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Bacterial-Responsive B Lymphocytes Induce Periodontal Bone Resorption

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Host immune responses play a key role in periodontal diseases. We have found that B lymphocytes in human periodontal lesions bear abundant receptor activator of NF-κB ligand (RANKL), a major factor in the regulation of osteoclast differentiation. The purpose of this study was to evaluate Actinobacillus actinomycetemcomitans-responsive B lymphocytes in their level of RANKL expression and their effects on periodontal bone resorption. Congenitally athymic Rowett rats received injections of formalin-fixed A. actinomycetemcomitans into the gingival papillae, and donor B cells from normal rats immunized with A. actinomycetemcomitans were transferred via tail vein injection. We demonstrated that B cells from A. actinomycetemcomitans-immunized animals had greater levels of RANKL expression and induced a significantly higher level of osteoclast differentiation from RAW 264.7 cells than did nonimmune B cells that were not Ag specific. This activity was eliminated by incubation with the RANKL decoy receptor osteoprotegerin fusion protein. A. actinomycetemcomitans-binding B cell (ABB) and RANKL-expressing B cells were recovered from the gingival tissues of recipient rats transferred with ABB, but not from recipients of PBS nonimmune B cells or A. actinomycetemcomitans nonbinding B cells. Also, recipients of ABB exhibited increased osteoclast formation on the alveolar bone surface and significant periodontal bone resorption. This effect was antagonized by injection of osteoprotegerin fusion protein into the local gingival tissues. In summary, this study suggests that B lymphocytes can contribute to increased periodontal bone resorption in the absence of T lymphocytes. This effect is associated with the up-regulation of RANKL expression. The Journal of Immunology, 2006, 176: 625–631.

Host immune responses have been suggested to play a key role in periodontal diseases (1, 2). T and B lymphocytes appear to be prominent in chronic periodontal lesions in humans and rodents (3–5). In particular, a progressive lesion has been associated with increased infiltration of B lymphocytes and plasma cells (6, 7). Lymphocyte prominence in periodontal lesions prompted the suggestions that this segment of cellular influx could be responsible for regulation of immune response in periodontal tissues (1, 8, 9). We have developed a model of periodontitis in gnotobiotic Rowett rats (10, 11) infected with Actinobacillus actinomycetemcomitans, a pathogenic microbe linked to periodontal disease. In previous studies, we demonstrated that adoptive transfer of an enriched (1) or cloned (12) population of A. actinomycetemcomitans-specific Th2 cells to A. actinomycetemcomitans-infected rats resulted in decreased bone loss compared with that in animals receiving nonspecific Th2 cells. Recently, we have also investigated the role of Th1 cells in periodontal disease and our results suggested that local Ag-specific activation of Th1 cells appeared to trigger inflammatory bone resorption (11, 13, 14). However, the potential role of B lymphocytes in the progression of periodontal disease, particularly in the generation of inflammatory periodontal bone resorption, has not been elucidated.

Receptor activator of NF-κB ligand (RANKL) is a major factor in the regulation of osteoclast differentiation (15). RANKL, its receptor RANK, and a decoy receptor osteoprotegerin (OPG) are three key molecules that regulate osteoclast recruitment and function (16). We have found that B lymphocytes in human periodontal lesions bear abundant surface RANKL (17). Understanding of the production and regulation of such factors in Ag-specific B cells, both in vitro and in vivo, will allow insights into the mechanisms underlying the involvement of B cell immune response in diseased periodontal tissues.

Understanding the mechanism that results in bone loss due to periodontal disease is in itself an important area of research. However, this information is likely applicable to other inflammatory diseases. Moreover, the mechanism of bone resorption in these diseases is strikingly different from normal physiologic remodeling (18, 19). The purpose of this study was to characterize A. actinomycetemcomitans-responsive B lymphocytes, and to evaluate their level of RANKL expression and their effects on experimental periodontal bone resorption. Our results suggested that the presence of Ag-specific, RANKL-expressing B cells resulted in destructive periodontal bone resorption.

Materials and Methods

Animals

All animals were inbred Rowett rats maintained under pathogen-free conditions in laminar flow cabinets. Experiments using these animals were approved by the Forsyth Institute Internal Animal Care and Use Committee. Normal Rowett rats (nu/nu, male, 4- to 5-mo-old) were used as donors after i.p. immunization with 2 × 105 formalin-killed A. actinomycetemcomitans in PBS as previously described (12), and then the rats were

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Boosted i.p. with the same bacteria (2 × 10^10) 10 days later. Rats injected with PBS were used as control animals to also obtain B cells. Rats were sacrificed 4 days after the booster injection, and spleen cells were obtained and prepared for B cell isolation. B cells isolated from animals injected with A. actinomycetemcomitans were considered immune B cells (IB), whereas B cells from animals injected with PBS were considered nonimmune B cells (NIB). Congenitally athymic (nude) Rowett rats (nu/nu, female, 2.5- to 3-mo-old) were used as recipients for adoptive transfer of donor B cells via tail vein injection.

**B cell preparation, isolation, and characterization**

Single cell suspensions from spleen were applied to a prepared Ficoll-Hyphaque solution (density = 1.088; Sigma-Aldrich) and centrifuged at 2000 × g for 20 min in 20°C to remove erythrocytes and dead cells. Mononuclear cells at the interface were collected and were incubated with a combination of mouse anti-rat Abs (Serotec) to T cell/NK cell surface markers (αG TCR/CD6 for T cells, CD161 for NK cells) at a ratio of 5:4:1 followed by FITC-conjugated rat anti-mouse IgG (Jackson ImmunoResearch Laboratories). Labeled T cells/NK cells were separated from unlabeled cells that served as a purified B cell population excluding by the optimized size and granularity gating during FACS. We used these negative selections to avoid activation of B cells by Ab binding. After sorting two separate times, <1% cells were αG TCR/CD6/CD161-positive. The remaining 2–5% cells were likely PanB/CD45RA-negative B cells (PanB to CD45RA ratio 1:1; Serotec) followed by FITC-conjugated rat anti-mouse IgG (Jackson ImmunoResearch Laboratories). The percentage of PanB/CD45RA-positive B cells in this population was 95–98%. The remaining 2–5% cells were likely PanB/CD45RA-negative B cells that were not detected by our selected B cell markers.

**Generation of specific bacterial reactive B cells**

Purified B cells were incubated with propidium iodide (PI)-labeled formalin-killed A. actinomycetemcomitans (cell to bacteria ratio 1:20, 4°C for 2 h). After incubation, A. actinomycetemcomitans-binding B cells (ABB) were separated from A. actinomycetemcomitans-nonbinding B cells (ANB) by sorting on PI-positive cells using flow cytometry. After separation, the collected cells were trypsinized by the method of Snow et al. (20) to remove A. actinomycetemcomitans and then resuspended as a purified B cell population were collected and with mouse anti-rat Abs to B cell surface markers (PanB to CD45RA ratio 1:1; Serotec) followed by FITC-conjugated rat anti-mouse IgG (Jackson ImmunoResearch Laboratories). The percentage of PanB/CD45RA-positive B cells in this population was 95–98%. The remaining 2–5% cells were likely PanB/CD45RA-negative B cells that were not detected by our selected B cell markers.

**Overall experimental protocol for adoptive transfer**

In these experiments, we developed an Ag-specific B cell adoptive transfer Ag challenge model, similar to our previous rat T cell transfer model, in congenitally athymic (nude) rats to eliminate T cell effects on osteoclastogenesis (13). These experiments were performed to determine the osteoclast-mediated effects of various B cells participating in periodontal bone resorption. Nuclei rat recipients were randomly divided into four groups (n = 6 rats/group) that had received microinjection of formalin-killed A. actinomycetemcomitans in the right palate at three sites (10^7/site) as previously described (13), whereas PBS was injected into the symmetric sites of the left palate as control. The injection was performed on days 0, 2, and 4. On day 0, each group of rats (n = 6 rats/group) also received tail vein injection of PBS, PBS/donor cells NIB, ANB, or ABB (1 × 10^6 cells/ml PBS/animal). To determine the formation of osteoclasts in vivo, calcified maxillary tissue from each rat in each group (n = 3 rats/group) was prepared by cryostat sectioning. The slices were subjected to tartrate-resistant acid phosphatase (TRAP) staining, and osteoclasts were identified as multinucleated TRAP-positive cells lying on the alveolar bone surface. Also, gingival tissues were removed from each rat in each group (n = 6 rats/group), and the isolated gingival mononuclear cells were individually cocultured with RAW 264.7 cells (10^5 cells/well) on a 16-well plate coated with calcium phosphate apatite to assess the formation of resorption pits. In addition, bone resorption was measured in the defleshed jaws of all animals in the groups described (n = 6 rats/group). To determine whether the in vivo bone resorption observed was RANKL-dependent, four groups of rats were used (n = 6 rats/group) for another adoptive transfer experiment as earlier described. The four groups included in this experiment were PBS, ABB alone, ABB plus OPG-Fc, and ABB plus L6-Fc. Human OPG-Fc fusion protein or an irrelevant fusion protein, L6-Fc, was injected into the gingival papillae (1 μg/site) on days 1, 3, and 5 after adoptive transfer of ABB.

**RANKL expression by isolated B cells assessed by RT-PCR analyses**

Total RNA was extracted from the isolated B cells using an RNeasy kit (Qiagen). Isolated RNA (0.1 μg each) was reverse transcribed with SuperScript synthesis system in the presence of random primers (Invitrogen Life Technologies). The resultant cDNA was amplified by PCR with TaqDNA polymerase (Invitrogen Life Technologies) as described by the manufacturer. The primer sequences used for the amplification were as follows: RANKL, 5′-tcaggggtagctccagctgccctcgg-3′ and 5′-aaccagctttgtagctgccac-3′ (product size, 450 bp); OPB, 5′-aacaagcttgctggtctgc-3′ and 5′-agctgcttcgctgctgg-3′ (product size, 750 bp); GAPDH, 5′-tcagctcacatggacactgtc-3′ and 5′-ggctcctctctgcttcagta-3′ (product size, 520 bp). PCR conditions were 94°C, 30 s; 55°C, 30 s, 72°C, 1 min for 35 cycles for RANKL, and OPG, and 30 cycles for GAPDH.

**B cell capacity for induction of osteoclast differentiation**

The mouse macrophage/monoocyte cell line RAW 264.7 (catalog no. TIB-71; American Type Culture Collection) was seeded in 96-well plates at a density of 10^4 cells/well in 150 μl of DMEM supplemented with 10% FCS and 50 ng/ml murine soluble RANKL (R&D Systems). After 2 days the medium was replaced with fresh medium without soluble RANKL, and the RAW 264.7 cells were cocultured with purified B cells (1.5 × 10^5 cells/well) or with the culture supernatant (100 μl/well) of purified B cells for another 3 days. For some experiments, cocultures were performed in the presence of human OPG-Fc (a fusion protein provided by Dr. C. Dunstan, Amgen, Thousand Oaks, CA) at a concentration of 1 μg/ml, to confirm that the observed osteoclastogenesis of RAW cells were mediated through RANKL–RANK interaction. For TRAP activity assay, the adherent cells were lysed with 100 μl of CellLytic-M lysis reagent (Sigma-Aldrich) and incubated for 30 min in a reaction buffer containing 1 mg/ml p-nitrophenyl phosphate (Sigma-Aldrich). The reaction was stopped with 1 N NaOH, and absorbance was read by spectrophotometer at 405 nm. Cells were stained for TRAP activity using a Leukocyte Acid Phosphatase kit (Sigma-Aldrich) according to the manufacturer curves provided by dilution of an Ab-containing hyperimmune serum.

**Determination of serum Ab levels to A. actinomycetemcomitans by ELISA**

Formalin-killed A. actinomycetemcomitans were bound onto 96-well plates at 1 × 10^4 dilution. Rats were sacrificed on days 1, 3, and 5 after adoptive transfer of B cells to the recipients rats. Serum was determined by rabbit anti-rat IgG or mouse anti-rat IgM Ab (Serotec), followed by alkaline phosphatase-conjugated goat anti-rabbit IgG Ab or goat anti-mouse IgG Ab (Sigma-Aldrich). After incubation in p-nitrophenyl phosphate solution (1 mg/ml) for 30 min, the reaction was terminated by the addition of 1 N NaOH and the absorbance was determined by spectrophotometer at 405 nm. The Ab levels were expressed as ELISA units based on a reference curve curve provided by dilution of an Ab-containing hyperimmune serum.

**Flow cytometric analyses**

Mononuclear cells were isolated from spleen, cervical lymph nodes, and gingival tissues as previously described (22) and were characterized for IgG-positive cell percentage, RANKL expression, and A. actinomycetemcomitans-binding capacity by flow cytometry. Briefly, the percentage of IgG-positive B cells was determined by incubating the B cells with rabbit anti-rat IgG or mouse anti-rat IgM Ab (Serotec), followed by alkaline phosphatase-conjugated goat anti-rabbit IgG Ab or goat anti-mouse IgG Ab (Sigma-Aldrich). After incubation in p-nitrophenyl phosphate solution (1 mg/ml) for 30 min, the reaction was terminated by the addition of 1 N NaOH and the absorbance was determined by spectrophotometer at 405 nm. The Ab levels were expressed as ELISA units based on a reference curve provided by dilution of an Ab-containing hyperimmune serum.

**Tissue preparation and histochemical staining**

The maxillary samples from each group (PBS, NIB, ANB, and ABB, n = 3 rats/group) were dissected and fixed in 4% formaldehyde overnight at 4°C. The specimens were then washed consecutively in 5, 10, and 15% glycerol in PBS and decalcified in 10% EDTA/0.1 M Tris for 3 wk at 4°C.
Decalcified samples were cut in half through the midline of the palate, embedded in OCT compound (Miles) and frozen at −80°C for 1 h. Serial sections (6 μm) were cut by cryostat sectioning. For TRAP staining, the slides were incubated for 1 h in the TRAP staining solution at 37°C in the dark. The slides were then counterstained with 1% methyl green. Osteoclasts were identified as multinucleated TRAP-positive cells, lying on the alveolar bone surface. For quantitative image analysis, the number of osteoclasts per millimeter of the alveolar bone surface was measured using a computerized image analysis program (AxioVision 3.1; Carl Zeiss MicroImaging).

Assessment of bone resorption pit formation

RAW 264.7 cells (10³ cells/well) were plated in BioCoat Osteologic 16-well plates coated with calcium phosphate apatite (BD Bioscience) in 300 μl DMEM supplemented with 10% FBS and 50 ng/ml soluble RANKL. After 2 days, the medium was replaced with fresh medium without soluble RANKL. Mononuclear cells recovered from nude rat gingival tissues (7 days after adoptive transfer) were added onto the plate at 4 × 10⁵ cells/well and cocultured with RAW 264.7 cells for 5 days. After termination of culture, all adherent cells were removed from the Osteologic disk using 1 M NaOH, and digital images of the disk were taken under darkfield microscopy. The areas of resorption lacunae on the digital images were quantified using a computerized image analysis program (AxioVision 3.1; Carl Zeiss MicroImaging), and the percentage of the resorbed area was calculated relative to the total well area.

Measurement of bone resorption

After defleshing of the maxillary jaws, periodontal bone resorption was measured as previously described (13). Briefly, the distances from cemento-enamel junction (CEJ) to the alveolar crest (CEJ to AC) on the palatal side of each root were measured using a microscope with a reticule eyepiece under magnification of ×25. Recordings were made in the long axis of the root surfaces of all molar teeth. A total of seven recordings was evaluated in each quadrant, including three roots of the first molar and both roots of the second and the third molars. Measurements were made without prior knowledge of the group designation of the animals, and the recordings were verified by a second examiner. The bone resorption was calculated and expressed as percentage of bone loss with the equation: \((\text{total CEJ to AC distance of right maxilla} - \text{total CEJ to AC distance of left maxilla})/\text{(total CEJ to AC distance of left maxilla)} \times 100\).

Results

Characterization of isolated B cells in vitro

The level of RANKL mRNA expression in immune B cells and NIB was detected by RT-PCR. RANKL mRNA transcripts were greatly increased in immune B cells compared with NIB, indicating a higher level of RANKL expression in B cells from immunized animals (Fig. 1A). RANKL expression in the cultured immune B cells peaked at 3 days, and then decreased after 7 days (Fig. 1B). OPG mRNA transcript levels did not change throughout the conditions tested (Fig. 1, A and B). Cultured NIB did not demonstrate detectable RANKL mRNA transcripts (data not shown). The TRAP activity assay was performed to test these purified B cells for the induction of osteoclast differentiation. Osteoclast precursor cells (RAW 264.7 cells) demonstrated significantly increased TRAP activity when cocultured with immune B cells or with culture supernatants of immune B cells as compared with controls (Fig. 2, A and B; p < 0.001). Addition of human OPG-Fc into the culture significantly diminished such increases, suggesting that the observed induction of osteoclastogenesis of RAW cells is RANKL-dependent. No such effect was observed in RAW cells cocultured with NIB or in their supernatant when compared with the controls (Fig. 2, A and B). In a separate experiment, cocultures of RAW cells with immune B cells exhibited significantly larger number of multinucleated TRAP-positive cell formation compared with controls (Fig. 2, C and D; p < 0.001). Cocultures of RAW cells with supernatant of cultured immune B cells also demonstrated a greater number of multinucleated TRAP-positive cell formation compared with controls (p < 0.001). Similarly, these effects occurred in a RANKL-dependent fashion as demonstrated by the reduction of TRAP-positive cell formation in the presence of human OPG-Fc (Fig. 2). There were no significant difference in the formation of multinucleated TRAP-positive cells between RAW cells cocultured with NIB and controls (Fig. 2, C and D).

We further separated the immune B cells into ABB and ANB to characterize A. actinomycetemcomitans-specific B cells. The percentage of IgG⁺ cells, RANKL expression level, and A. actinomycetemcomitans-binding capacity were tested on the three different B cell populations, ABB, ANB, and NIB, using flow cytometric analyses (Fig. 3). Although the majority of B cells tested were IgG⁺ cells (70–80%), there was no difference in the percentage of IgG⁺ cells among the three cell populations. However, the percentage of RANKL-expressing IgG⁺ cells was significantly higher in ABB (30.6 ± 1.1%) than in ANB (17.6 ± 3.9%) and NIB (6.2 ± 3.2%). As expected, the percentage of A. actinomycetemcomitans-binding IgG⁺ cells was significantly higher in ABB (54.6 ± 1.5%) than in ANB (5.1 ± 1.4%) and NIB (5.0 ± 2.1%). NIB contain very few A. actinomycetemcomitans-binding cells and RANKL-positive cells.

Ab production after adoptive transfer

To determine the status of transferred B cells in vivo, serum IgG Ab to A. actinomycetemcomitans was measured 10 days after transfer of the different B cell populations (Fig. 4). Only rats receiving ABB showed a significantly higher level of serum IgG Ab to A. actinomycetemcomitans compared with rats receiving ANB and NIB (p < 0.001), indicating that only ABB-transferred rats retained significant Ab synthesizing capability. There were no changes in IgM Ab production throughout the tested period (data not shown). These results suggest that transferred immune B cells survive in recipient rats and produce mainly IgG Ab to A. actinomycetemcomitans during the indicated time period.

Tissue distribution of transferred B cells

Ten days after B cell transfer, the percentage of IgG⁺ cells, RANKL-expressing IgG⁺ cells, and A. actinomycetemcomitans-binding IgG⁺ cells were enumerated in tissues extracted from recipient rats using flow cytometric analyses. In spleen and cervical lymph nodes, the percentage of IgG⁺ cells, RANKL-expressing IgG⁺ cells, and A. actinomycetemcomitans-binding IgG⁺ cells...
was very low and there were no differences among animals transferred with different B cell populations (data not shown). However, in gingival tissues injected with *A. actinomycetemcomitans*, the percentage of IgG-positive cells, RANKL-expressing IgG-positive cells, and *A. actinomycetemcomitans*-binding IgG-positive cells was significantly elevated in ABB-transferred animals as compared with the percentage in animals transferred with ANB, NIB, or PBS (Fig. 5). These data verified that *A. actinomycetemcomitans*-expressing cells and/or their progeny may be recruited during the immune response to *A. actinomycetemcomitans* and accumulate in the gingival tissues where *A. actinomycetemcomitans*-specific Ags are encountered.

Formation of multinucleated TRAP-positive cells in vivo

To further investigate the effect of transferred B cells on periodontal bone resorption, the formation and localization of osteoclast cells were evaluated in vivo by TRAP staining of maxillary tissue sections. Multinucleated TRAP-positive cells were only observed in alveolar bone surfaces of ABB-transferred rats (3.5 ± 0.8 cells/mm). No TRAP-positive cells were observed in animals transferred with ANB, NIB, or PBS (Fig. 6).

Bone resorption pit formation

To test the ability of transferred B cells to induce bone resorption, RAW 264.7 cells were cocultured with mononuclear cells recovered from gingival tissues of recipient rats on a 16-well plate coated with calcium phosphate apatite (Fig. 7). RAW 264.7 cells cocultured with mononuclear cells recovered from gingival tissues of ABB recipients demonstrated significantly greater induction of bone resorption pit formation compared with cells from PBS, NIB, or ANB recipients (p < 0.01).

Alveolar bone resorption

Ten days after adoptive transfer, alveolar bone loss was measured on the palatal side of maxillary jaws to evaluate any effects of transferred B cells on the progression of periodontal bone resorption. As shown in Fig. 8A, rats transferred with ABB demonstrated significantly elevated alveolar bone resorption (24.4 ± 6.2%) as compared with rats transferred with ANB (7.6 ± 2.2%) or NIB (6.7 ± 2.8%) (p < 0.05). There were no significant differences in the percentage of bone loss among ANB, NIB, and PBS recipients. In a separate experiment, human OPG-Fc fusion protein or the irrelevant protein L6-Fc was injected into the gingival papillae (1 μg/site) after adoptive transfer of ABB cells (Fig. 8B). ABB-induced alveolar bone resorption (13.3 ± 2.0%) was significantly inhibited by local injection with OPG-Fc (2.0 ± 2.8%), but was not affected by the injection with unrelated control fusion protein L6-Fc (10.4 ± 1.1%) (Fig. 8B). These results confirmed that the observed bone loss in ABB transferred animals is RANKL-dependent.

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**FIGURE 2.** Induction of osteoclast differentiation by NIB and IB. RAW 264.7 cells were first cultured with 50 ng/ml murine soluble RANKL for 2 days, then were cocultured with isolated B cells (A and C) or with culture supernatant of isolated B cells (B and D) for another 3 days. Cocultures were performed in the presence or absence of human OPG-Fc at a concentration of 1 μg/ml. A and B, TRAP activity was detected in a reaction buffer containing 1 mg/ml ρ-nitrophenyl phosphate and the absorbance was read spectrophotometrically at 405 nm. C and D. For TRAP staining, cells were stained using a Leukocyte Acid Phosphatase kit (Sigma-Aldrich) and multinucleated TRAP-positive cells were counted. Data are mean ± SE of n = 6 rats (**, p < 0.01).

**FIGURE 3.** Properties of different B cell populations. After collection of isolated B cells, the percentage of IgG-positive cells, surface RANKL expressing cells, and *A. actinomycetemcomitans* (Aa) binding cells were evaluated by flow cytometry. At least 20,000 B cells were counted. Data are mean ± SE in n = 5 rats (*, p < 0.05; **, p < 0.01).

**FIGURE 4.** Determination of serum IgG Ab to *A. actinomycetemcomitans* in recipient rats by ELISA. PBS, NIB, ANB, or ABB cells were transferred to recipients through tail vein injection, and serum IgG Ab to *A. actinomycetemcomitans* (Aa) was determined 10 days after transfer of different B cells. Data are mean ± SE of n = 3 rats (**, p < 0.01).
Host immune responses have been implicated in the onset and progression of periodontal diseases. We have extensively studied the role of immune responses in rodent experimental periodontal disease (10–13). In this study, we characterized B cells on their RANKL expression and on the induction of osteoclast differentiation. B cells from *A. actinomycetemcomitans*-immunized animals had greater levels of RANKL expression and induced a significantly higher level of osteoclastogenesis in RAW cells. Further tests on ABB, ANB, and NIB confirmed that *A. actinomycetemcomitans*-responsive B cells were mainly IgG cells and had markedly increased levels of surface RANKL. These results suggested that Ag-responsive B cells could contribute to RANKL accumulation in animals immunized with *A. actinomycetemcomitans*, which may lead to an enhancement of the induction of osteoclast differentiation.

Because *A. actinomycetemcomitans* bacteria can also trigger host humoral immune response (24), it is essential to evaluate the level of residual bacteria potentially attached to B cells before adoptive transfer. Residual bacteria on the B cell surface were detected by PCR amplification of *A. actinomycetemcomitans*-specific DNA fragment coding for surface membrane protein OMP29. Few than 5/10^2 *A. actinomycetemcomitans* per 10^6 B cells were detected (data not shown). Our previous experiments indicated that rats transferred with such a number of fixed *A. actinomycetemcomitans* showed no Ab response (our unpublished observations), indicating that the immune responses and effect on bone resorption observed in this study were due to the transferred B cells and not to residual *A. actinomycetemcomitans*.

The production of serum IgG Ab to *A. actinomycetemcomitans* could be detected 10 days after cell transfer. This result clearly indicated that enriched ABB actually survived in the recipients' gingival tissues for at least 10 days.
A. actinomycetemcomitans- and RANKL-expressing B cells were recovered from gingival tissues (Fig. 5), particularly at the A. actinomycetemcomitans-microinjected sites, suggested that Ag-specific B cells or their descendants may home to gingival sites exposed to Ags and produce Ab in recipients.

It has been well established that alveolar bone resorption in periodontal disease is mediated by enhanced osteoclastogenesis (25). RANKL is a TNF-related cytokine that has not only been reported to be involved in physiological osteoclastogenesis, but also in pathological bone loss (23, 26). Of considerable importance is the recent finding that B cells are key participants in the RANKL-mediated bone resorption (27). Our results showed that the recipients adoptively transferred with A. actinomycetemcomitans-responsive B cells exhibited significantly more bone resorption when compared with other groups. A. actinomycetemcomitans-binding and RANKL-expressing B cells were recovered from the gingival tissues of recipient rats transferred with A. actinomycetemcomitans-responsive B cells. These data suggest that B lymphocytes can contribute to increased periodontal bone resorption during immune response to A. actinomycetemcomitans in periodontal disease and this effect is associated with the up-regulation of RANKL expression.

Our previous studies using immunohistochemical analyses and confocal microscopy indicated abundant RANKL expression on T cells and B cells in the gingival lesions of patients with chronic periodontitis, and both cell types taken from gingival tissues can induce osteoclastic development and associated bone-pit formation (our unpublished observations). Interestingly, the percentage of RANKL-positive T cells (50–60%) in diseased periodontal lesions is substantially less than the percentage of RANKL-positive B cells (>90%). It is not clear whether this difference indicates that B cells can contribute more extensively to bone resorption than T cells in the lesions. In our adoptive transfer studies Th2 cells ameliorated bone resorption and resulted in marked increased in Ab (12, 28). In the more recent studies several Th1 Ag-specific clones gave rise to significant bone resorption that could be ablated with CTLA4-Ig and OPG-Fc (13, 22). In this study we focused on Ag-specific B cells and studied their potential role in experimental periodontal disease in vivo using an adoptive transfer model. The congenitally athymic rats were selected as recipients in our experiments because they do not develop a thymus and have deficient T cell-B cell function (29–31). Therefore, the effects of adoptively transferred B cells on inflammatory periodontal bone resorption can be directly evaluated. Our results demonstrated that congenitally athymic recipients of Ag-specific RANKL-expressing B cells exhibited markedly increased osteoclast formation on the alveolar bone surface and quite significant periodontal bone resorption. Therefore, Ag-specific RANKL-expressing B lymphocytes can directly mediate bone resorption in the absence of T lymphocytes. The information obtained in these studies should contribute to development of therapeutic strategies to treat inflammatory periodontal bone disease and other systemic bone disorders associated with abnormal osteoclast functions related to B cells in tissue inflammation.

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Disclosures

The authors have no financial conflict of interest.

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