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Effect of adoptive transfer of antigen-specific B cells on periodontal bone resorption

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Background and Objectives: Host immune responses to periodontal pathogens have been considered to contribute to the alveolar bone destruction in periodontitis. However, the role of B lymphocytes in the pathogenesis of periodontal bone loss is not clear.

Methods: We examined the effect of adoptive transfer of antigen-specific B cells from rat spleens on experimental periodontal bone resorption. Donor rats were immunized intraperitoneally (i.p.) with formalin-killed *Actinobacillus actinomycetemcomitans*. Antigen-specific B cells were prepared from splenocytes by first binding $CD43^+$ cells to Petri dishes coated with anti-CD43 antibody to remove T cells, and non-binding cells were passed through a nylon wool column to deplete accessory cells. The retained cells were then collected and bound to *A. actinomycetemcomitans*-coated Petri dishes for enrichment of *A. actinomycetemcomitans*-binding B cells (AAB). *A. actinomycetemcomitans* non-binding B cells (ANB) and B cells from non-immunized donor rats (NIB) were also collected from these procedures. Each type of B cell was injected into a group of recipient rats that were then orally infected with live *A. actinomycetemcomitans*.

Results: At termination, the antibody levels to *A. actinomycetemcomitans* in serum and gingival wash fluids were significantly higher in the recipients transferred with AAB when compared to the recipients transferred with ANB or NIB. A markedly elevated number of antibody-forming cells were observed in the spleens of the recipients transferred with AAB, and these recipient rats also exhibited significantly increased bone resorption when compared to the other groups.

Conclusions: It is suggested that B cells can contribute to periodontal bone resorption and that antigen-triggering of B cells is required for the bone resorption.

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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). The progressive lesion has been associated with increased infiltration of B lymphocytes and plasma cells (6, 7), although it is clear that T cells are also present (1, 8). Lymphocyte prominence in periodontal lesions prompted the suggestion that this segment of cellular influx could be responsible for regulation of immune response in periodontal tissues (1, 9, 10). In previous studies, we demonstrated that adoptive transfer of an enriched (1) or cloned (11) population of *Actinobacillus actinomycetemcomitans*-specific Th2 cells to *A. actinomycetemcomitans*-infected rats resulted in decreased bone loss compared with that in animals receiving non-specific Th cells. Recently, we have also investigated the role of Th1 cells in periodontal disease and our results suggested that local antigen-specific activation of Th1-type T cells appeared to trigger inflammatory bone resorption (12–14). However, the potential role of B lymphocytes in the progression of periodontal disease, particularly in periodontal bone resorption, has not been studied.

We have developed a model of periodontitis in gnotobiotic Rowett rats infected with *A. actinomycetemcomitans* (12,15). In this study, we used a previously developed method to enrich *A. actinomycetemcomitans*-binding B cells from immunized donors (AAB), *A. actinomycetemcomitans* non-binding B cells from immunized donors (ANB) and B cells from non-immunized donors (NIB). We then investigated the role of antigen (*A. actinomycetemcomitans*)-specific B cells in experimental periodontal bone resorption by adoptively transferring B cell populations of different specificity into recipient rats followed by oral infection with live *A. actinomycetemcomitans*. Our results indicated that the extensive presence of *A. actinomycetemcomitans* antigen-specific B cells resulted in experimental destructive periodontal bone resorption.

Material and methods

Animals and immunization

All animals were male Rowett rats maintained under pathogen-free conditions in laminar flow cabinets (inbred at the Forsyth Institute over 20 years) (12). Experiments using these animals were approved by the Forsyth Institute's Internal Animal Care and Use Committee (IACUC). Four-month-old rats were used as donors, and recipients were 30 ± 2 days old. Donor rats were first immunized intraperitoneally (i.p.) with 2×10^8 formalin-killed *A. actinomycetemcomitans* in phosphate-buffered saline as described previously (11), and were boosted i.p. with the same bacteria (2×10^7) 2 weeks later. Rats were killed 4 days after the booster injection, and spleen cells were isolated and prepared for cell transfer.

B cell enrichment

Single cell suspensions from spleen were applied to a prepared Ficoll-Hypaque solution (density = 1.088; Sigma Diagnostics, St. Louis, MO, USA) and centrifuged (2000 *g*, 20 min, 20°C) to remove erythrocytes and dead cells. Mononuclear cells at the interface were collected and resuspended in RPMI 1640 complete medium containing 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine and 10 mM HEPES buffer. T cells were removed by incubating the mononuclear cells on Petri dishes precoated with 20 μg/ml of monoclonal anti-CD43 antibody (W3/13, Sera-Laboratory, Indianapolis, IN, USA) in sodium bicarbonate buffer (pH 9.6) at 37°C for 1 h. This antibody has been widely used as a rat T lymphocyte marker (16–18). Nonadherent cells were collected as CD43 negative cells and were passed over a prewarmed (37°C) nylon wool column (1×10^8 cells/0.6 g nylon wool) (19) to remove the accessory cells (macrophages and other antigen presenting cells (APCs)). After incubation at 37°C for 45 min, the column was extensively washed with 5% fetal calf serum in RPMI 1640 medium, and the nylon wool was aseptically removed and teased in medium in Petri dishes to release retained cells. The retained cells were collected and served as an enriched B cell population. The collected B cells were reacted with rabbit anti-rat immunoglobulin G (IgG) (20) followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (affinity purified $F(ab')^2$, Biosource International, Inc., Camarillo, CA, USA). After staining, cells were examined by fluorescence microscopy (at least 1000 cells were counted) and the percentage of IgG-positive cells of total cells examined was 83.3 ± 4.8%.

Actinobacillus actinomycetemcomitans panning

Petri dishes coated with 8×10^8/ml formalin-killed *A. actinomycetemcomitans* were kept at 37°C for 3.5 h, then incubated at 4°C for at least 2 days. Adherence of *A. actinomycetemcomitans* to the Petri dishes was confirmed by enzyme-linked immunosorbent assay (ELISA), using rabbit anti-*A. actinomycetemcomitans* antibody (21), followed by alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Zymed Laboratories, Inc., San Francisco, CA, USA). Enriched B cells were incubated on *A. actinomycetemcomitans*-coated dishes at 37°C for 1 h. *A. actinomycetemcomitans* non-binding B cells (ANB; non-specific B cells from immunized donors) were collected by washing with prewarmed (37°C) RPMI supplemented with 5% fetal calf serum. Then, *A. actinomycetemcomitans* binding B cells (AAB; specific B cells from immunized donors) were collected by vigorous pipetting in cold RPMI. The collected B cells were reacted with rabbit anti-rat IgG (20) followed by FITC-conjugated affinity purified goat anti-rabbit IgG [$F(ab')^2$, Biosource International]. The percentage of IgG-positive cells was 93.5 ± 1.1%.

Actinobacillus actinomycetemcomitans binding assay

For the detection of B cells that bind to *A. actinomycetemcomitans*, the collected B cells (10^6) were mixed with formalin-killed *A. actinomycetemcomitans* (2×10^7) on ice for 1 h and then fixed with 1% paraformaldehyde. The cells were examined by fluorescent microscopy after reaction with rabbit anti-*A. actinomycetemcomitans* antibody (21) followed by FITC-conjugated affinity purified goat anti-rabbit IgG antibody [$F(ab')^2$, Biosource International]. At least 1000 cells were counted in each experiment. Only cells binding three or more visible bacteria were counted. The percentage of *A. actinomycetemcomitans*-positive cells in AAB vs. ANB was 16 ± 0.13% vs. 0.08 ± 0.05%. In a separate experiment, CD43 positive cells (T cells) were examined for the ability to bind *A. actinomycetemcomitans* using the method described above. The percentage positive cells binding *A. actinomycetemcomitans* was 0.05 ± 0.05%. This strongly suggested that T cells do not bind to *A. actinomycetemcomitans* and that binding is specific to B cells.

Trypsin treatment

After *A. actinomycetemcomitans* panning, both AAB and ANB cells were treated with trypsin by the method of Snow *et al.* (22) to remove attached bacteria. Briefly, cells were incubated in 0.05% trypsin-EDTA in RPMI medium

(37°C, 30 min). Thereafter, cells were washed with 100% fetal calf serum and resuspended in RPMI medium supplemented with 10% fetal calf serum. Residual bacteria on the B cell surface were detected by fluorescence microscopy as described above and the number were calculated ($< 10^4$ *A. actinomycetemcomitans*/10^6 B cells). These cells were cultured in complete RPMI medium overnight (37°C, 5% CO_2) for recovery of Ig receptors and antibody synthetic capability.

Antibody formation in recipient rats after transfer of B lymphocytes

An initial experiment was performed to determine whether transferred B cells are active in recipient rats. In this experiment, recipient rats (30 ± 2 days old) were divided into four groups of 2–3 rats/group/time point. The recipient rat groups received either $7.5–10 \times 10^5$ live specific B cells in 500 µl phosphate-buffered saline, or the same number of specific B cells fixed with 1% paraformaldehyde, or the calculated maximum number of residual *A. actinomycetemcomitans* potentially carried with the specific B cells after trypsin digestion (approximately 8×10^3 *A. actinomycetemcomitans*) or saline. Rats were bled from the retro-orbital venous plexus at 3, 7, 14, 21 and 28 days after cell transfer. Serum IgG antibody to *A. actinomycetemcomitans* was assessed at these intervals.

Adoptive transfer and oral infection

Recipient rats received specific B cells (1×10^6 per animal) through tail vein injection and were divided into three groups based on the B cells transferred. The first group received AAB, $n = 17$, the second group received ANB, $n = 11$, and the third group received NIB, $n = 18$. One day after cell transfer, each rat was orally infected with live *A. actinomycetemcomitans* (2×10^8) as described previously (11) for five consecutive days. The presence of infection at the time of tissue harvest was verified by immunofluorescence with rabbit anti-*A. actinomycetemcomitans* serum (21) as previously described (11). The animals were fed with sterilized L356 diet (Harlan Teklad, Madison, WI, USA) and were given autoclaved water *ad libitum*. Six months after cell transfer and oral infection, rats were killed and tissue samples were collected for evaluation as described below.

Determination of antibody levels to *A. actinomycetemcomitans* in serum and gingival wash fluid

Peripheral blood was collected by cardiac puncture. Gingival tissue was prepared as previously described (12). Briefly, after surgical removal of marginal gingivae (approximately 1 mm in width), the tissues were placed in a 60 mm Petri dish containing 2 ml RPMI medium with 10 units/ml heparin and were cut into 1 mm^3 pieces and then were compressed through a 60 gauge stainless steel screen. After centrifugation, the supernatant was collected and was termed 'gingival wash fluid'. IgG and IgM antibody to *A. actinomycetemcomitans* in serum and in gingival wash fluid were determined by ELISA (23), using rabbit anti-rat IgG or IgM antisera, followed by alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Zymed Laboratories). After incubation in *p*-nitrophenyl phosphate disodium solution (1 mg/ml) for 30 min, the reaction was terminated by the addition of 1 N NaOH and the absorbance was determined spectrophotometrically at 405 nm. Antibody titer was expressed as ELISA units (EU) relative to an antibody-containing serum standard.

Solid-phase enzyme-linked immunospot assay

In order to detect the *A. actinomycetemcomitans*-specific antibody-secreting cells in spleen, the solid-phase enzyme-linked immunospot (ELISPOT) assay was performed as previously described (24). Briefly, spleen cells were incubated in a 24-well culture plate precoated with *A. actinomycetemcomitans* for 4 h (37°C, 5% CO_2). After washing with phosphate-buffered saline, cells were incubated with biotin-conjugated goat anti-rat IgG antibody (Accurate Chemicals & Scientific Corporation, Westbury, NY, USA) followed by peroxidase-conjugated streptavidin (Zymed). Enzyme substrate containing 1% agarose in 0.15 M phosphate buffer, pH 7.0 supplemented with paraphenylenediamine (0.5 mg/ml) was added to the wells and spot forming cells (SFC) on each plate were counted microscopically and expressed as SFC per 10^6 spleen cells.

Horizontal bone loss

After defleshing of mandibular and maxillary jaws, a microscope with a reticule eyepiece was used to measure the distance from the cementoenamel junction to the alveolar crest under 25 × magnification. Recordings were made in the long axis of both buccal and lingual root surfaces of all molar teeth as previously described (25). There are six recordings for the first molar, which has three roots, and four recordings for the second and third molars, each of which have two roots. The sum of the recordings for each tooth surface was used as a measure of the total bone loss expressed in millimeters. Measurements were made without prior knowledge of the group designation of the animals.

Results

Antibody production after transfer of *A. actinomycetemcomitans*-specific B cells

To test if transferred B cells produce antibody to *A. actinomycetemcomitans in vivo*, live AAB (1×10^6 cells in 500 µl phosphate-buffered saline) were transferred to 30 ± 2-day-old recipient rats via tail vein injection. Blood was obtained from the retro-orbital venous plexus 3, 7, 14, 21 and 28 days after cell transfer, and serum IgG antibody to *A. actinomycetemcomitans* was determined by ELISA. Antibody was detected within 7 days after AAB transfer and the level peaked at 14 days (Fig. 1). The antibody level remained high until 28 days after AAB transfer. In contrast, killed AAB did not give rise to any antibody

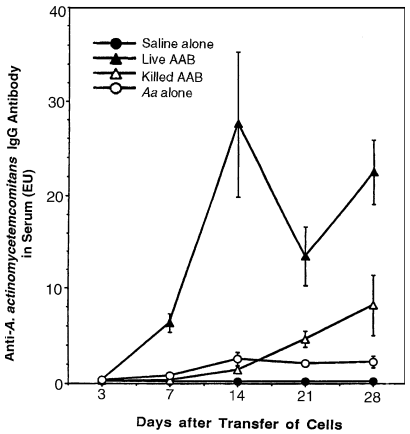

Fig. 1. Kinetics of antibody production in the serum of recipients after transfer of B lymphocytes. Live *A. actinomycetemcomitans*-binding B cells (AAB) ($7.5–10 \times 10^5$), 1% paraformaldehyde-fixed AAB ($7.5–10 \times 10^5$), *A. actinomycetemcomitans* (8×10^3), or saline was transferred into groups of recipient rats ($n = 2–3$) via tail vein injection. After the indicated time, venous blood was taken from the retro-orbital plexus and the level of anti-*A. actinomycetemcomitans* IgG antibody in serum was determined by ELISA. The absorbance was determined spectrophotometrically at 405 nm and the antibody level was expressed as ELISA units (EU) relative to an antibody-containing serum standard (Mean ± SE).

production at 14 days after cell transfer and demonstrated a slight increase of antibody production starting at 21 days. This modest late response could be due to the residual surface-bound *A. actinomycetemcomitans* on the fixed AAB and can be contrasted with animals immunized with the minimal dose of *A. actinomycetemcomitans* devoid of cells. As controls, rats transferred with saline did not show a detectable level of antibody production during the same period. These results suggest that transferred AAB survived in recipient rats and produced antibody to *A. actinomycetemcomitans* during the indicated period. Furthermore, rats were also transferred with a corresponding number of fixed *A. actinomycetemcomitans* (8×10^3 per animal) that represent the amount of the residual bacteria potentially still attached to trypsinized AAB. As shown in Fig. 1, the *A. actinomycetemcomitans* transferred group produced little or no antibody.

Antibody production at termination of specific B cell transfer and *Actinobacillus actinomycetemcomitans* infection

In order to compare the effects of transferred B cells of different specificity, we measured the levels of serum IgG and IgM antibody to *A. actinomycetemcomitans* in the serum of different recipient animals at the termination of the transfer and infection experiment (Fig. 2). Only the group receiving AAB demonstrated significantly elevated IgG and IgM antibody as compared to either the group receiving ANB ($p < 0.008$ for IgG; $p < 0.0001$ for IgM) or the group receiving NIB ($p < 0.0001$ for both IgG and IgM). Little or no anti-*A. actinomycetemcomitans* antibody was detected in the groups receiving ANB or NIB.

We also investigated IgG and IgM antibody levels in the gingival wash fluid indicative of the local response to oral infection after adoptive transfer. IgG antibody was significantly elevated in the AAB transferred group when compared with either ANB or NIB transferred group ($p < 0.0001$) (Fig. 3). However, no differences in IgM antibody levels were detected among all the tested groups (data not shown). Together, these findings sug-

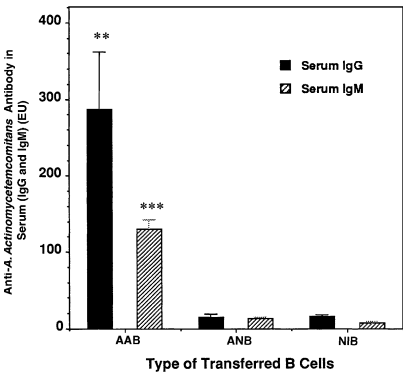

Fig. 2. Immunoglobulin G (IgG) and IgM antibody levels in serum after cell transfer. Different B cell populations [*A. actinomycetemcomitans*-binding B cells (AAB), $n = 17$; *A. actinomycetemcomitans* non-binding B cells (ANB), $n = 11$; and non-immunized donor B cells (NIB), $n = 18$] were isolated as described in 'Material and methods' and transferred into recipient rats via tail vein injection. One day after cell transfer, each rat was orally infected with live *A. actinomycetemcomitans* (2×10^8) for 5 consecutive days. Six months after adoptive transfer, at termination of the experiment, anti-*A. actinomycetemcomitans* IgG and IgM antibody levels in the serum were measured by enzyme-linked immunosorbent assay (ELISA). The absorbance was determined spectrophotometrically at 405 nm and the antibody levels were expressed as ELISA units (EU) relative to an antibody-containing serum standard. (mean ± SE, SNK multiple comparisons test, $^{**}p < 0.008$, $^{***}p < 0.0001$).

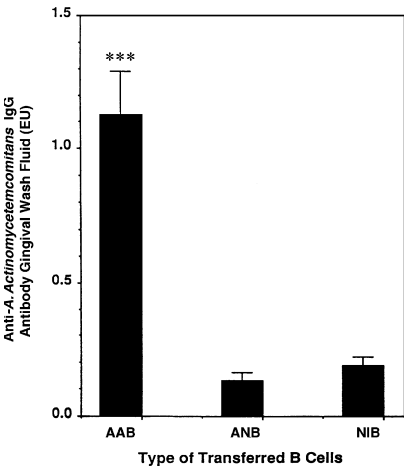

Fig. 3. Serum IgG antibody level in gingival wash fluid after cell transfer. The experimental procedure was as described in Fig. 2. Six months after adoptive transfer, gingival tissues from marginal gingivae were surgically removed and were cut into 1 mm^3 pieces and then were compressed through a 60-gauge stainless steel screen. Anti-*A. actinomycetemcomitans* IgG antibody levels in the 'gingival wash fluid' were measured by enzyme-linked immunosorbent assay (ELISA). The absorbance was determined spectrophotometrically at 405 nm and the antibody level was expressed as ELISA units (EU) relative to an antibody-containing serum standard. IgG antibody in the AAB group was significantly elevated compared to groups receiving ANB or NIB. (mean ± SE, SNK multiple comparisons test, $^{**}p < 0.0001$). AAB, *A. actinomycetemcomitans*-binding B cells; ANB, *A. actinomycetemcomitans* non-binding B cells; NIB, non-immunized donor B cells.

gested that AAB cells and/or their progeny may be recruited during the immune response to *A. actinomycetemcomitans* infection and contribute to the high level of specific antibody in the gingival tissue. In addition, it appears from Fig. 3 that specific B cells are functional in the gingival tissues for the duration of the experiment (6 months).

Specific antibody-producing cells in spleen

The existence of anti-*A. actinomycetemcomitans* antibody-producing cells in the recipient spleens was investigated by the ELISPOT assay (Fig. 4). Only the spleen cells from AAB recipients exhibited a substantial number of SFC ($17 \pm 7/10^6$ spleen cells). This was significantly higher than those in ANB recipients ($0.4 \pm 0.07/10^6$ spleen cells, $p < 0.02$) or in NIB recipients ($0.3 \pm 0.2/10^6$ spleen cells, $p < 0.01$), which demonstrated less than one SFC per 10^6 spleen cells for each group (Fig. 4). These data verified that transferred B cells survived in recipient animals and that specifically reactive B lymphocytes are retained in the spleen.

Total bone resorption

Horizontal bone resorption was measured on all mandibular and maxillary jaws, in order to evaluate any effects of transferred B cells on the progression of periodontal bone resorption. As shown in Fig. 5, animals transferred with AAB exhibited the most significantly elevated level of total bone resorption when compared to animals transferred with ANB or NIB ($p < 0.01$). There was no significant difference in total bone loss between ANB and NIB recipients.

Discussion

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 (11–13, 15). Although we previously emphasized the role of T lymphocytes, in this study we focused on antigen-specific B cells and investigated their potential for a role in experimental periodontal disease *in vivo* using an adoptive transfer model.

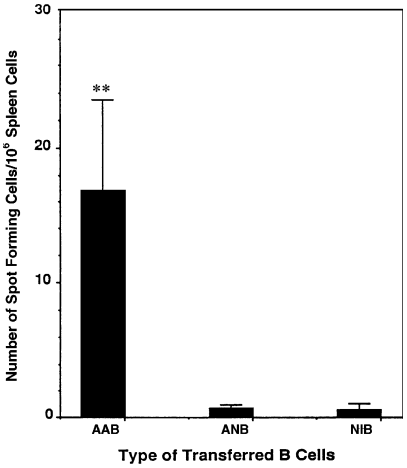

Fig. 4. Actinobacillus actinomycetemcomitans-specific antibody-producing cells in the spleen of recipients. The experimental procedure was as described in Fig. 2. Six months after adoptive transfer, spleen cells from recipient rats were incubated in a 24-well culture plate precoated with *A. actinomycetemcomitans*. After washing, biotin-conjugated goat anti-rat IgG antibody was added, followed by peroxidase-conjugated streptavidin. Enzyme substrate containing 1% agarose in 0.15 M phosphate buffer, pH 7.0 supplemented with paraphenylenediamine (0.5 mg/ml) was added to the wells and spot forming cells on each plate were counted and expressed as spot forming cells per 10^6 spleen cells. The numbers of spot forming cells in the spleens of the AAB transferred group were significantly elevated compared to the other groups. (mean ± SE, SNK multiple comparisons test, $^{**}p < 0.01$). AAB, *A. actinomycetemcomitans*-binding B cells; ANB, *A. actinomycetemcomitans* non-binding B cells; NIB, non-immunized donor B cells.

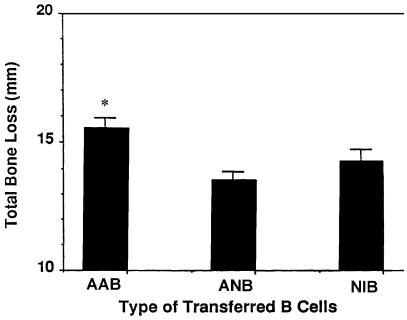

Fig. 5. Total horizontal bone loss after cell transfer. The experimental procedure was as described in Fig. 2. Mandibular and maxillary jaws of all recipient rats were defleshed and a microscope with a reticule eyepiece was used to measure the distance from the cementoenamel junction to the alveolar crest under 25 × magnification. Recordings were made in the long axis of both buccal and lingual root surfaces of all molar teeth as described in 'Material and methods'. The sum of the recordings expressed in millimeter was used as an indication of the total bone loss. The bone resorption in the AAB group was significantly elevated compared to the other groups. (mean ± SE, SNK multiple comparisons test, $^{**}p < 0.01$). AAB, *A. actinomycetemcomitans*-binding B cells; ANB, *A. actinomycetemcomitans* non-binding B cells; NIB, non-immunized donor B cells.

Recipients of live AAB transferred-cells produced elevated levels of antibody within 7 days after cell transfer, whereas the formalin-killed AAB transferred cells did not show comparable levels of antibody production even after 28 days of cell transfer (Fig. 1). This result clearly indicated that enriched AAB actually survived in the circulation and produced antibody in the recipients. This observation also confirmed that AAB recovered by our method represent antigen-specific B cells.

We detected high IgG and IgM antibody levels to *A. actinomycetemcomitans* in serum and high IgG antibody levels to *A. actinomycetemcomitans* in gingival wash fluid only in the AAB transferred recipients (Figs 2 and 3), further supporting the necessity of antigen-specific B cells for antibody formation and retention of B cells. This observation was confirmed by the increased number of spot forming cells among the splenocytes of the AAB transferred recipients (Fig. 4). These findings suggested that antigen-specific B cells or their descendant cells may home to gingival sites or distribute to secondary lymphoid organs such as spleen and lymph nodes, and produce antibody in recipients. Since *A. actinomycetemcomitans*-specific B cells can be considered as memory B cells (26), such B cells are able to present antigen

to T cells and thereby enhance the production of antibody (27).

In this study, the AAB cell recipients exhibited significantly more bone resorption compared to the other groups (Fig. 5). In conjunction with the observed increase in gingival antibody to *A. actinomycetemcomitans* in AAB transferred animals, it is conceivable that an extensive accumulation of antigen-specific B cells may be found in the gingival/periodontal tissues and these may be responsible for the increased periodontal bone resorption. However, these results may not be considered as evidence that antigen-specific B cells directly caused periodontal bone resorption. Previously, we examined the fate of similarly transferred $CD4^+$ T cells and suggested that *A. actinomycetemcomitans*-sensitized T cells can be stimulated in recipient rats after oral *A. actinomycetemcomitans* infection and may take part in the increase of gingival lymphocytes (12). Therefore, further studies are warranted under conditions wherein T cells are devoid of function so that the direct effects of antigen-specific B cells can be evaluated.

Recently, on the basis of adoptive transfer experiments (12, 28, 29) and cell recoveries from diseased tissues (13, 30), we have suggested that Th1 and $CD8^+$ T cells might be destructive via the potential stimulation of macrophage secretion of interleukin-1 (IL-1) and tumor-necrosis factor-α (TNF-α) and subsequent indirect stimulation of osteoclastogenic bone destructive activity (14). On the other hand, Th2 cells can be protective via production of IL-4 (31, 32) and IL-10 (2, 33, 34), which seem to inhibit Th1 cell function and protect against periodontal bone resorption.

In relation to the current experiments, it is suggested that the destructive effect associated with the transferred B cells may be related to production of cytokines that can enhance Th1 responses (35). For example, production of IL-12 by B cells and macrophages initiates cell-mediated immunity by inducing differentiation of Th1 cells from uncommitted T cells and stimulates the growth and functional activity of natural killer and Th1 cells (36). Thus, B cell production of IL-12 might be responsible for induction of a Th1 response and also production of IL-1 and TNF-α by macrophages and other cells, giving rise to increased bone resorption and decreased bone formation (13, 37, 38). This speculation emphasizes the necessity to further investigate the role of IL-12 and other cytokine products in periodontal disease pathogenesis. Although possible IL-12 production by the transferred B cells was not analyzed in this study, the survival of these cells or their progeny was indicated by the presence of SFC specific for *A. actinomycetemcomitans* in the spleens of AAB transferred rats.

Additionally, it has been established that alveolar bone resorption in periodontal disease is mediated by enhanced osteoclastogenesis (39). Recent studies suggested that macrophage colony stimulating factor and receptor activator of NF-κB ligand (RANKL) are two major factors in the regulation of osteoclast differentiation (40,41). In particular, RANKL, its receptor RANK, and a decoy receptor osteoprotegerin are three key molecules that regulate osteoclast recruitment and function (42). Understanding of the production and regulation of such factors in antigen-specific B cells, both *in vitro* and *in vivo*, will allow us to gain insights into the molecular mechanisms underlying the B cell induced alveolar bone resorption. Of considerable importance is the recent finding that B cells are key participants in the direct RANKL-mediated osteoclastic bone resorption (43). Further studies are clearly needed to investigate the mechanism of B cell involvement in periodontal bone resorption. Such studies should help to establish therapeutic strategies to treat periodontal disease and other systemic bone diseases associated with osteoclast functions related to B cells in tissue inflammation.

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