Pathogenesis of induced rat periapical lesions

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Studies of the mechanisms of pathogenesis of periapical lesions were undertaken using a rat model of surgical pulp exposure. In this model, periapical lesions develop rapidly between days 0 and 15 (active phase) and more slowly thereafter (chronic phase). A Gram-negative anaerobic flora, similar to that seen in human beings, are quickly established. Lesions contain a mixed inflammatory cell infiltrate consisting of T cells, neutrophils, B cells, macrophages, and plasma cells. Helper T cells predominate during the active phase, whereas suppressor T cells are more frequent in the chronic phase. Extracts of periapical lesions contain bone-resorbing activity, the highest levels of which are present when lesions are actively expanding. Most bone-resorbing activity is mediated by the cytokine interleukin-1α, as determined by biochemical criteria and antibody neutralization studies. Prostaglandin$_2$ accounts for 10% to 15% of resorptive activity. Cells that express interleukin-1α were identified in pulp beginning on day 2 after exposure and in periapical tissue beginning on day 7, as determined by in situ hybridization and immunostaining. Macrophages, fibroblasts, neutrophils, and osteoclasts were positive for interleukin-1α mRNA and protein. Cells that express tumor necrosis factor α were also detected, whereas cells expressing interleukin-1β or tumor necrosis factor β were absent. Finally, periapical bone destruction was inhibited by 60% by treatment with interleukin-1 receptor antagonist. These studies establish a key role for interleukin-1α in the pathogenesis of periapical lesions in the rat model. (ORAL SURG ORAL MED ORAL PATHOL 1994;78:494-502)

Bacterial infection of the dental pulp results first in pulpal destruction and ultimately in the development of a periapical lesion with the concomitant resorption of bone. Until recently, little was known concerning the intermediate pathways that link the infection with osteoclastic bone resorption. Candidate stimulators of bone resorption that have been proposed include bacterial components such as lipopolysaccharides (LPS)[1,2], prostaglandins (PGE),[3,4] bradykinin,[5] antigen-antibody complexes,[6] and proinflammatory cytokines[7] produced by host cells in response to the infection. Proinflammatory cytokines, including interleukin-1α (IL-1α),[8] IL-1β,[9] and tumor necrosis factor alpha (TNFα),[10] are of particular interest because they are strongly expressed by many host cell types in response to bacterial infection and are potent stimulators of bone resorption. In this review, the results of recent experiments from this laboratory that elucidate the mechanisms of periapical bone destruction will be presented.

RAT MODEL OF PERIAPICAL PATHOGENESIS

In all studies we have used the rat model of periapical lesion development initially described by Kakehashi et al.,[11] Yu and Stashenko,[12] and Wang and Stashenko.[13] In this model, surgical exposure of molars and infection of the dental pulp from the oral environment reproducibly results in the development of periapical lesions and the destruction of bone. Lesions expand rapidly between day 7 and day 15 to 20 (active phase) with slowed expansion thereafter (chronic phase). Periapical destruction may be estimated by high-resolution radiography and image analysis, or it may be accurately quantified by sectioning and histomorphometry (Fig. 1). The defined kinetics of bone destruction in this model have permitted us to study the microbiology of root canal infection and to investigate the presence or absence of candidate bone resorptive mediators in the temporal context of active disease.[14] Our working hypothesis has been that putative mediators of bone destruction should be present in biologically significant concentrations in pulpal and periapical tissues just before and during the rapid phase of lesion expansion.

MICROBIOLOGY OF THE RAT MODEL

The root canal flora have been extensively characterized in human beings and nonhuman primates.[15-17] These studies have demonstrated that the infecting bacteria are predominantly gram-negative and strictly anaerobic. To determine whether the microbiota and hence the potential pathogenic mechanisms are similar in the rat and human models, we have recently characterized the root canal flora present during the active phase of lesion development.[18] After pulp ex-

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Fig. 1. Photomicrograph of periapical lesion and attendant bone destruction 20 days after pulp exposure in the rat model. Note total necrosis of coronal pulp and partial necrosis of radicular pulp with intracanal abcess. (Hematoxylin-eosin stain; original magnification ×100.)

posure on day 0, teeth were extracted after 7 days (n = 10 animals) and 15 days (n = 10), and the microflora present in roots were isolated and characterized with the use of standard anaerobic methods. Anaerobic bacteria increased significantly between day 7 (24.3 ± 5.7%) and day 15 (47.3 ± 7.5%) ($p < 0.01$), and the proportion of gram-negative organisms increased from day 7 (24.3 ± 6.1%) to day 15 (46.9 ± 6.8) ($p < 0.01$) (Table I). On day 7 the predominant bacteria included: *Streptococcus* and *Bacteroides*; on day 15, *Streptococcus, Bacteroides, Prevotella, Neisseria,* and *Peptostreptococcus. S. oralis, S. mitis, S. rattus, B. pneumosintes,* and *B. ureolyticus* were frequently isolated at both time points. No colonies were isolated from unexposed control teeth, which confirms the lack of superficial contamination.

Although 3 to 4 different species were isolated/tooth on both day 7 and 15, the overall diversity of the isolates was increased on day 15. These results demonstrated that the root canal flora became increasingly anaerobic with the emergence of *Peptostreptococcus, Bacteroides, Prevotella,* and *Neisseria* during the period of rapid lesion expansion in this model (Fig. 2). This constellation of species is similar to that found

Table I. Comparison of bacterial counts on day 7 and day 15 after pulp exposure

	Experimental period	
	Day 7	*Day 15*
Total CFU	1532.5 ± 641.2*	1491.5 ± 373.6
Number of colonies identified/tooth	17.1 ± 3.9	18.5 ± 4.0
Number of different species isolated from tooth	3.4 ± 0.4	3.5 ± 0.4
Anaerobes (%)	24.3 ± 5.7	47.3 ± 7.5†
Gram (−) organisms (%)	24.3 ± 6.1	46.9 ± 6.8†

*Mean ± SE.
†$p < 0.02$ vs day 7 (Wilcoxson 2-sample test).

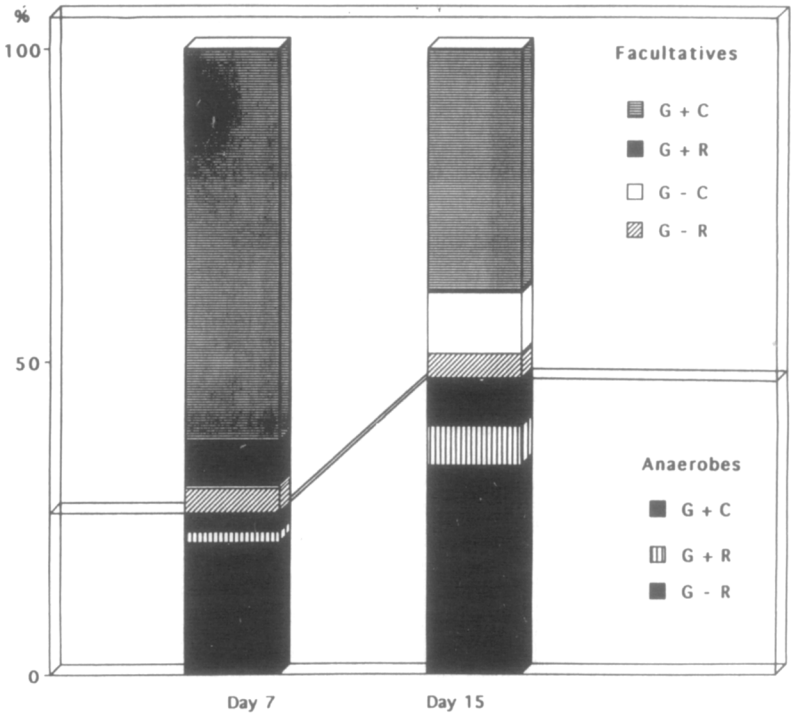

Fig. 2. Proportions of facultative and anaerobic bacteria and morphotypes from root canals on day 7 and day 15 after pulp exposure. *G*, gram staining; *C*, cocci; *R*, rods. (Reprinted from Tani-Ishii et al.[18])

Table II. Presence of cytokine-specific mRNA in rat periapical tissues by polymerase chain reaction

Days after pulp exposure	*Number of animals*	*Cytokine mRNA*	
		IL-1α	*IL-1β*
5	5	0/5*	0/5
7	5	2/5	0/5
10	5	4/5	0/5
15	5	5/5	0/5
20	5	4/5	2/5
30	5	3/5	2/5

*Positive animals/total.

previously in the root canals of human beings and monkeys and suggests that the rat model is closely similar to primates with respect to root canal microbiology.

INFLAMMATORY CELL INFILTRATE IN DEVELOPING AND CHRONIC PERIAPICAL LESIONS

To determine the inflammatory cell types associated with active lesion formation, a kinetic study was carried out to identify cells present in developing rat periapical lesions.[12] On days 15, 20, 30, and 90 after induction, inflammatory cells from periapical lesions were isolated and enumerated on the basis of morphologic and phenotypic criteria. At all time points, lymphocytes were the predominant cell type (50% to 60%), followed by polymorphonuclear leukocytes (25% to 40%), macrophage-monocytes, plasma cells, and blasts. No significant time-related differences in morphologic types were found. Phenotyping with monoclonal antibodies specific for all T cells (W3/13+) and Class II antigen positive cells (OX4+) revealed that T cells constituted the major non-polymorphonuclear leukocytes inflammatory cell component and outnumbered Class II positive B cells at all time points. The cell infiltrate in these lesions was similar to that found in chronic human periapical tissues[19, 20] as well as in diseased human periodontal disease.[21] Further phenotypic analysis revealed that helper T cells (T_H) predominated in periapical lesions during the active phase of periapical lesion development, whereas suppressor T cells (T_S) predominated in more chronic lesions.[22] At the earliest time point studied after induction (day 15), T_H cells (W3/25+) outnumbered T_S cells (OX8+) and resulted in a T_H/T_S ratio of 1.7. By day 20 and beyond, this ratio was less than 1.0, compared with a peripheral blood value of approximately 2.0. A reduced T_H/T_S ratio has been observed in human periapical lesions,[23] as well as in gingiva from human periodontitis.[21]

This result indicates that immunoregulatory changes occur during periapical lesion development. Its significance for periapical pathology may be interpreted in several ways. It may indicate that T_H-mediated activities may be of importance in periapi-

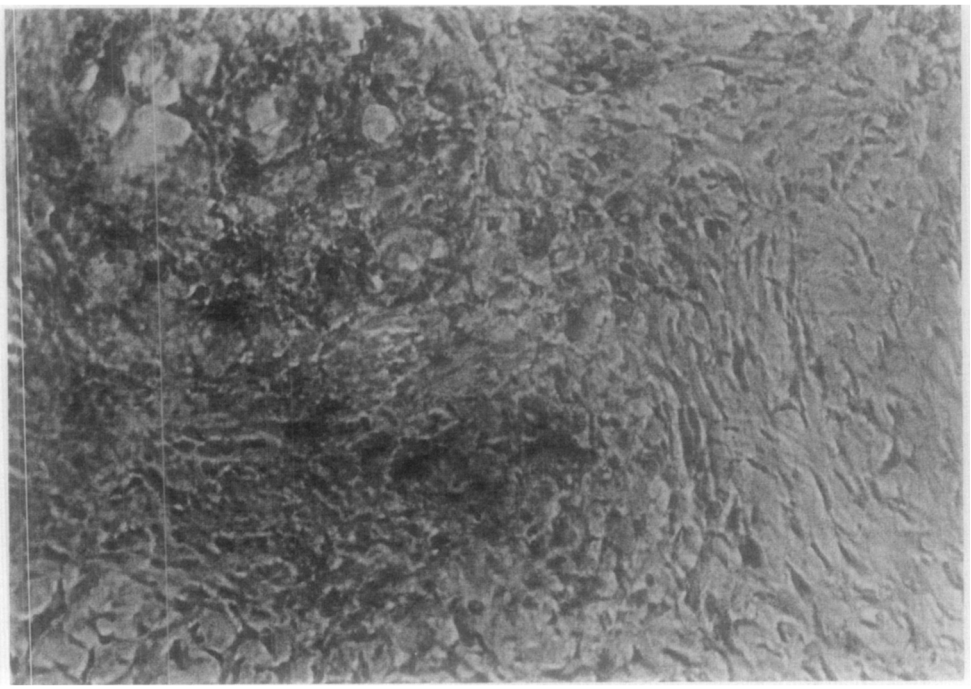

Fig. 3. In situ hybridization with digoxygenin-labeled cRNA antisense probe for IL-1α to section of periapical tissue on day 30 after exposure. Numerous stained cells with the morphology of macrophages and fibroblasts are present. (Original magnification ×400.)

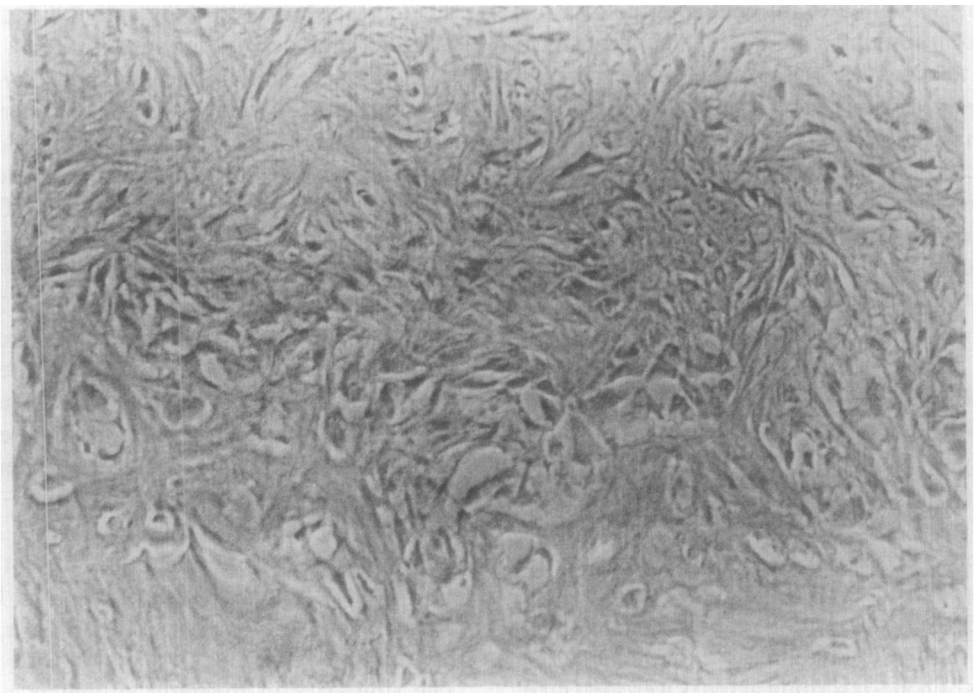

Fig. 4. In situ hybridization with IL-1α cRNA sense negative control probe. (Original magnification ×400.)

cal lesion expansion, whereas T_S functions may be involved in lesion stabilization. In this respect, potential T_H-mediated mechanisms relevant to bone resorption include (1) production of γ-interferon, which activates macrophages to produce bone-resorptive mediators IL-1α, IL-1β, and TNFα; (2) production of the bone-resorptive cytokine TNFβ (lymphotoxin); (3) production of IL-2, IL-4, IL-5, and IL-6 (helper T

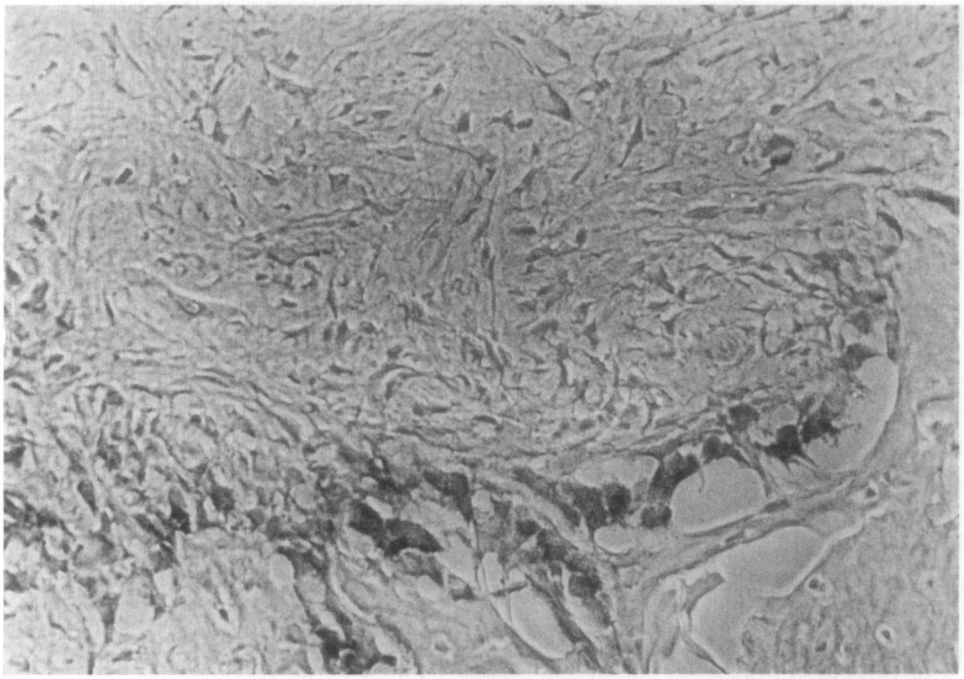

Fig. 5. Staining of osteoclasts by in situ hybridization with an antisense cRNA probe for IL-1α. Note staining of multinucleated on-bone cells. Section of periapical tissue on day 30 after pulp exposure. (Original magnification ×400.)

factors), which stimulate antibody production and ultimately form immune complexes. The latter have been shown to stimulate bone resorption in the cat.[6]

Alternatively, the preponderance of T_H cells during the active phase may simply reflect the genesis of the local antibacterial T-cell response, which initially involves proliferation of T_H cells stimulated by Class II histocompatibility antigen positive antigen-presenting cells. T_S cells are subsequently induced by antigen and T_H cells[24] and may come to predominate at later time points.

CHARACTERIZATION OF BONE-RESORBING ACTIVITY IN PERIAPICAL LESIONS

To identify the bone-resorptive mediators actually present during periapical lesion expansion, we have analyzed extracts of periapical tissues for bone-resorbing activity.[13] Extracts obtained on day 15 contained significant levels of bone-resorbing activity as determined by ^{45}Ca release from prelabeled long bones from fetal rats. In kinetic experiments, the highest levels of bone-resorbing activity were detected in tissues on days 10 and 15, declining thereafter on day 20 to near baseline levels by day 30. Identical levels of resorbing activity were found in the presence or absence of polymyxin B, an inhibitor of bacterial LPS. Normal rat dental pulp and periodontal ligament contained no activity. These findings demonstrated that bone-resorbing activity is temporally related to bone destruction in this model.

In characterization studies, bone-resorbing activity was abrogated by proteinase K treatment but not by polymyxin B, which indicates that the active moiety was a host-derived protein rather than bacterial LPS.[8] Activity was destroyed by heating to 70° C but not 56° C, an inactivation profile identical to that of IL-1. The bulk of activity chromatographed to peaks of molecular weight 30 to 80 kDa, 15 to 20 kDa, and approximately 0.5 kDa. The low molecular weight peak corresponded to PGE_2 and accounted for 10% to 15% of total resorbing activity. Treatment of extracts with anticytokine antisera demonstrated that only anti-IL-1α significantly neutralized bone-resorbing activity (−46% for day 15 extracts and −72% for day 30 extracts). This result indicates that IL-1α is the predominant resorptive mediator present during both active and chronic phases of periapical lesion development. Antisera against IL-1β, TNFα, and TNFβ had minimal inhibitory effects at both time points. The similarity in mediator profile in active versus chronic lesions suggests that quantitative rather than qualitative differences in mediators are likely to account for lesion progression.

Day 15 periapical tissue explants also produced significantly more IL-1α (approximately 90%) than IL-1β (10%) in vitro. Finally, the levels of IL-1α were

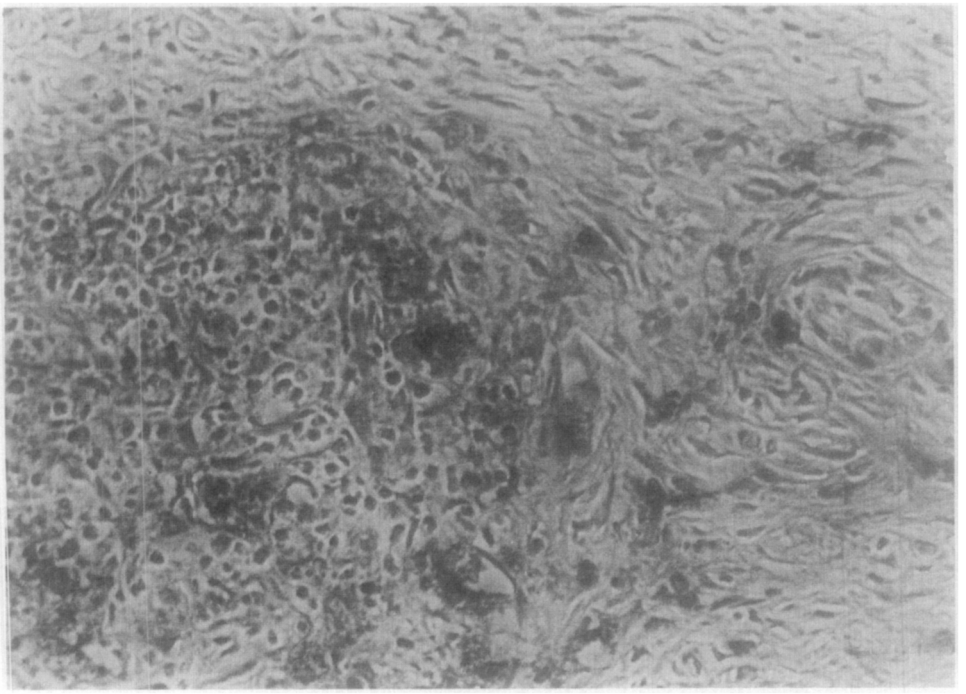

Fig. 6. Immunohistochemical staining of IL-1α positive cells in day 30 periapical tissue. Reactivity of the primary antibody was revealed with the use of an immunoglucose oxidase conjugate (Vector Laboratories, Burlingame, Calif.) as previously described.[12] (Original magnification ×400.)

highest on day 10 and 15 after induction, as determined by radioimmunoassay. Taken together, these results indicate a central role for IL-1α in stimulating bone resorption in this model. In addition, PGE_2 may also participate in this process, both by its direct effects and through synergism with IL-1α.[25]

CYTOKINE mRNA EXPRESSION IN DEVELOPING PERIAPICAL LESIONS

Cytokine gene expression has also been analyzed at the level of mRNA[26, 27] and protein[28] in developing lesions. Total cell RNA was isolated from periapical tissue obtained on days 5, 7, 10, 15, 20, 30, and 60 after pulp exposure. mRNA for IL-1α and IL-1β was amplified by reverse transcription polymerase chain reaction. IL-1α mRNA was expressed beginning on day 7 (2 of 5 animals) and increased on days 10 and 15 (5 of 5), corresponding to the active phase (Table II). IL-1α mRNA persisted to day 60. In contrast, IL-1β mRNA was expressed only after the active phase (days 20, 30, and 60) at lower levels than IL-1α. Culture supernatants from days 15, 30, and 60 periapical tissue explants contained IL-1 activity as determined by the thymocyte costimulation assay, >95% of which was neutralized by an anti-IL-1α antiserum.

Cytokine gene expression has also been assessed by *in situ* hybridization, which detects mRNA for specific cytokines within individual cells.[27] Digoxygenin-labeled sense (control) and antisense cRNA probes were generated from the T7 and T3 promoters of pBluescript containing cDNA for IL-1α or IL-1β. Significant numbers of IL-1α mRNA-expressing cells were present in periapical lesions beginning on day 7 after pulp exposure (Fig. 3), whereas only rare IL-1β positive cells were seen (not shown). No specific labeling was observed with either of the sense probes (Fig. 4). IL-1α mRNA positive cells had morphologic characteristics consistent with macrophages, fibroblasts, and of interest, osteoclasts (Fig. 5). Studies are in progress to evaluate TNFα mRNA by this technique.

IMMUNOHISTOCHEMISTRY OF CYTOKINE EXPRESSION

The expression of bone-resorptive cytokine protein by cells in inflamed pulp and periapical lesions has also been quantified immunohistochemically.[28] Maxillae and mandibles were obtained on days 0, 2, 4, 7, 15, and 30 after pulp exposure. Jaws were defleshed, decalcified, sectioned, and immunostained for the presence of IL-1α, IL-1β, TNFα, and TNFβ with the use of an immunoglucose oxidase second antibody conjugate. IL-1β and TNFβ-producing cells were not observed in pulp or periapical lesions from day 0 to day 30. In contrast, IL-1α-expressing cells were nu-

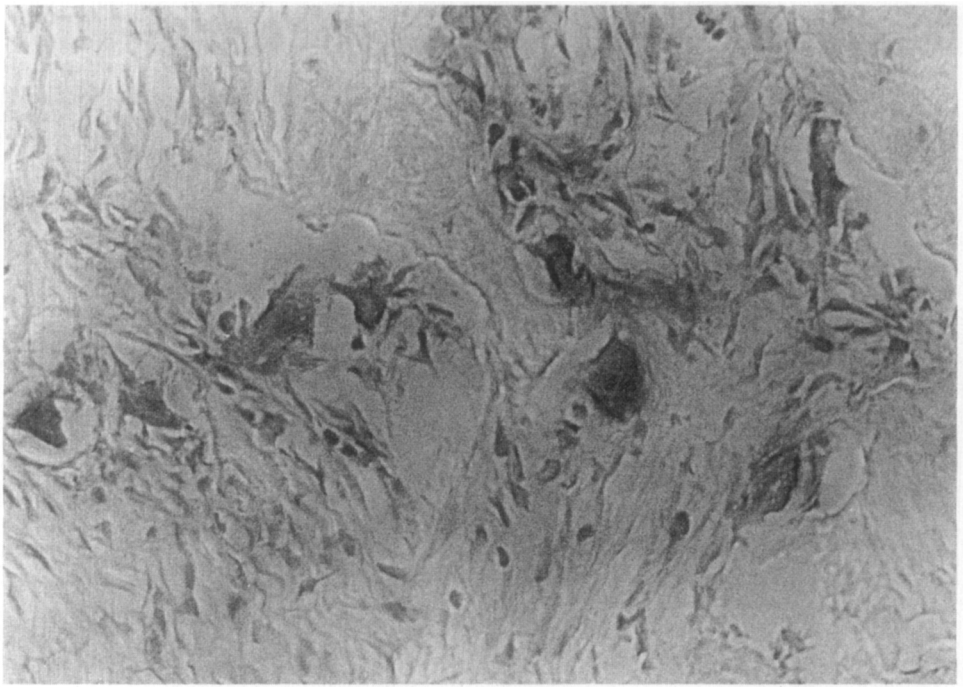

Fig. 7. Immunohistochemical staining of IL-1α positive osteoclasts in day 30 periapical tissue. Immunoglucose oxidase detection (Original magnification ×400.)

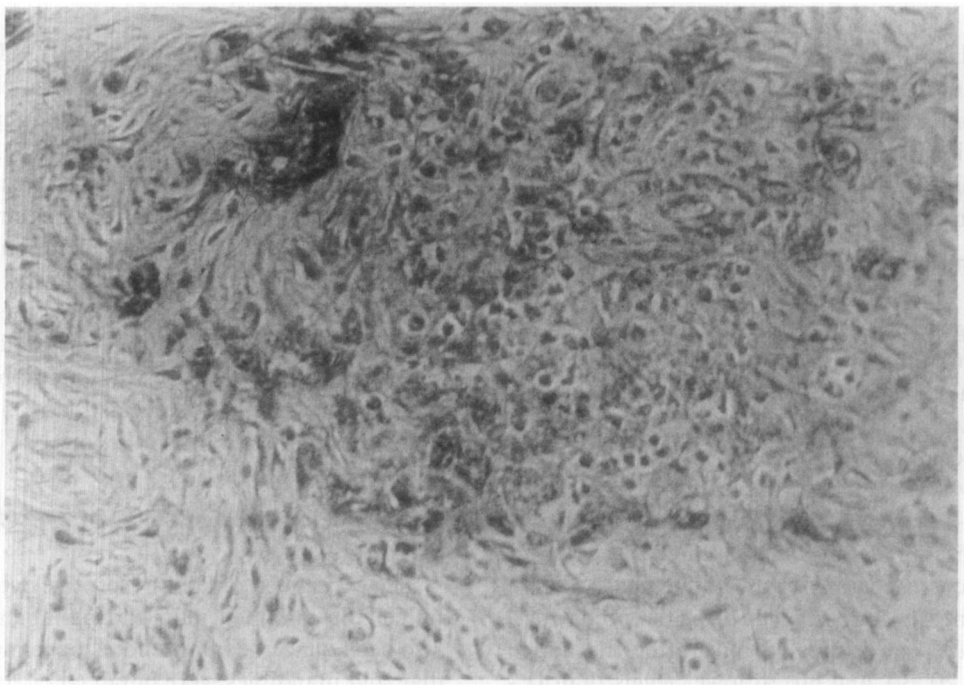

Fig. 8. Immunohistochemical staining of TNFa positive cells in day 30 periapical tissue. Immunoglucose oxidase detection. (Original magnification ×400.)

merous and were present in pulp beginning on day 2 and in periapical lesions beginning on day 7. Cells positive for these mediators were identified mainly as macrophages and fibroblasts, with occasional expression by neutrophils (Fig. 6).[29] Osteoclasts were also IL-1α positive (Fig. 7)[27] whereas lymphocytes were uniformly negative.[30] Of interest, TNFα-producing cells were also detected in pulp and periapical tissue

Table III. Effect of IL-1ra infusion on periapical bone resorption

Treatment	*Animals (n)*	*Net cross-sectional area resorbed (mm^2)*	
		Histomorphometry	*Radiography*
Vehicle control	6	0.052 ± 0.036*	0.056 ± 0.032
IL-1ra	6	0.021 ± 0.018 (−60)†	0.017 ± 0.012 (−70)

*Mean ± S.D.
†(% inhibition vs control).

by immunohistochemical examination (Fig. 8), whereas there was little evidence for their presence in bone-resorbing activity neutralization studies. The explanation for this discrepancy is currently under further investigation. Cytokine-producing cells were usually located together around abscesses and were increased in concert with enlargement of the area of periapical bone resorption. Taken together, these results on cytokine mRNA and protein expression provide strong confirmatory evidence that IL-1α is the primary mediator of bone resorption in periapical lesions in the rat model system.

INHIBITION WITH IL-1 RECEPTOR ANTAGONIST

Recently, a direct demonstration of the importance of IL-1α in periapical pathologic examination was provided by specific inhibition studies in vivo. The IL-1 receptor antagonist (IL-1ra) is a naturally occurring homologue of IL-1 that binds to IL-1 receptors but fails to activate receptor-bearing cells.[31, 32] The IL-1ra is thus a competitive inhibitor of IL-1 that was the first described example of a cytokine, which functions to block the action of another cytokine. Recently, we have used the IL-1ra to examine the role of IL-1α in the periapical lesion model in vivo. Groups of rats were implanted with 14 day Alzet minipumps that contained recombinant human IL-1ra or saline solution as a control. The following day periapical lesions were induced by pulp exposure. Animals were killed on day 18, and the size of lesions quantified by histomorphometry. The results demonstrated that, at least in this preliminary study, lesions were inhibited by 60% in animals receiving IL-1ra compared with controls (Table III, Fig. 9). This experiment confirms the functional significance of IL-1α in periapical pathogenesis in the rat model.

CYTOKINES IN HUMAN PERIAPICAL LESIONS

We have also examined human periapical lesions for the presence of proinflammatory cytokines. In contrast to the rat, the predominant form of IL-1 expressed in human beings is IL-1β rather than IL-1α.[9] This appears to represent a species difference in that

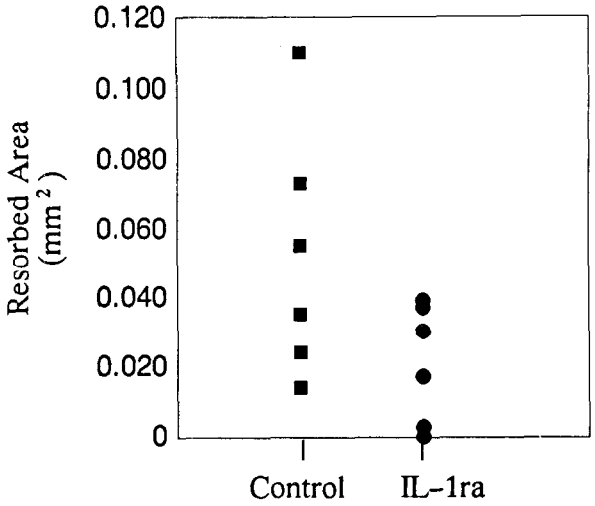

Fig. 9. Effect of IL-1ra on periapical bone destruction. The area of resorption was quantified histomorphometrically. Symbols represent the mean of right and left mandibular first molars from each animal on day 20 after pulp exposure.

IL-1α is generally observed to be the predominant form of this mediator present in rodents versus IL-1β in human beings. Nevertheless, this finding indicates that IL-1 is also likely to represent a final common pathway mediator for periapical destruction in humans.

CONCLUSION

Our studies using the rat model have implicated the bone resorptive cytokine IL-1α as a key mediator in periapical bone destruction. PGE_2 is also present and probably contributes to resorptive activity, both through its direct effects, and by means of synergistic interactions with cytokines. In addition to its stimulating resorption, IL-1 is a potent inhibitor of bone formation[33, 34] through suppressive effects on osteoblast synthesis of bone matrix proteins. The continued presence of IL-1α at high levels in chronic phase lesions may serve to prevent reparative bone formation. Immunohistochemical studies demonstrating the presence of TNFα-positive cells suggests that this mediator, which also inhibits bone formation, may further contribute to this process.[35, 36]

The rat model of periapical pathogenesis is closely similar in its microbiologic, immunologic, and histopathologic characteristics to periodontal disease. The primary differences are the more rapid kinetics of destruction and the localized nature of periapical disease. The effectiveness of the IL-1ra and prostaglandin synthetase inhibitors[4] in reducing periapical bone destruction illustrates how therapeutic strategies for preventing infection-stimulated bone resorption can emerge from analyses of basic pathogenic mecha-

nisms. Furthermore, such strategies may also prove useful in periodontal disease and in other disorders that involve chronic inflammation and bone resorption.

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