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IL-1 and TNF Antagonists Inhibit the Inflammatory Response and Bone Loss in Experimental Periodontitis[1]

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Periodontal disease is the most frequent cause of tooth loss in humans and is the most prevalent disease associated with bone loss, including osteoporosis. Periodontal destruction is initiated by bacteria that colonize the tooth surface, leading to inflammation and bone resorption. To assess the roles of IL-1 and TNF in this process, studies were conducted in a *Macaca fascicularis* primate model of experimental periodontitis. Function-blocking soluble receptors to IL-1 and TNF were applied by local injection to sites with induced periodontal destruction and compared with similar sites injected with vehicle alone. The results indicate that injection of soluble receptors to IL-1 and TNF inhibited by approximately 80% the recruitment of inflammatory cells in close proximity to bone. The formation of osteoclasts was reduced by 67% at the experimental sites compared with that at the control sites, and the amount of bone loss was reduced by 60%. All results were statistically significant ($p < 0.01$). These findings indicate that a significant component of the pathologic process of periodontitis is due to IL-1/TNF activity, since inhibiting IL-1/TNF reduces both inflammatory cell recruitment and bone loss. The data also suggest that inflammation associated with gingivitis is actively protective, since blocking further up-regulation of the host response with IL-1/TNF inhibitors does not cause periodontal damage. Furthermore, these results coupled with recent evidence that IL-1 and TNF participate in endocrine-associated osteoporosis suggest that multiple pathologies involving excessive loss of bone may operate through a common mechanism involving IL-1 and/or TNF. ***The Journal of Immunology,*** **1998, 160: 403–409.**

Periodontal disease is the leading cause of tooth loss among adults. It is defined as a plaque-induced inflammation of the periodontal tissues that results in a loss of support of the affected teeth (1, 2). This process is characterized by destruction of the periodontal attachment apparatus, loss of crestal alveolar bone, apical migration of the epithelial attachment, and formation of periodontal pockets (1, 3). Although the presence of periodontal pathogens is a prerequisite, the progression of periodontal disease is dependent on the host response to pathogenic bacteria that colonize the tooth surface (1, 2). A number of bacterial products are capable of affecting the host response (4). These products initiate a local host response that involves the generation of PGs and cytokines, the recruitment of inflammatory cells, the elaboration of lytic enzymes, and the activation of osteoclasts.

Several mediators have been proposed to induce periodontal disease. Prominent among these are the PGs, particularly PGE_2 (5). Although PG inhibitors can reduce periodontal bone loss, the effects of PG are unlikely to account for all the inflammatory changes associated with periodontal disease (6). Thus, IL-1 is another mediator that could potentially participate in this process (7). IL-1 stimulates a wide variety of biologic effects. There are two active forms of IL-1, IL-1α and IL-1β, both of which bind to type I and type II IL-1R (8–10). A third member of the IL-1 family has been identified as an IL-1R antagonist. It binds to IL-1R without agonist activity (8, 9). The IL-1 type I receptor is capable of transducing signals and, hence, mediates the biologic effects of IL-1 (10–12). The type II receptor, which may be membrane bound or released in soluble form, has been proposed to serve as an IL-1 binding protein and, thus, antagonizes IL-1 (11, 13). IL-1 possesses inflammatory, metabolic, physiologic, hemopoietic, and immunologic properties (9). It is a powerful bone mediator in vitro and in vivo, capable of inducing osteoclastic bone resorption (14, 15). By direct or indirect mechanisms, IL-1 can induce the proliferation of osteoclast precursors and the differentiation and activation of mature osteoclasts (14–16).

TNF refers to two related proteins, TNF-α and TNF-β, that have a high degree of structural and sequence homology and share the same receptors (17). Like IL-1, TNF interacts with two types of receptors, termed TNF-R1 and TNF-R2 (18, 19). Most of TNF's deleterious effects have been attributed to TNF-R1 (20, 21). Many, but not all, of the biologic properties of TNF overlap with those of IL-1. Both stimulate bone resorption by inducing the proliferation of osteoclast progenitors and, indirectly, by stimulating the resorbing activity of mature osteoclasts (15, 22). It is noteworthy that IL-1 and TNF are often coproduced in vivo and can act synergistically to stimulate bone-resorptive activity (23). TNF antagonists can be found in soluble forms, known as TNF binding proteins (24), which are derived from shedding the extracellular domains of either TNF-R1 or TNF-R2 (17, 18, 25).

The use of soluble receptors to antagonize the effects of specific cytokines has offered a valuable tool with which to study the roles of these factors. Soluble receptors for IL-1 have been used in pathogenic models to study the host response (8). Soluble type I IL-1R was shown to inhibit local allergen-induced inflammation (26), ocular inflammation (27), and LPS-induced acute pulmonary inflammation (28). Similarly, soluble TNFR or chimeric molecules linking soluble TNFR to the Fc region of Igs have been used in experimental models of endotoxemia or bacteremia (29, 30). The

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chimeric molecules were shown to be more efficacious than monomeric soluble TNF receptors in protecting mice from lethal endotoxemia (30) and obesity-linked insulin resistance (31). Furthermore, the utility of using both IL-1 and TNF antagonists together has been recently demonstrated in protecting against toxic endotoxemia (28, 32).

Several animal models have been proposed to study the events associated with inflammatory periodontal disease. A model that allows initiation of the disease process at a known time involves the placement of silk ligatures around the posterior teeth of nonhuman primates. This model has several distinct advantages (33, 34). 1) Ligature-induced alveolar bone loss has clinical and microbiologic features similar to those found in humans and has been shown to be induced by bacteria. 2) The model provides a predictable sequence of events leading to the loss of alveolar bone. Knowing when alveolar bone loss actually occurs is an essential feature that is not possible in many other widely accepted models. 3) The immune system of the *Macaca fascicularis* is very similar to that of humans. 4) Bone loss is associated with the presence of an inflammatory infiltrate in this model as it is in humans. Thus, the host response in ligature-induced periodontal tissue destruction in nonhuman primates is most similar to that observed in humans compared with other animal models. 5) Histologic analysis of the results can be conducted, while similar analysis cannot be performed in humans.

In the studies presented here, we demonstrate that a significant component of experimental periodontitis is the recruitment of inflammatory cells in close proximity to bone, and that this depends, to a large extent, on IL-1/TNF activity. Thus, the mechanism of periodontal disease is likely to involve a "field effect" characterized by an overexuberant inflammatory response to penetration of bacterial products into the gingiva, rather than direct bacteria-induced bone loss. We also propose that inflammation associated with gingivitis is actively protective, since blocking further upregulation of the host response with IL-1/TNF inhibitors does not cause further injury to the host.

Materials and Methods

Soluble human rIL-1R type I consisting of the extracellular portion of the type I receptor (35) and the soluble receptor to TNF consisting of the extracellular domain of TNF receptor-2 linked to the Fc portion of a human IgG1 (30) were provided by the Immunex Corp (Seattle, WA). All animal manipulations were performed according to a protocol approved by the institutional animal care and use committees at the Boston University Medical Center and the University of Texas Health Science Center at San Antonio. Fourteen animals (*Macaca fascicularis*) used in this study ranged from 3 to 7 yr of age and were obtained from Primate Import (Port Washington, NY). Experimental periodontitis was induced by tying *Porphyromonas gingivalis*-soaked silk ligatures around the posterior mandibular teeth according to the procedure described previously (36). Placement of these sutures induces a bacteria-mediated inflammation and alveolar bone loss. Three animals received vehicle alone (sterile PBS), and two animals received soluble receptors to IL-1 (6.6 μg/injection) plus soluble receptors to TNF (6.6 μg/injection) three times each week for 6 wk following suture placement. Injections of vehicle alone or antagonists were placed locally in the interdental papillae between the first and second molar teeth (100 μl in each papilla). Additional animals were included in the study but did not receive blockers. These animals served to identify the induced progression of events over time. Three animals were killed at each time point 0, 2, and 4 wk after placement of sutures. Immediately after sacrifice, animals were perfused with 400 ml of PBS followed by an equal volume of 4% paraformaldehyde in PBS (0.15 M NaCl and 0.05 M Na_2PO_4, pH 7.5). This perfusion was made into the carotid artery; the jugular vein was opened to allow drainage.

Specimen preparation

Mandibular posterior sextants were surgically dissected and immediately fixed in 4% paraformaldehyde for 2 days at 4°C. After fixation, specimens were washed and subdivided into smaller blocks by cutting the center of each tooth along its long axis. This provided an interproximal area consisting of two tooth surfaces, periodontal ligament, gingiva, and interdental alveolar bone, as illustrated in Figure 2. Each sample was then decalcified for 8 to 10 wk in 15% glycerol/EDTA, pH 7.0, with constant stirring at 4°C. Decalcification was established radiographically. Specimens were then embedded in low melting (56°C) paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin.

Histomorphometric analysis

Two interdental regions between the first and second molars of the right and left mandibular quadrants were examined. This provided an interproximal region consisting of two tooth surfaces, periodontal ligament, gingiva, and interdental alveolar bone, as illustrated in Figure 2. Two representative tissue sections from each interdental area were analyzed. A minimum of five fields were examined in each area of interest for every section. Results from two sections were averaged to provide values for each area of interest from a given interdental specimen. All data were analyzed in a double-blind fashion using coded specimens. In some cases specimens were analyzed multiple times with three different examiners. In all cases, interexaminer and intraexaminer variations were $<10\%$. Images were captured at $\times 500$ magnification, stored on an optical disk, and analyzed randomly at a later time. The areas of interest included the superficial and deep gingival connective tissue at the midpoint of the interdental papillae and the periodontal ligament space adjacent to the two teeth present in each specimen. In each area of interest, the total number of inflammatory cells and the total number of osteoclasts were manually counted with the assistance of an image analysis system. Inflammatory cells included polymorphonuclear leukocytes, mononuclear leukocytes, and plasma cells. Polymorphonuclear leukocytes were distinguished by a multilobed nucleus and a granular cytoplasm. Plasma cells were ovoid with an eccentric nucleus and a clear perinuclear zone. Lymphocytes, macrophages, and monocytes presented similar histologic appearances, with round, intensely stained nuclei and were counted as mononuclear leukocytes. Osteoclasts were recognized as large multinucleated cells in direct contact with bone surfaces, with a pale foamy cytoplasm and irregularly shaped nuclei. The data were then presented as the number of inflammatory cells per tissue area or the number of osteoclasts per length of bone surface.

The amount of bone loss was assessed from images of hematoxylin- and eosin-stained sections that were captured at $\times 100$ magnification. The amount of normal bone present was determined first in animals that had not received ligatures (zero time point). The corresponding amount of bone in each experimental sample was then determined. The percent bone loss was determined by dividing the amount of bone found on experimental samples (vehicle or blockers) by the amount of bone in normal controls (zero time point).

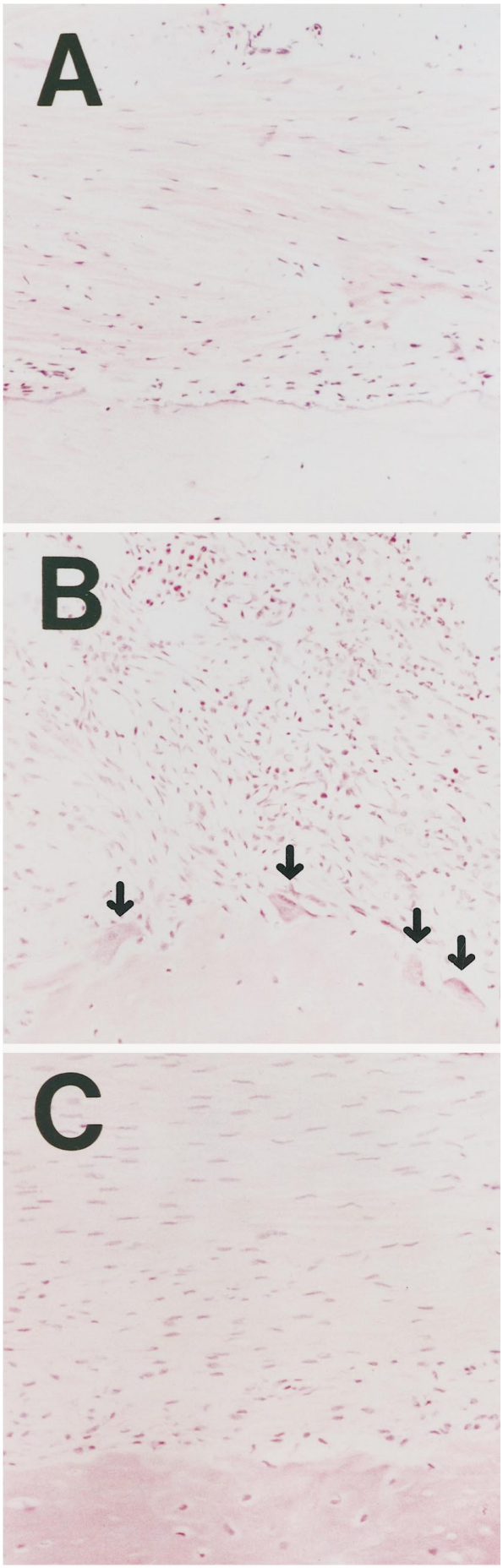
Statistical analysis

ANOVA was used to compare changes in each of the variables over time (0, 2, 4, and 6 wk). Student's *t* test was used to establish significance between control and experimental animals at the 6 wk point for each parameter.

Results

Experimental alveolar bone loss was induced in Macaca fascicularis monkeys by tying *Porphyromonas gingivalis*-soaked silk ligatures around the posterior teeth. For the 6 wk point, three animals received local injection of vehicle alone into the interdental gingiva, while two received local injection of IL-1- and TNF-soluble receptors. At the zero time point, there were few inflammatory cells in close proximity to bone, with virtually no osteoclasts lining bone (Fig. 1*A*). At 6 wk, there was considerable infiltration of this region by inflammatory cells in animals treated with vehicle alone (Fig. 1*B*). Several multinuclear osteoclastic cells in Howship's lacunae were present, consistent with the induction of periodontal bone loss. In addition, a large number of mononuclear cells were observed in close proximity to bone. In animals that received local injection of IL-1 and TNF blockers there was a large reduction in the number of mononuclear cells and osteoclasts (Fig. 1*C*).

Histomorphometric analysis of the 6 wk specimens was performed using computer-assisted image analysis in well-defined areas of interest (Fig. 2). The interdental gingival connective tissue was divided into two compartments: one in close proximity to the



oral epithelium (box 1), and the other in close proximity to the bone surface (box 2). The third connective tissue compartment examined was the coronal portion of the periodontal ligament (box 3). These areas represent different anatomic regions, which allowed us to determine the distribution of inflammatory cells in relation to the various structures of the periodontium. These areas are likely to be critical in the destructive process of the supporting apparatus in periodontal disease.

Superficial gingival connective tissue is typically infiltrated by inflammatory cells in response to bacteria that colonize the surfaces of teeth and oral epithelium, even in clinically healthy conditions. Figure 3 demonstrates that IL-1/TNF blockers significantly inhibit the recruitment of inflammatory cells associated with experimental periodontitis in this area. At 6 wk the number of inflammatory cells in the interdental gingival connective tissue in close proximity to oral epithelium (box 1) was 48.6/mm^2 in control sites. This number was reduced by 56% with the injection of IL-1- and TNF-soluble receptors. The decrease in inflammatory cells in close proximity to bone (box 2) was even more dramatic. There were 7.2 inflammatory cells/mm^2 in the control group and 1.6/mm^2 when soluble receptors were applied. Thus, blocking IL-1 and TNF activity decreased by 78% the recruitment of inflammatory cells in close proximity to bone. The number of inflammatory cells present in the periodontal ligament (box 3) is also shown in Figure 3. There were 17.5 inflammatory cells/mm^2 in the control sites and 2.9 cells/mm^2 in the presence of IL-1 and TNF function-blocking Abs, representing an 83% decrease. The number of osteoclasts per millimeter of bone surface is shown in Figure 4. When animals were injected with vehicle alone, there were approximately 0.75 osteoclasts/mm. In animals that received IL-1- and TNF-soluble receptors there were approximately 0.25 osteoclasts/mm. Thus, the number of osteoclasts decreased by 67% when IL-1 and TNF activities were inhibited. The decrease in osteoclast formation correlated well with decreased bone loss when soluble receptors were applied (Fig. 4). Injection of IL-1 and TNF blockers inhibited periodontal bone loss by 60%.

The progression of inflammatory cell recruitment, the formation of osteoclasts, and bone loss in experimental periodontitis were examined at 0, 2, 4, and 6 wk. The number of inflammatory cells in the superficial gingival connective tissue near oral epithelium evaluated at the zero time point was relatively high, consistent with a chronic inflammation typically found in gingivitis (Fig. 5*A*). After placement of silk sutures, there was a time-dependent increase in the number of inflammatory cells. In the gingiva in close proximity to bone there were virtually no inflammatory cells present at

FIGURE 1. Inflammatory cell recruitment and osteoclast formation are inhibited by antagonists to IL-1 and TNF in periodontal bone loss. The gingival connective tissue in close proximity to bone was examined from three groups of animals. One group was killed just before ligature placement and showed no alveolar bone loss (*A*). The second group represents control animals that had *P. gingivalis*-soaked silk ligatures tied around the posterior teeth and received local injection of vehicle alone (*B*). The third group had *P. gingivalis*-soaked silk ligatures tied around the posterior teeth and was treated simultaneously by local injection of IL-1/TNF blockers for 6 wk (*C*). It can be seen that in the 0 wk control there are no osteoclasts and few inflammatory cells in this area (*A*). In the 6 wk group treated with vehicle alone, osteoclasts and inflammatory cells are evident (*B*). No osteoclasts and few inflammatory cells are present in the 6 wk experimental group that received IL-1/TNF blockers while silk ligatures were in place (*C*), demonstrating the effectiveness of the blockers in blocking the inflammatory response associated with this model of experimental periodontitis. Sections were stained with hematoxylin and eosin. Original magnification, ×200.

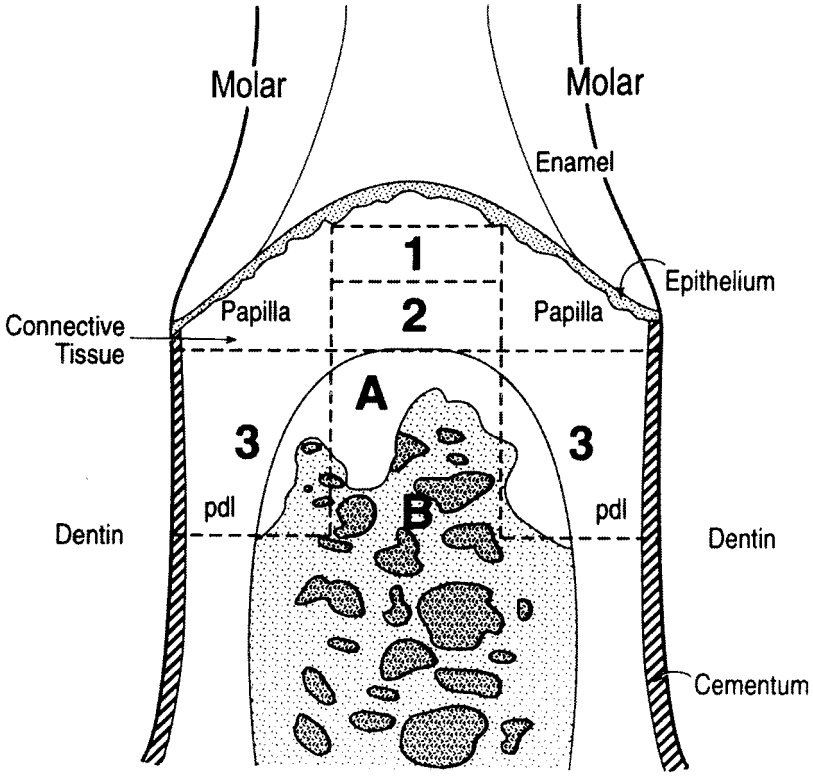


FIGURE 2. Diagrammatic representation of the periodontium. Areas of interest are represented by the superficial interdental gingival connective tissue (box 1), the deep interdental gingival connective tissue in close proximity to bone (box 2), and the coronal periodontal ligament area (box 3). The area of alveolar bone loss is represented by *A*, and the area of intact alveolar bone is represented by *B*.

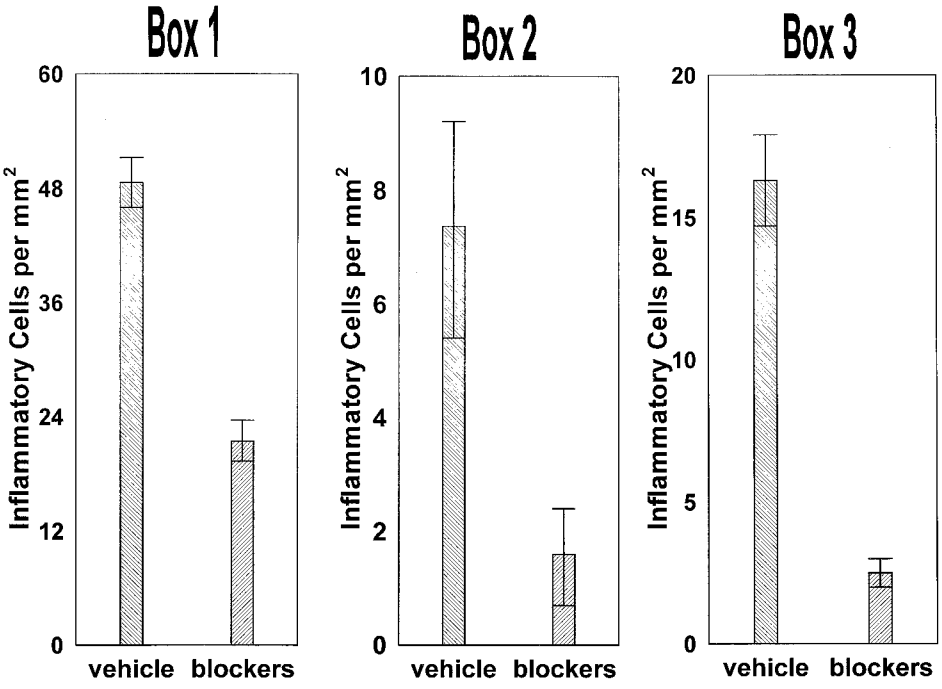


FIGURE 3. IL-1 and TNF antagonists inhibit inflammatory cell recruitment during periodontal bone loss in superficial gingival connective tissue, deep gingival connective tissue, and periodontal ligament. Localized periodontal bone loss was induced by application of *P. gingivalis*-soaked silk ligatures. Animals received local injection of either vehicle alone or IL-1/TNF antagonists. Hematoxylin- and eosin-stained sections were counted with the aid of an image analysis system. The data are presented as the number of inflammatory cells per square millimeter of connective tissue. The reductions in inflammatory cells were 56, 78, and 83% in each of the areas of interest, respectively. The reduction in each area of interest was statistically significant ($p < 0.01$).

the zero time point, consistent with the absence of periodontitis (Fig. 5*B*). Following induction of experimental periodontitis, there were significant numbers of inflammatory cells in this compartment at 2 wk, which increased further at 4 and 6 wk. The number of inflammatory cells in the periodontal ligament was relatively low at the zero time point and increased significantly after placement of silk ligatures in a time-dependent manner (Fig. 5*C*). Likewise, there were virtually no osteoclasts present before experimental periodontitis was induced (Fig. 5*D*). Osteoclast number increased significantly at 2 wk and reached a maximum at 6 wk.

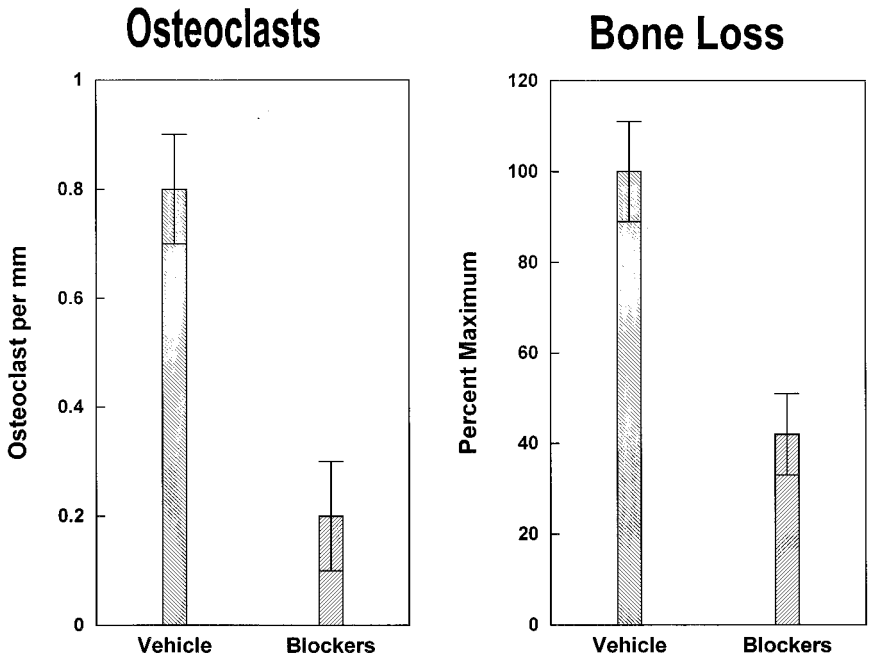


FIGURE 4. IL-1 and TNF antagonists inhibited osteoclast formation and periodontal bone loss. Localized periodontal bone loss was induced by application of *P. gingivalis*-soaked silk ligatures. Animals received local injection of either vehicle alone or IL-1/TNF antagonists. Determination of osteoclast number and histomorphometric analysis of bone loss were determined on hematoxylin- and eosin-stained sections with the aid of an image analysis system. The data are presented as the number of osteoclasts per millimeter length of bone, and bone loss is presented as a percentage of the maximum. Statistically significant reductions in osteoclasts (67%) and bone loss (60%) were observed ($p < 0.01$).

The amount of bone loss exhibited the same time-dependent pattern observed for osteoclast formation; bone loss was observed as early as 2 wk and increased further at 6 wk (Fig. 5*E*). It is clear from comparing the different parameters that there is a consistent pattern of increase over time and an association between inflammatory cell recruitment close to bone, osteoclast activity, and bone loss.

Discussion

Periodontal disease is the most frequent cause of tooth loss in humans and is the most prevalent disease associated with bone loss, including osteoporosis. The process of periodontal disease is initiated by bacteria. It has been proposed that bacterial stimulation induces a host response that leads to the loss of tooth attachment, osteoclast formation, and bone loss (2). It has previously been reported that PG activity can account for some of the bone loss associated with experimental periodontitis (6). We found that as much as 80% of the inflammatory cell recruitment and 60% of the bone loss that occurred could be accounted for by blocking IL-1 and TNF. The magnitude of this inhibition was unexpected given the primary role previously ascribed to PGs in this process. The dramatic effect of IL-1/TNF inhibitors could be due to their role as primary mediators in the inflammatory response, while PGs could potentially have a more secondary role.

Studies by Kimbel and co-workers provide convincing evidence that IL-1 activity contributes to osteoporosis associated with endocrine changes (16). In these studies, IL-1R antagonist was shown to substantially reduce bone loss in the period immediately following ovariectomy in rats, indicating that IL-1 activity plays an important role in estrogen-deficient bone loss. Furthermore, the application of blockers to both IL-1 and TNF was more effective than blockers of either alone in preventing rapid bone loss immediately following ovariectomy. This suggested that both cytokines act in synergy to induce bone loss resulting from estrogen deficiency.

In our study we tested the roles of IL-1 and TNF in bone resorption associated with bacterial inflammation using a nonhuman primate model of experimental periodontitis. By using soluble receptors that block IL-1 and TNF, we demonstrated that approximately 80% of the inflammatory cell recruitment that occurs in close proximity to bone and 67% of osteoclast formation can be accounted for by IL-1 and/or TNF activity. Thus, the generation of IL-1 and/or TNF may provide a common mechanism to explain bone resorption in a variety of pathologies with diverse causes ranging from bacterial stimulation to endocrine-associated bone loss.

By examining time-dependent changes in inflammatory cell recruitment in different compartments of the periodontium and the effect of IL-1/TNF blockers, our studies clearly demonstrate that a critical event in periodontal disease progression is the recruitment of inflammatory cells to an area close to alveolar bone, and that this recruitment requires IL-1/TNF activity. Before the onset of periodontal disease there is considerable infiltration of the superficial gingival connective tissue near the oral epithelium. This is characteristic of gingivitis, which is typified by chronic inflammation without bone loss. When alveolar bone loss is induced, there is a dramatic increase in leukocyte recruitment in close proximity to bone. In the presence of blockers to IL-1 and TNF, new recruitment of leukocytes is substantially decreased. This suggests that periodontal disease is initiated when the inflammatory stimulus spreads to the deep gingival connective tissue, stimulating the recruitment of leukocytes. Thus, blocking IL-1 and TNF activities may inhibit bone loss both directly and indirectly; the latter occurring via decreased recruitment of mononuclear cells in the area of bone. This is in contrast to the observed periodontal changes in leukocyte adhesion-deficient patients, who fail to mount an effective basal inflammatory response and frequently present with fulminant periodontal destruction (37). The apparent difference can be explained by the presence in normal individuals of a protective inflammatory infiltrate present in the superficial gingiva, characteristic of gingivitis, that protects the host against pathogenic micro-organisms. We speculate that enhanced bacterial challenge that occurs in experimental periodontitis stimulates the levels of proinflammatory cytokines (IL-1/TNF), resulting in an exacerbated recruitment of inflammatory cells that is detrimental to the host. The corollary of this observation is that no damage occurs when further up-regulation of the host response is blocked by IL-1/TNF antagonists, indicating that

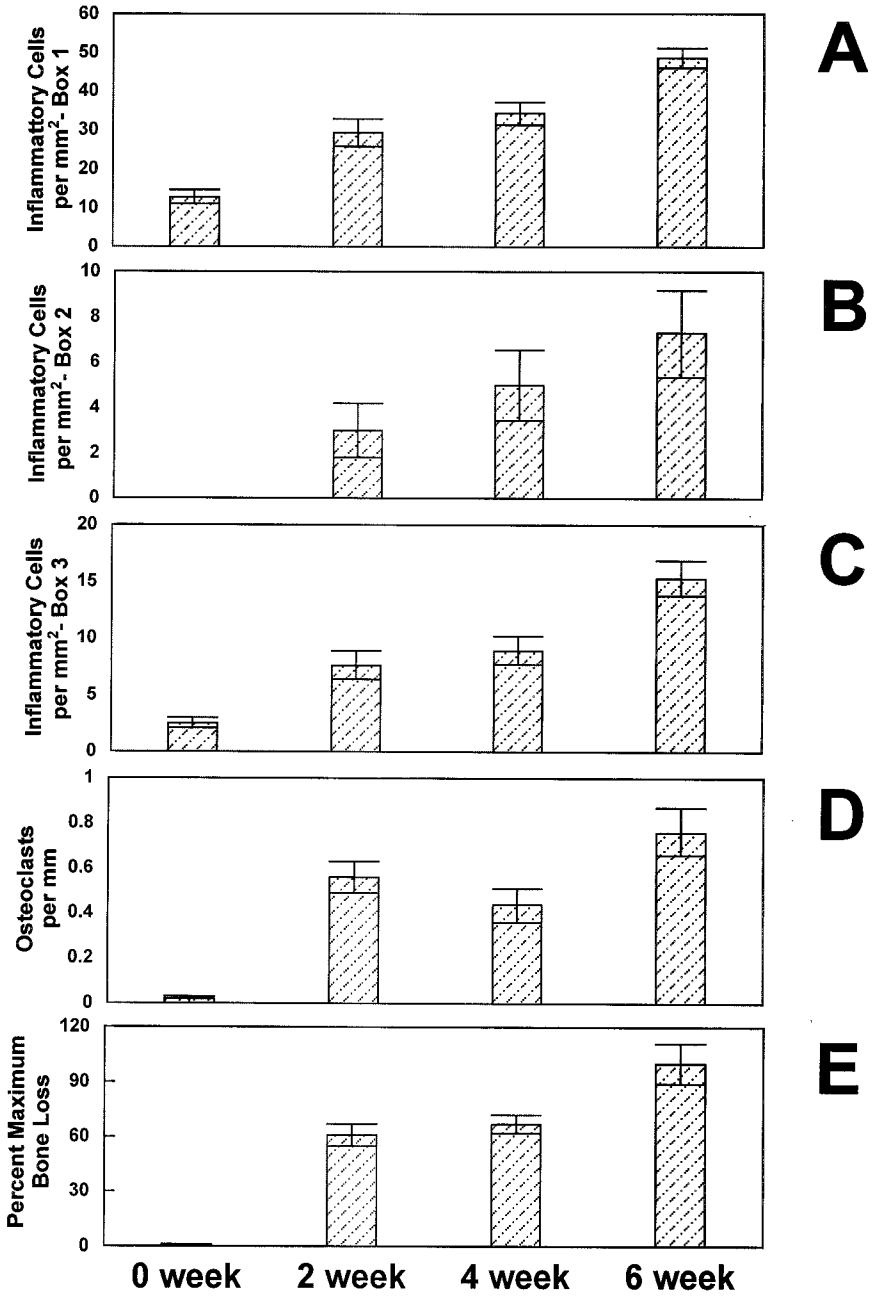


FIGURE 5. Induction of periodontal bone loss by *P. gingivalis*-soaked ligatures causes a time-dependent increase in inflammatory cell recruitment (*A–C*), osteoclast formation (*D*), and bone loss (*E*). Inflammatory cell and osteoclast number as well as bone loss were counted as described in *Materials and Methods*. Animals were killed 0, 2, 4, and 6 wk following placement of ligatures. A statistically significant increase in each parameter was observed starting at 2 wk compared with the negative control value at 0 wk ($p < 0.01$).

the steady state chronic inflammation associated with gingivitis is protective against the bacterial insult initiated by the placement of silk ligatures. However, when the basal inflammatory response is magnified in the absence of IL-1/TNF blockers, tissue destruction occurs. In leukocyte adhesion deficiency patients, an effective basal inflammatory response typical of normal gingivitis is not present. Thus, the failure to mount an effective basal polymorphonuclear cell/monocyte response typical of gingivitis is likely to facilitate bacterial destruction of the periodontium in leukocyte adhesion deficiency patients.

It is thought that epithelial leakage allows penetration of bacteria or their products into the gingival connective tissue, which, in turn, induces a host response (38, 39). As suggested above, the steady state inflammation present in the superficial gingiva represents a highly effective host response to bacteria that colonize the teeth. If this chronic host response was not effective, it is possible that the blockage of IL-1 and TNF would lead to greater bacterial penetration and direct bacteria-induced inflammation close to bone. In fact, what we observed was the opposite; IL-1 and TNF blockers reduced inflammation and osteoclast activity close to bone. This suggests that the mechanism of periodontal disease involves a field effect, in which the penetration of bacteria into the superficial gingiva is effectively dealt with by inflammatory cells chronically present in the superficial connective tissue. Perturbation of this equilibrium may lead to excess production of IL-1 and/or TNF, which, in turn, activates a cascade leading to the generation of secondary inflammatory mediators, inflammatory cell recruitment close to bone, osteoclast formation, and bone loss. Our studies along with others using inhibitors of PG synthesis establish that the major loss of alveolar bone in periodontal disease results from collateral damage due to an excessive host response rather than to direct bacteria-induced bone loss or other primary mediators. Bone loss represents a significant medical problem, particularly in the elderly, as exemplified by periodontal disease and

osteoporosis. Even though these processes have considerably different etiologies, our results and those reported by Kimble and colleagues (16) suggest that both operate through a common mechanism involving IL-1 and/or TNF activity.

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