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Topical application of lipopolysaccharide into gingival sulcus promotes periodontal destruction in rats immunized with lipopolysaccharide

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Background and Objective: The causes of periodontitis are bacteria and the host immune system, but the role of the immune system in the onset and progression of periodontal disease is still unclear. Our previous report showed that the formation of an immune complex in the gingival sulcus induces periodontal destruction. This study was carried out to investigate how the immune system, particularly immunization, is involved in periodontal destruction.

Material and Methods: Animals immunized intraperitoneally with lipopolysaccharide (LPS) were used as the immunized group. The nonimmunized group received only phosphate-buffered saline. LPS was applied daily onto the palatal gingival sulcus in both groups 1 d after the booster injection. Serum levels of anti-LPS IgG were determined. Loss of attachment and the level of alveolar bone were histopathologically and histometrically investigated. RANKL-bearing cells and the expression of C1qB were immunohistologically evaluated.

Results: The serum levels of anti-LPS IgG were elevated in the early experimental period in the immunized group. There were significant increases in loss of attachment, level of alveolar bone and the number of RANKL-bearing cells in the immunized group. C1qB was observed in the junctional epithelium and adjacent connective tissue. The nonimmunized group showed similar findings at and after the time when the serum level of anti-LPS IgG was elevated.

Conclusion: Topical application of LPS as an antigen induced periodontal destruction when the serum level of anti-LPS IgG was elevated in rats immunized with LPS. The presence of C1qB suggests that the formation of immune complexes is involved in this destruction.

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Periodontitis is an inflammatory and infectious disease induced by bacterial oral biofilm. The major clinical characteristics of the disease are site-specific periodontal pocket formation and alveolar bone resorption (1). Periodontal tissue destruction is caused by the host immune system and by an inflammatory response against bacteria invading the periodontal tissue rather than as a direct effect of bacteria (2–5). However, the role of the immune system in the onset and progression of periodontal disease has remained unclear. We recently reported that the formation of immune complexes in the gingival sulcus, with lipopolysaccharide (LPS) as an antigen and its specific antibody, induced attachment loss and bone resorption in rats (6). Clinically, antigens are actually present in the gingival sulcus as exogenous substances; however, antibodies are endogenous. There are some reports showing a marked increase in the serum titers of immunoglobulin G (IgG) against periodontopathic bacteria in patients with chronic periodontitis and a positive correlation between the progression of the disease and the elevation of serum antibody against the oral bacteria colonizing the gingival sulcus (7–9). Other studies have shown the presence of immune complexes in the gingiva in periodontitis (10,11). Therefore, we thought that the topical application of an antigen onto the gingival sulcus could induce periodontal destruction when a specific antibody was present in the gingival crevicular fluid. However, it was impossible to measure the level of antibody in the gingival crevicular fluid of rats and consequently the experiment was performed during the period when the serum antibody level was elevated. In the present study, LPS was applied topically as an antigen onto the gingival sulcus of rats immunized with LPS. We detected the existence of immune complexes by immunohistological staining for C1qB and then the subsequent periodontal destruction was investigated histopathologically and immunohistologically.

Material and methods

Experimental design

Sixty male 9-wk-old Lewis rats were divided into two groups – an immunized group and a nonimmunized group – consisting of 30 rats each. Another six rats were used as a control group. The animals in the immunized group received intraperitoneal injections of 0.3 mL of 150 μg of *Escherichia coli* LPS (O111: B4; Sigma, St Louis, MO, USA) suspended in phosphate-buffered saline (PBS) emulsified in complete Freund's adjuvant, followed by a booster injection of LPS emulsified in incomplete Freund's adjuvant 28 d later (12). The control group and the nonimmunized group received intraperitoneal injections of 0.3 mL of PBS emulsified in complete Freund's adjuvant, followed by a booster injection of incomplete Freund's adjuvant 28 d later. At baseline, 1 d after the booster injection, the immunized group and the nonimmunized group were challenged daily with topical application of *E. coli* LPS onto the gingival sulcus, and the control group was challenged with PBS only.

The topical application of LPS in the present study was modified from our previous report (6). In the present study, animals received topical application of LPS alone as antigen (i.e. no subsequent topical application of antibody onto the gingival sulcus) because antibody was endogenous in the immunized group. Briefly, the rats were anesthetized with isoflurane, and the immunized group and the nonimmunized group received topical applications of *E. coli* LPS (50 μg/μL), resuspended in PBS using a micropipette, onto the palatal gingival sulcus of the left maxillary first molar, while the control group received PBS only. In total, 21 μL (3 μL × 7 times, with a 5-min interval between each application) of LPS suspended in PBS was administered within a 30-min time-period, daily, for 30 days. Rats were killed at baseline and 24 h after the 5th, 10th, 20th and 30th applications in the immunized group and the nonimmunized group. In the control group, rats were killed 24 h after the 30th application.

All rats were purchased from Charles River Japan (Tokyo, Japan) and were 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 the Guidelines for Animal Experimentation of Nagasaki University and with approval from the Institutional Animal Care and Use Committee.

Indirect ELISA for antibody detection

Blood samples were collected from the retro-orbital venous plexus of rats at baseline and 24 h after the 10th, 20th and 30th topical applications in all groups. The serum levels of anti-LPS IgG were determined in individual serum samples by ELISA. Each well of 96-well microtiter plates were coated with 100 μL of a 2.5 μg/mL solution of LPS from *E. coli* in 0.02 M carbonate buffer and were incubated overnight at 4°C. After being washed with 0.05 % Tween in PBS (PBST), the wells were blocked with PBS containing 0.1% bovine serum albumin. After further washing, 100 μL of the sera (1 : 1000 dilutions) to be tested was 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 : 5000 dilution; Zymed Laboratories, San Francisco, CA, USA) in PBS to each well and incubating for 1 h at room temperature. The plates were washed with PBST, and 75 μL of the 3,3′,5,5′-tetramethylbenzidine solution (R&D Systems, Minneapolis, MH, USA) was added and the plates were incubated at room temperature. The enzyme reaction was stopped with 25 μL/well of 1 M H_2SO_4. The plates were read at 450 nm.

Preparation of tissues

The left maxilla of each rat was removed immediately after death and was fixed in 4% paraformaldehyde in

PBS at 4°C for 10 h, decalcified with 10% EDTA for 3 wk and then embedded in paraffin using the AMeX method (acetone, methyl benzoate and xylene) (13). Bucco-lingually oriented serial sections (4 μm thickness) at the level of the central roots of the upper first molar were obtained.

Histopathological and immunohistological staining

Ten groups of serial sections, each containing five subsections, were obtained from each specimen. The first subsections from each group of serial sections were stained with hematoxylin and eosin for histopathological observation.

In order to identify the osteoclasts, the second subsections from each group were stained with TRAP (14). Briefly, a staining solution was made by mixing 0.5 mL of pararosanilin solution (1 g of pararosanilin in 20 mL of distilled water and 5 mL of concentrated hydrochloric acid), 0.5 mL of 4% sodium nitrite solution, 10 mL of 0.1 M acetate buffer, pH 5.0, and 10 mg of naphthol AS-BI phosphate (Sigma) dissolved in 8 mL of distilled water. The mixture was adjusted to pH 5.0 using concentrated 1 M NaOH and filtered through Whatman Grade 1 filter paper (Whatman Fisher Scientific, Houston, TX, USA). Furthermore, after adding 150 mg of L-(+)-tartaric acid to a 10 mL aliquot of the mixture solution, to give 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.

To detect the production of RANKL, the third subsections were used for the immunohistological staining of RANKL-bearing cells. Sections were deparaffinized and treated with 0.1% trypsin for 15 min at 37°C. Endogenous peroxidase activity was blocked with 0.3% H_2O_2/methanol for 30 min, followed by incubation in normal rabbit serum for 30 min at room temperature. These sections were then immersed in goat polyclonal anti-RANKL (N-19) (sc-7628; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), overnight at 4°C. Sections were then incubated for 30 min with biotinylated rabbit anti-goat polyclonal immunoglobulin (Dako, Glostrup, Denmark). These sections were finally incubated with peroxidase-conjugated streptavidin (Dako) for 30 min, then incubated with diaminobenzidine tetraoxide solution and counterstained with hematoxylin.

To detect immune complexes, C1qB was immunohistologically stained. Another six rats that were treated identically to the immunized or non-immunized groups for 10 d were prepared for this experiment. They were killed 1 h after the topical application and their maxilla were used for immunohistological staining. After sections were deparaffinized, endogenous peroxidase 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 overnight in rabbit polyclonal anti-C1qB (AVIVA Systems Biology, San Diego, CA, USA). The sections were then incubated with biotinylated goat anti-rabbit polyclonal immunoglobulin (Dako) for 30 min, with peroxidase-conjugated streptavidin (Dako) for 30 min and then placed in diaminobenzidine tetraoxide solution and counterstained with hematoxylin.

Histometrical study

The tissue sections stained with hematoxylin and eosin were used to measure the distances from the cemento–enamel junction (CEJ) to the coronal portion of the junctional epithelium (JE) (Fig. 1, X) to measure the depth of the periodontal pocket and from the CEJ to the alveolar bone crest (Fig. 1, Y) to evaluate the bone destruction. The distance was measured using image analysis software (IMAGE J; US National Institutes of Health, Bethesda, MD, USA). The numbers of RANKL-bearing cells in two 125-μm square regions around the surface of the alveolar bone crest were counted (Fig. 1).

Statistical analysis

Data were statistically analyzed using STATVIEW software (Abacus Concepts Inc., Berkeley, CA, USA). Differences between the nonimmunized group and the immunized group were evaluated using the Mann–Whitney *U*-test. $p < 0.05$ was considered statistically significant.

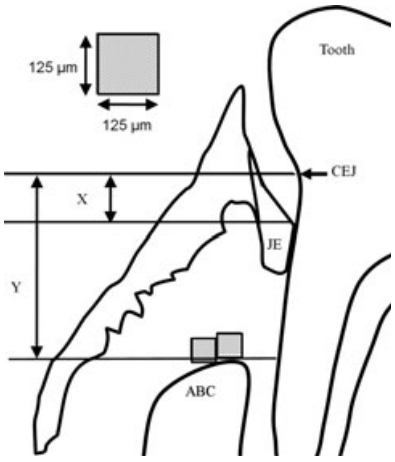

Fig. 1. Schema of rat periodontal tissue for histometrical analysis. ABC, alveolar bone crest; CEJ, cemento–enamel junction, JE, junctional epithelium; X, distance between the CEJ and the coronal portion of JE attachment; Y, distance between the CEJ and the ABC. Counting area of RANKL-positive cells: the observed area is indicated by two squares (125 μm × 125 μm) around the surface of the alveolar bone crest.

Results

Serum level of anti-LPS IgG

The serum levels of anti-LPS IgG were elevated in the immunized group on day 5, but not in the nonimmunized group. The level in the immunized group was maintained until day 30. That in the nonimmunized group was gradually elevated on days 20 and 30 (Fig. 2). No serum level of the anti-LPS IgG was detected in the control rats (data not shown).

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). In both experimental groups on day 0, the findings were similar to those of the control rats. On

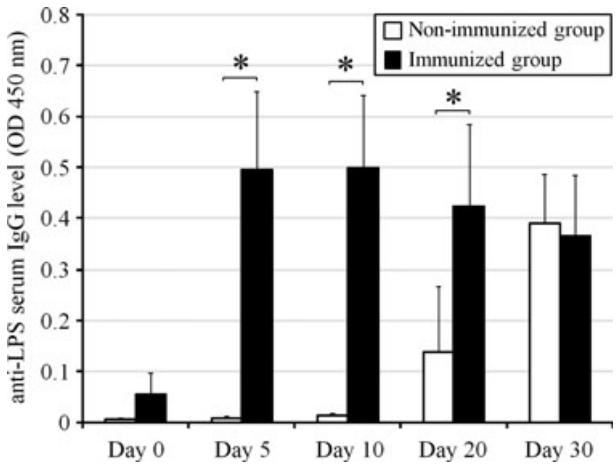

Fig. 2. Serum levels of anti-lipopolysaccharide immunoglobulin G (anti-LPS IgG) in the immunized and nonimmunized groups, determined by measuring the absorbance at 450 nm (A_{450}). Each bar represents the mean ± standard deviation. *Significantly different from the nonimmunized group. Mann–Whitney *U*-test, $p < 0.05$.

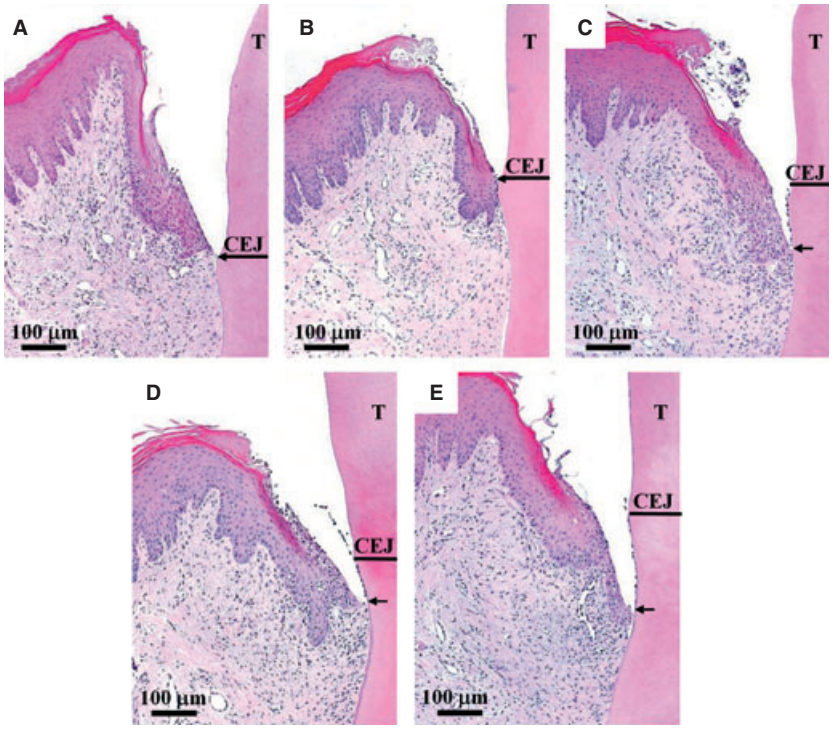

Fig. 3. Histopathological findings of the palatal side of the first molar stained with hematoxylin and eosin. (A) Control rats. (B) The nonimmunized group on day 10. (C) The immunized group on day 10. (D) The nonimmunized group on day 20. (E) The immunized group on day 20. The immunized group shows greater loss of attachment than the nonimmunized group on days 10 and 20. CEJ, cemento–enamel junction; T, teeth. The black arrow indicates the coronal portion of the junctional epithelium (JE) attachment. Scale bar = 100 µm.

days 5 and 10 the infiltration of a few inflammatory cells and slight apical migration of the JE were observed in the nonimmunized group. On the other hand, in the immunized group on day 10, periodontal pocket formation was obvious in the palatal side of the gingiva where LPS was applied (Figs. 3B and C). Coronal JE cells were located at the apical site away from the CEJ. Inflammatory cells, dominantly neutrophils, had infiltrated the JE and the surrounding connective tissue. However, the findings of the buccal side showed no changes. Apical migration of the JE, and pocket formation, were also observed in the nonimmunized group on day 20 (Fig. 3D). In the immunized group on day 20, apical migration of the JE, inflammatory infiltration and pocket formation increased compared with day 10 (Fig. 3E). The palatal alveolar bone surface became irregular, but only a few TRAP-positive cells at the edge of the alveolar bone were seen. On day 30, apical migration of the JE and pocket formation had increased in the nonimmunized group. In the immunized group, deeper periodontal pocket formation and many TRAP-positive cells at the edge of the reduced alveolar bone were seen. In addition to pocket formation, bone resorption was induced at the palatal side where LPS was applied, but not at the buccal side (Fig. 4).

Immunohistological and histometrical investigations

The distances from the CEJ to the coronal portion of the JE or to the alveolar bone crest were increased from day 10 in the immunized group and from day 20 in the nonimmunized group. Loss of attachment and the level of alveolar bone were significantly greater in the immunized group than in the nonimmunized group on days 10, 20 and 30 (Fig. 5A, B).

On day 0 in the connective tissue near the alveolar bone crest and the bone surface, the number of RANKL-bearing cells in the immunized group was similar to the number in the nonimmunized group. However, there was a significant elevation of the number of RANKL-bearing cells in the immunized group on days 5 and 10. On days 20 and 30 there was no significant difference between the two groups (Fig. 6).

Although we could not detect C1qB in all of the sections from the blocks at 24 h after topical application of LPS,

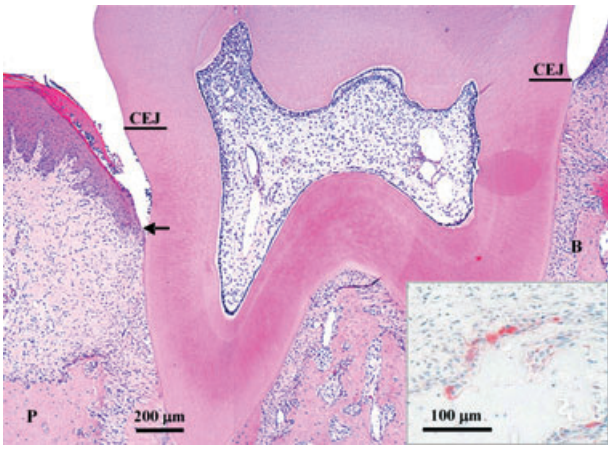

Fig. 4. Histological findings of the palatal side of the first molar stained with hematoxylin and eosin in the immunized group on day 30. A deeper periodontal pocket was present at the palatal site (black arrow) than at the buccal site. B, buccal side; CEJ, cemento–enamel junction; P, palatal side. Scale bar = 200 μm. The insert is a higher magnification of the reduced alveolar ridge, in which some osteoclasts showed a positive reaction to the TRAP stain. Scale bar = 100 μm.

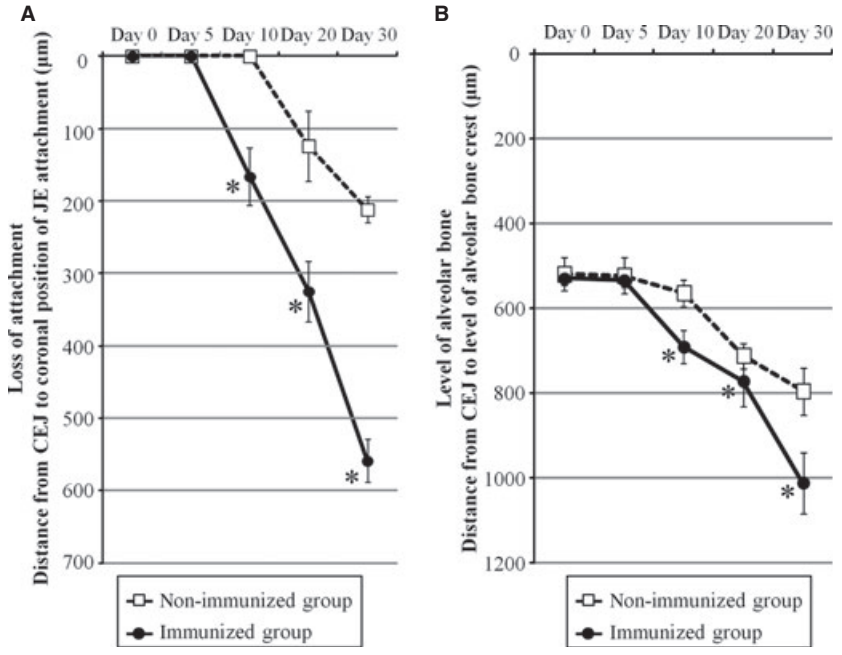

Fig. 5. Histometrical analysis of loss of attachment and level of alveolar bone. (A) The distance from the cemento–enamel junction (CEJ) to the coronal portion of the junctional epithelium (JE) attachment (loss of attachment) was analyzed histometrically. (B) The distance from the CEJ to the alveolar bone crest (level of alveolar bone) was analyzed. Each bar represents the mean ± standard deviation. *Significantly different from the nonimmunized group. Mann–Whitney *U*-test, $p < 0.05$.

C1qB could be observed in the sections from the blocks at 1 h. C1qB was detected in the JE and in the connective tissue near the JE in the immunized group, but not in the nonimmunized group(Fig. 7A, B).

Discussion

In chronic periodontitis, the antibody titers to the periodontal pathogen in the gingival crevicular fluid increase (7–9,15). The antibody titers in the serum and the gingival crevicular fluid in periodontitis patients showed a positive correlation (9,16,17). Therefore, because of the elevation of the level of specific antibody in serum, we thought that the level of specific antibody in the gingival crevicular fluid would also increase in the immunized group owing to the formation of immune complexes between the specific antibody in the gingival crevicular fluid and the LPS antigen in the rat gingival sulcus. Although immune complexes could not be directly detected in the present study, the anti-LPS IgG level increased in serum and C1qB was also detected in the JE and in the connective tissue near the JE on day 10 in the immunized group, but not in the nonimmunized group. Furthermore, loss of attachment and the level of alveolar bone were increased from day 10 in the immunized group compared with the nonimmunized group. Based on the presence of C1qB, it was suspected that immune complex formation may induce inflammatory cell infiltration, periodontal pocket formation and bone resorption (6). The nonimmunized group, with a low level of antibody in the early stage of the experiment, showed no periodontal pocket formation, even though LPS was topically applied. In the nonimmunized group, periodontal pocket was directly correlated to the serum level of anti-LPS IgG, although pocket depth was shallower than in the immunized group. These findings suggest that the response of antibody to antigen plays an important role in periodontal pocket formation, although the levels of serum cytokines, such as interleukin-1beta and tumor necrosis factor-alfa, should also be considered to potentially play a role. As the antibody levels in gingival crevicular fluid were inferred to be the same among each side of the teeth under systemic immunization in the present study, there should be more LPS as an antigen in the palatal gingival sulcus, and consequently attachment loss and bone resorption were induced on the palatal side. These findings were site-specific, which is a characteristic feature of periodontitis.

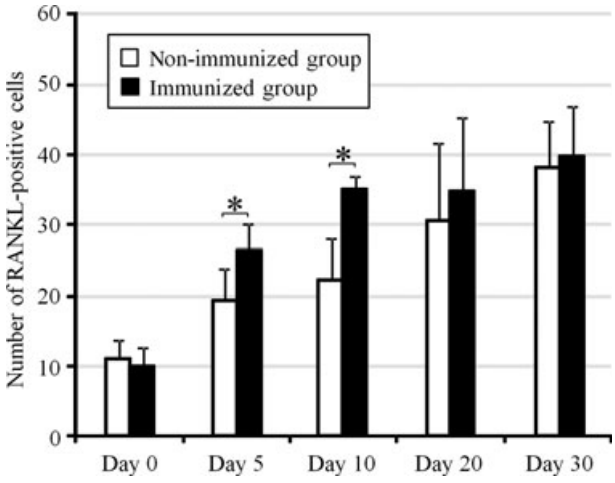

Fig. 6. Number of RANKL-positive cells near the alveolar bone crest. Each bar represents the mean ± standard deviation. *Significantly different from the nonimmunized group. Mann–Whitney *U*-test, $p < 0.05$.

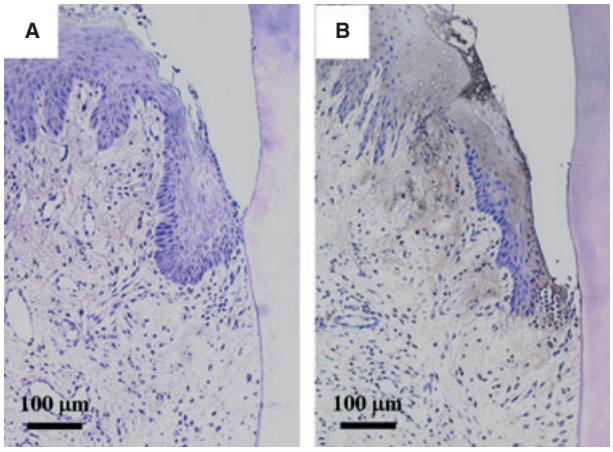

Fig. 7. Localization of C1qB (indicated by dark-red staining) on the palatal side of the first molar. C1qB was not observed in the nonimmunized group on day 10 (A). C1qB was observed in the junctional epithelium (JE) and in the adjacent connective tissue of the immunized group on day 10 (B, dark-red stained area). Scale bar = 100 μm.

In general, to observe immune complexes immunohistologically, antigen and antibody are investigated individually. However, in the present study, it was difficult to detect LPS and LPS antibody immunohistologically, Therefore, we performed immunohistological staining of C1qB to observe localization of immune complexes. It is known that C1qB is a component of complement factor C1 and that it binds to the Fc region of immunoglobulin involved in the formation of immune complexes. C1qB is usually used to detect the presence of immune complexes by immunohistological staining (11). Unfortunately, little tissue produces C1qB constitutively for use as a positive control and therefore we were unable to confirm the specificity of the C1qB antibody used in this study. However, in our previous study we had applied LPS topically as an antigen and its specific or nonspecific antibody onto the gingival sulcus in rats and induced the formation of immune complexes in the gingival tissue (6). To detect the immune complexes, we had used this C1qB antibody and C1qB had been observed only in the group in which LPS and its specific antibody were applied. Therefore, we considered that we could confirm the existence of C1qB immunohistologically and detect immune complexes.

RANKL, a member of the tumor necrosis factor ligand family, is an important factor in bone resorption (18). It has been reported in *in vitro* studies that RANKL expression was enhanced in rat splenocytes from *Aggregatibacter actinomycetemcomitans*-immunized mice in response to *A. actinomycetemcomitans* (19,20). The activation of the classical complement pathway is initiated by immune complex formation (21). The complement components C3a and C5a anaphylatoxins increase the production of RANKL in osteoblasts in the presence of interleukin-1β (22). On days 5 and 10 in the present study, the number of RANKL-positive cells in the connective tissue near the alveolar bone crest and bone surface in the immunized group was increased compared with the number in the nonimmunized group. Furthermore, bone resorption was more prevalent in the immunized group than in the nonimmunized group on and after day 10. In the present study, C3a and C5a might have been involved in the increase of RANKL expression in osteoblasts.

Clinically observed periodontitis shows chronic inflammation associated with abundant plasma cell infiltration (23,24). In the present study, the predominant infiltrations of neutrophils were observed in the JE and in the surrounding connective tissue. The neutrophil is the first line of cellular host response against invading bacteria, but it has also been implicated in tissue degradation through the release of reactive oxygen species and proteolytic enzymes (25,26). An *in vitro* study showed that myeloperoxidase/H_2O_2, elastase and cathepsin G production from active neutrophils caused lysis of the gingival epithelial cells (27). From day 10 in the immunized group, inflammatory cells, chiefly neutrophils, infiltrated the JE and the surrounding connective tissue where C1qB was immunohistologically detected. This discrepancy between the present study and the clinical finding is probably

because the progression in naturally occurring periodontitis is much slower, perhaps with repeating cycles of aggravation and quiescence, than in this experimental model.

In conclusion, the topical application of LPS as an antigen induced the infiltration of inflammatory cells in the JE and the surrounding connective tissue and of RANKL-positive cells on the bone surface in rats systemically immunized with LPS. In consequence, periodontal pocket formation and bone resorption were increased. These findings suggest that immune complex formation and complement activation are associated with periodontal pocket formation and alveolar bone resorption; however, the details of the mechanism remain unclear. Further studies are needed to clarify the mechanism.

Acknowledgements

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