of incomplete Freund's adjuvant (DIFCO; Detroit, MI, USA) and bacterial suspension. The mixtures included 500 μL of incomplete Freund's adjuvant and 2.5 mg/500 μL of bacterial suspension of *S. aureus* or *A. actinomycetemcomitans*. The same mixture was injected as a booster after 28 d. The non-immunized groups received intraperitoneal injections of 500 μL of PBS and 500 μL of incomplete Freund's adjuvant. One day after the booster injection, the immunized groups and non-immunized groups were challenged daily with a topical application of bacterial suspension on to the gingival sulcus. The topical application of bacterial suspension in the present study was modified from a previous report (3,4). Briefly, animals were anesthetized using isoflurane, then a total of 21 μL (3 μL × seven times) of bacterial suspension was administered daily to the immunized groups and non-immunized groups by micropipette on to the palatal gingival sulcus of the maxillary first molar for 30 min. Rats in the immunized groups and non-immunized groups were killed 1 h after the 10th application. Five rats were used as untreated controls.

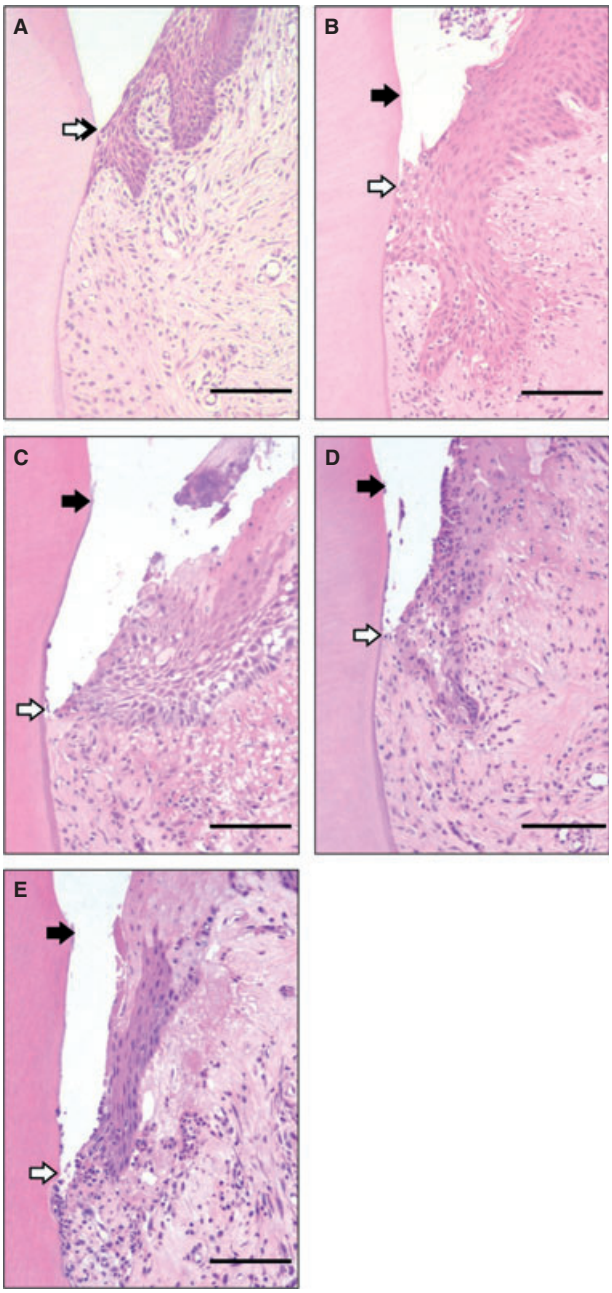

Fig. 3. Histopathological findings of loss of attachment on the palatal side of the first molar. The immunized groups show greater loss of attachment than the non-immunized groups. A number of inflammatory cells, mainly neutrophils, were more observed in junctional epitheliumand adjacent connective tissue than in the non-immunized group. (A) Control group; (B) *S. aureus*-non-immunized group; (C) *S. aureus*-immunized group; (D) *A. actinomycetemcomitans*-non-immunized group; (E) *A. actinomycetemcomitans*-immunized group Black arrow: cemento-enamel junction. White arrow: loss of attachment. Scale bar = 100 μm.

Indirect enzyme-linked immunosorbent assay for antibody detection

Blood samples were collected from the retro-orbital venous plexus of rats in all groups just before the first and sixth application and just before being killed. Serum levels of anti-*S. aureus* or anti-*A. actinomycetemcomitans* immunoglobulin G (IgG) antibodies were determined by enzyme-linked immunosorbent assay from individual serum samples. Microtiter plates with 96 wells were coated with 100 μL/well of bacterial antigens in 0.01 M carbonate buffer and were incubated overnight at 4°C. Bacterial antigens were prepared by each bacterial suspension. Bacterial suspensions were dialyzed using dialysis membrane tubing (Spectrum Laboratories, Rancho Domingez, CA, USA). First, we immersed the tubing in 1 L of 2% sodium bicarbonate/1 mM EDTA and boiled the tubing for 10 min. After the tubing was rinsed thoroughly with deionized distilled water, we boiled the tubing in deionized distilled water for 10 min. Then, the tubing was stored at 4°C in 50% ethanol/1 mM EDTA before use. The sonicated supernatants of the bacterial suspension were dialyzed against MilliQ water. We used 5.0 μg/mL of *S. aureus* antigens and 2.0 μg/mL of *A. actinomycetemcomitans* antigens in 0.01 M carbonate buffer for each capture. After being washed with PBS containing Tween-20 (PBST), the wells were blocked with 0.1% bovine serum albumin/PBS. After further washing, 100 μL of the sera of *S. aureus* (1 : 4000 dilutions) and the sera of *A. actinomycetemcomitans* (1 : 8000 dilutions) to be tested were added, and the plates were incubated for 1 h at room temperature and then washed with PBST. Antibody reactivity was determined by adding peroxidase-conjugated goat anti-rat IgG (1 : 2000 dilutions, Invitrogen, Tokyo, Japan) in PBS to each well and incubating for 1 h at room temperature. The plates were washed with PBST and 75 μL of the TMB solution (R&D Systems, Minneapolis, MN, USA) was added and incubated at room temperature. The enzyme reaction was stopped with 25 μL/well of 2 N H_2SO_4. The plates were read at 450 nm.

Preparation of tissues

The maxilla of each rat was removed immediately after death and fixed in 4% paraformaldehyde in PBS at 4°C for 10 h, decalcified with 10% EDTA for 3 wks and then embedded in paraffin using the AMeX method (11). Tissues were fixed in acetone overnight, then cleared in methyl benzoate and xylene, consecutively, and embedded in ordinary paraffin at 60°C. Buccolingually oriented serial sections (4-μm thickness) at the level of the central roots of the upper first molar were obtained.

Histopathological and histometrical analysis

Five groups of serial sections, each containing ten subsections, were obtained from each specimen. The first subsections from each group of serial sections were stained with hematoxylin and eosin (H&E) for histopathological observation. The tissue sections stained with H&E were used to measure the distances from the cemento-enamel junction (CEJ) to the coronal portion of the junctional epithelium (JE) to measure the length of the loss of attachment (Fig. 1 X) and from the CEJ to the alveolar bone crest to evaluate bone destruction (Fig. 1 Y). The distance was measured using image analysis software (Image J; U.S. National Institutes of Health, Bethesda, MD, USA). To identify osteoclasts, the second subsections from each group were stained with tartrate-resistant acid phosphatase (TRAP) (12). Briefly, a staining solution was made by mixing 0.5 mL of pararosaniline solution (1 g pararosaniline in 20 mL distilled water and 5 mL concentrated hydrochloric acid), 0.5 mL of 4% sodium nitrite solution, 10 mL of 0.1 M acetate buffer at pH 5.0, and 10 mg of naphthol AS-BI phosphate (Sigma, St. Louis, MO, USA) dissolved in 8 mL distilled water. The mixture was adjusted to pH 5.0 using concentrated NaOH and filtered through No.1 Whatman filter paper. Furthermore, after adding 150 mg L-(+)-tartaric acid to a 10 mL aliquot of the mixture solution to a final tartrate concentration of 0.1 M, the solution was adjusted to pH 5.0 with concentrated NaOH. After the second subsections were incubated within the stain solution for 30 min at 37°C, they were counterstained with hematoxylin. The numbers of TRAP-positive multinuclear cells adjacent to the surface of the alveolar bone crest were counted (Fig. 1).

Immunohistological staining of C1qB

To detect immune complexes, C1qB was immunohistologically stained. The third subsections were deparaffinized, and endogenous peroxidase

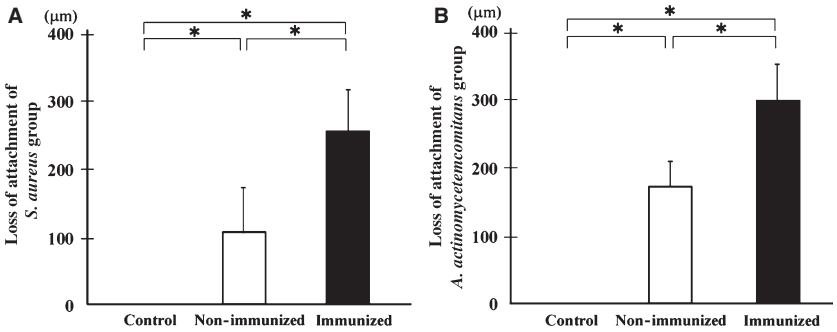

Fig. 4. Histometrical analysis of loss of attachment. The loss of attachment is greater in both immunized groups. (A) *S. aureus* group; (B) *A. actinomycetemcomitans* group. Each bar represents the mean ± SD. Fisher least significant difference, $P < 0.05$.

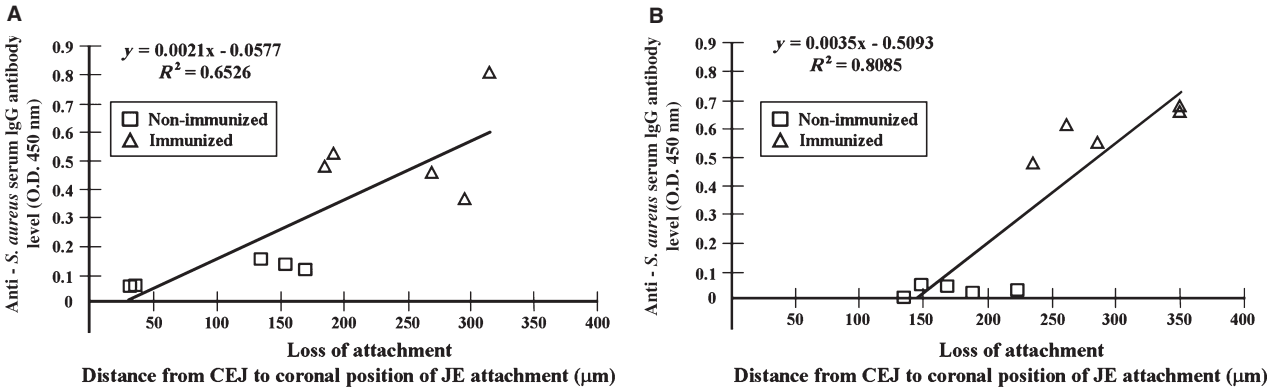

Fig. 5. Correlation between serum levels of anti-*S. aureus* or anti-*A. actinomycetemcomitans* IgG antibodies and loss of attachment. *A. actinomycetemcomitans* group shows strong correlation. (A) *S. aureus* group; (B) *A. actinomycetemcomitans* group. CEJ, cemento-enamel junction; JE, junctional epithelium.

activity was blocked with 0.3% H_2O_2/methanol for 30 min, followed by incubation in normal goat serum for 30 min at room temperature. These sections were then immersed in rabbit polyclonal anti-C1qB (AVIVA Systems Biology, San Diego, CA, USA) overnight. Sections were then incubated with biotinylated goat anti-rabbit polyclonal immunoglobulin (Dako, Glostrup, Denmark) for 30 min, then with peroxidase-conjugated streptavidin (Dako) for 30 min, and then incubated with diaminobenzidine tetraoxide solution and counterstained with hematoxylin.

Statistical analysis

Data were statistically analyzed using KaleidaGraph (Synergy Software, Perkiomen, PA, USA). Differences between each non-immunized group and each immunized group were evaluated with the Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant. Differences among the control, non-immunized and immunized groups for each bacteria were evaluated by statistical analysis, including three-way analysis of variance and the Fisher protected least significant difference test for *post hoc* pair-wise comparisons. $P < 0.05$ was considered statistically significant. Correlation between serum levels of anti-*S. aureus* or anti-*A. actinomycetemcomitans* IgG antibodies and loss of attachment were analyzed by linear correlation analysis.

Results

The serum levels of anti-*S. aureus* and anti-*A. actinomycetemcomitans* antibody

The serum levels of anti-*S. aureus* and anti-*A. actinomycetemcomitans* IgG antibodies were elevated in the immunized groups after fifth applications but not in non-immunized groups. The immunized groups showed significantly higher values than the non-immunized groups after the 10th application (Fig. 2).

Histopathological findings

Control rats showed an apical portion of the JE located at the CEJ with few inflammatory cells infiltrating into the JE and the surrounding connective tissue (Fig. 3A). Loss of attachment was observed in the *S. aureus*-immunized and *S. aureus*-non-immunized groups (Fig. 3B), although a necrotic layer and a lot of inflammatory cells appeared in the JE and adjacent connective tissue only in the *S. aureus*-immunized group (Fig. 3C). The same tendency was shown in the *A. actinomycetemcomitans*-non-immunized and *A. actinomycetemcomitans*-immunized groups (Fig. 3D,E). The large loss of attachment was observed in both *S. aureus*-immunized and *A. actinomycetemcomitans*-immunized groups.

Histometrical analysis

The largest loss of attachment was observed in both *S. aureus*-immunized and *A. actinomycetemcomitans*-immunized groups. Furthermore, there was

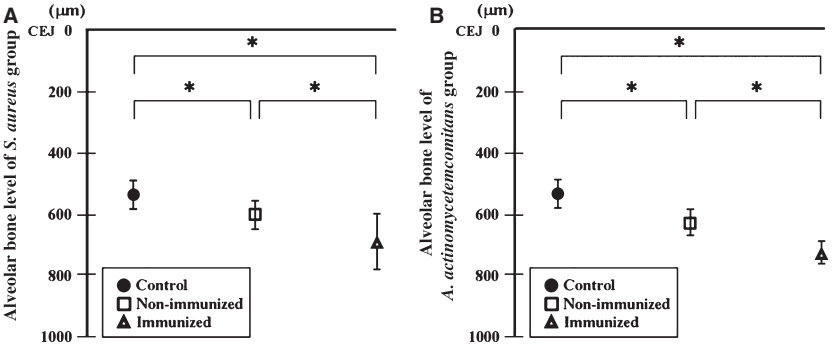

Fig. 6. Histometrical analysis of alveolar bone level. The alveolar bone level is significantly lower in both immunized groups. (A) *S. aureus* group; (B) *A. actinomycetemcomitans* group. Each bar represents the mean ± SD. *Significant difference among the three groups. Fisher least significant difference, $P < 0.05$. CEJ, cemento-enamel junction.

a statistically significant difference between the non-immunized groups and control group (Fig. 4). The serum levels of anti-*A. actinomycetemcomitans* IgG antibody and loss of attachment of each rat showed a positive correlation (Fig. 5). The lowest alveolar bone level was observed in both the *S. aureus*-immunized and *A. actinomycetemcomitans*-immunized groups. There were also statistically significant differences between the non-immunized groups and the control group (Fig. 6). TRAP-positive multinuclear cells were observed in contact with alveolar bone resorption lacunae in all experimental groups (Fig. 7B–E). There were statistically significant differences in the number of TRAP-positive multinuclear cells between the *S. aureus*-immunized group and the control group and among the *A. actinomycetemcomitans*-immunized group, *A. actinomycetemcomitans*-non-immunized group and control group (Fig. 7F,G).

Immunohistological finding of C1qB

C1qB was not detected in the control group or in the non-immunized groups (Fig. 8A). Deposits of C1qB were found in the JE and adjacent connective tissue in both immunized groups (Fig. 8B,C).

Discussion

In previous studies, the levels of antibodies to periodontal pathogens such as *Porphyromonas gingivalis* and *A. actinomycetemcomitans* were higher in patients with periodontitis than in healthy subjects. (13,14). In addition, the antibody levels in the serum and in GCF showed a positive correlation (15–17). In our study, considering the elevation of the serum-specific antibody level on day 10, it is likely that the specific antibody level in GCF would also increase in both immunized groups, although it was difficult to measure specific antibody levels in rat GCF. The loss of attachment levels in both the *S. aureus*- and *A. actinomycetemcomitans*-immunized groups were significantly greater than in both the *S. aureus*- and *A. actinomycetemcomitans*-non-immunized groups. In addition, the infiltration of inflammatory cells and reduced alveolar bone level were observed in both immunized groups. It seems that anti-*S. aureus* or anti-*A. actinomycetemcomitans* IgG antibodies in GCF and *S. aureus* or *A. actinomycetemcomitans* as an antigen formed immune complexes in the immunized groups and resulted in inflammation and tissue destruction. In particular, the anti-*A. actinomycetemcomitans* IgG antibody level had a strong correlation with the loss of attachment.

In general, to observe the immune complex immunohistologically, antigen and antibody are investigated individually. However, it is difficult in the present study, because rat IgG as an antibody was an endogenous substance and bacterial fragments as antigens expanded through gingival tissues. When activation of the

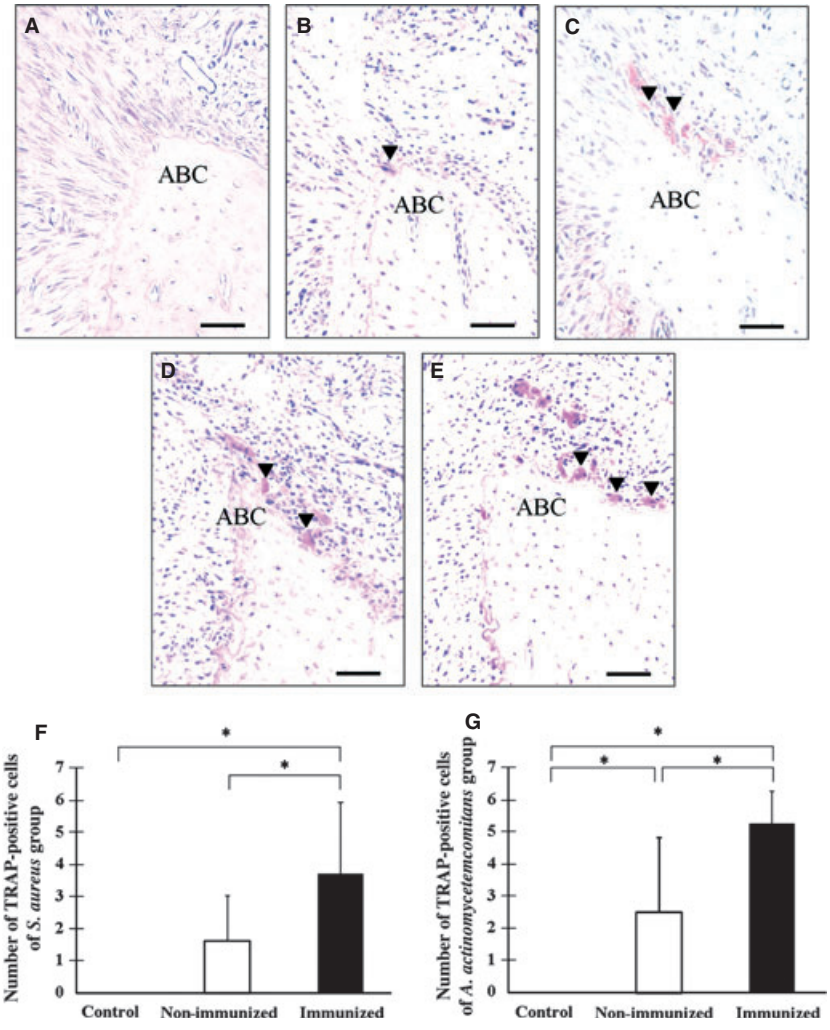

Fig. 7. Histopathological findings of TRAP staining and the number of TRAP-positive cells. The formation of osteoclasts was observed in the experimental groups (B,C,D,E). In the control group, osteoclasts were not observed (A). There were statistically significant differences in the number of TRAP-positive multinuclear cells between the *S. aureus*-immunized group and the control group (F) and among the *A. actinomycetemcomitans*-immunized group, the *A. actinomycetemcomitans*-non-immunized group and control group (G). Fisher least significant difference, $p < 0.05$. (A) Control group; (B) *S. aureus*-non-immunized group; (C) *S. aureus*-immunized group; (D) *A. actinomycetemcomitans*-non-immunized group; (E) *A. actinomycetemcomitans*-immunized group (F) *S. aureus* group; (G) *A. actinomycetemcomitans* group. ABC, alveolar bone crest; TRAP, tartrate-resistant acid phosphatase. Arrowhead: TRAP-positive multinuclear cells. Scale bar = 100 μm.

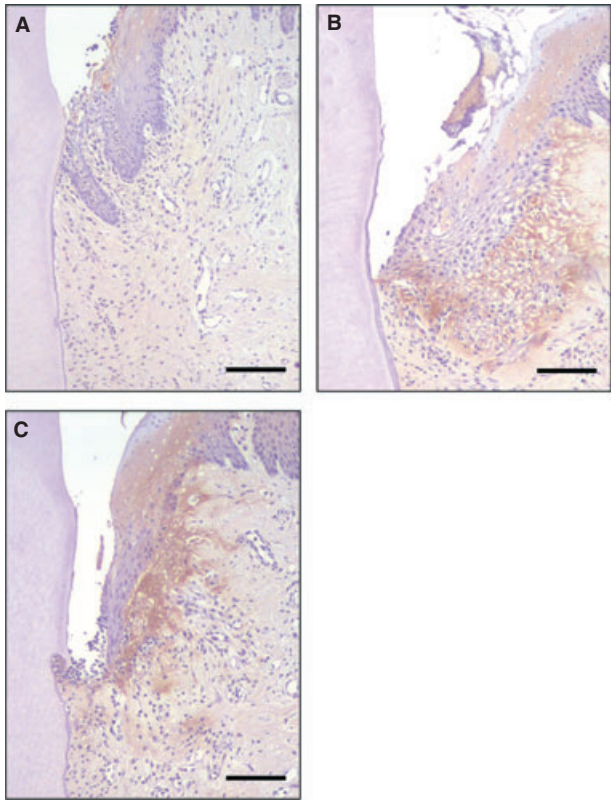

Fig. 8. Localization of C1qB on the palatal side of the first molar. C1qB stained with dark red color was observed in junctional epithelium and adjacent connective tissue of the *S. aureus*-immunized group (B) and *A. actinomycetemcomitans*-immunized group (C). C1qB was not observed in the *S. aureus*-non-immunized group (A) and in the *A. actinomycetemcomitans*-non-immunized group. (A) *S. aureus*-non-immunized group (B) *S. aureus*-immunized group (C) *A. actinomycetemcomitans*-immunized group Scale bar = 100 μm.

classical pathway of complement is initiated, the C1 component binds to antigen-bound antibodies. So, we immunohistologically confirmed C1qB to detect the localization of immune complexes in rat gingiva. We observed deposits of C1qB within the JE and adjacent connective tissue in the *S. aureus*- and *A. actinomycetemcomitans*-immunized groups. On the other hand, the *S. aureus*- and *A. actinomycetemcomitans*-non-immunized groups had almost no such deposits. Immune complexes formed in the JE and adjacent connective tissue activate a complement cascade, and then neutrophil chemotaxis factors such as C3a and C5a are released. This results in neutrophil infiltration within those tissues. Although neutrophils exhibit defensive activity against bacteria (18–20). They have also been implicated in tissue degradation through the release of reactive oxygen species and proteolytic enzymes (21). A recent report (22) showed that aging-associated periodontitis is accompanied by lower expression of Del-1, an endogenous inhibitor of neutrophil adhesion. Therefore, the neutrophil activation is also thought to cause periodontal destruction in this study.

The losses of attachment in both the *S. aureus*- and *A. actinomycetemcomitans*-non-immunized groups were significantly greater than that in the control group in this study. Although the protein activities of *S. aureus* would be lost after heat treatment, heat-treated *S. aureus* still shows potent stimuli for cytokine production such as tumor necrosis factor-α, interleukin (IL)-8 and IL-10 in human monocytes (23–25). Peptidoglycan and lipoteichoic, components of the gram-positive bacterial cell wall, are the triggers of proinflammatory cytokine production and elicit the production of inflammatory effector substances such as nitric oxide via Toll-like receptors (26,27). Peptidoglycan is able to induce bone resorption by stimulating Toll-like receptor 2 (28,29). Considering this, such bacterial components were likely to induce loss of attachment even in the *S. aureus*-non-immunized group. On the other hand, live *A. actinomycetemcomitans* induces a high cytokine response, which remained after heat treatment. Heat-treated *A. actinomycetemcomitans* induces IL-1β, tumor necrosis factor-α, IL-6, IL-8, and IL-10 via Toll-like receptors 2 and 4 (30). Among *A. actinomycetemcomitans*'s components, lipopolysaccharide has heat stability and the potential for bone resorption (31), and is able to lead JE tissue to necrosis (21). Thus, we thought that lipopolysaccharide was the main influence in both *A. actinomycetemcomitans* groups. Furthermore, gram-positive or -negative peptidoglycan worked synergistically with lipopolysaccharide to induce bone resorption and osteoclastogenesis (32,33). Peptidoglycan may also influence the stimulation of osteoclastic bone resorption in *A. actinomycetemcomitans* groups.

Although we used both bacteria at the same dry weight, their sizes were different. For this reason, we did not compare each bacterial group's results with each other. The concentration of the bacterial suspension we used was high. However, even when the concentration of antigens is low, immune complexes are formed in periodontal tissue and then periodontal destruction would be induced little by little. Our results suggest that not only gram-negative but also gram-positive bacteria are important to analyze the mechanism of onset of loss of attachment in periodontitis.

Acknowledgements

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