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Review article

Macrophages in periapical lesions

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Abstract – Macrophages are major constituents of periapical granulomas. They have a central protective role in both innate immunity and adoptive, antigen-specific immune response. Macrophage activation may occur in periapical granulomas by cytokines produced by antigen-activated T-lymphocytes; by bacterial endotoxin, as part of the innate immunity; or by both these processes. Recent studies in athymic animals have shown that periapical granulomas may develop independently of T-lymphocytes. This observation reveals the major role that the activated macrophage may have in the formation of periapical lesions. Only a few of the macrophages in the periapical granuloma are activated. Current studies indicate that these activated cells are the source of the bone-resorbing cytokines in the periapical granuloma. Understanding the central role of the activated macrophage in the formation as well as the perpetuation of periapical lesions may lead to the development of new diagnostic and therapeutic tools in endodontics.

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Periapical lesions are an expression of the host response which actively prevents dissemination of bacteria from the infected root canal into the surrounding bone. The cells involved in this process include B- and T-lymphocytes, plasma cells and the "professional" phagocytes: macrophages and PMNs. The engagement, phagocytosis and killing of bacteria are the main tasks of the periapical host response; nevertheless, formation of these lesions is associated with bone loss in the area surrounding the root apex. The resulting radiolucent periapical lesion is one of the main clinical manifestations of this inflammatory response, and its progress or healing is commonly evaluated by the size and morphology of the lesion as shown on a radiographic image.

Qualitative and quantitative studies of the cellular composition of periapical granulomas have been profoundly influenced by the methodology available at the time. Initial attempts to characterize the cells participating in these lesions were based on the classic morphology of the cells. Electron microscopy and histochemistry followed later. With the introduction of immunohistological methods, the first attempts to specifically identify plasma cells in periapical lesions by their immunoglobulin content were reported. In the last decade, intensive use of monoclonal antibodies against subsets of T-lymphocytes, B-lymphocytes, macrophages, dendritic cells, as well as plasma cells and PMNs, resulted in a major breakthrough in the understanding of the immunobiology of periapical host response, in both naturally occurring human periapical lesions and those experimentally induced in the rat.

Cells with a distinct morphology such as PMNs, mast cells and osteoclasts have always been identifiable. This was also the case with lymphocytes as a group, but not with macrophages. In earlier studies only cells with a classic macrophage morphology could be identified as such. Currently available monoclonal antibodies make it possible to identify macrophages of diverse morphology and recognize subsets of these cells.

The purpose of the present review is to examine the role of macrophages in the formation and maintenance of these lesions, as it gradually emerges from the vast literature on this subject.

Presence of macrophages in periapical lesions

The presence of macrophages in human periapical inflammatory lesions has been a common and frequently reported finding. Macrophages constitute up to 46% of the periapical inflammatory cells found in tissue sections of human periapical granulomas (1). When Stern et al. (2) dispersed periapical granulomas to cell suspensions, 30% of the resulting cells were macrophages. Macrophages were also found to be the predominant inflammatory cell when Kopp & Schwarting (3) used monoclonal antibodies to identify them in human periapical lesions. Piattelli et al. (4) have similarly reported that macrophages outnumber T-lymphocytes in human periapical granulomas.

In the rat model, Kawashima et al. (5) recently demonstrated that macrophages are the predominant immunocompetent cells throughout the development of the lesion. The kinetics of their presence in these experimental periapical lesions were studied by Akamine et al. (6) who followed the periapical lesions for as long as 150 days. Macrophages increased in numbers during the first 10 days, maintained this level through day 60, and declined gradually thereafter.

Potential role of macrophages in the periapical granuloma

Macrophages have central roles in (a) innate, nonspecific immunity; (b) the onset, regulation and outcome of antigen-specific, acquired, immunity; and (c) the regulation of connective tissue destruction and repair.

Macrophages are professional phagocytic cells that can internalize and kill bacteria by several mechanisms, some of which are part of the innate immunity while others require the presence of specific antibodies against the bacterium and should be considered part of the effector arm of specific, acquired immunity. Bacteria that are new to the host may activate the complement system by the alternative pathway, resulting in their opsonization by the C3b component. This in turn will result in their phagocytosis by the macrophages via a C3b-receptor-mediated process. Other bacteria may attach to the macrophages through lectin-mediated mechanisms, leading to lectinophagocytosis, which is independent of the common receptor-ligand binding (7). Once specific antibodies to a bacterium are present, either developing through the course of the current infection or as result of a former encounter with this bacterium, a most efficient form of phagocytosis will occur, involving dual opsonization by IgG and C3b and the engagement of both the Fc and C3b macrophage receptors.

It is the innate immunity that enables the host to survive the initial steps of infection, while the acquired, specific, immunity allows it to efficiently eliminate the invading microorganisms. Macrophages present in the periapical granuloma contribute by their function as phagocytes to effectively preventing the dissemination of bacteria from the infected root canal.

Macrophages may also serve as "antigen-presenting cells" in the essential initial steps of the induction of acquired immunity. They process the antigen and present it to the antigen-specific clones of T-helper lymphocytes by a process involving the recognition by the lymphocytes of an MHC II molecule on the macrophages. Additionally, they produce the cytokine IL-1, which is an essential complementary signal for the activation of these lymphocytes. Macrophages that carry MHC II molecules, and thus may serve as antigen-presenting cells, have been identified in periapical granulomas in both humans and the rat model (termed also HLA-DR or Ia antigen-positive cells) (3, 8).

Macrophages are considered a main source of the cytokines IL-1α IL-1β and TNFα, which contribute to the initiation and regulation of the inflammatory process. Additionally, they produce a plethora of other active molecules, including metallo-proteases (collagenase, elastase), and prostaglandins, which may also contribute to the destructive outcome of the periapical inflammatory process. Some of these products directly damage connective tissue constituents, while others, including the cytokines produced by the macrophages, activate other cells to either (a) destructive action such as osteoclast activation and bone resorption or (b) the constructive process of repair by activating fibroblast proliferation and collagen production by these cells.

Though it is commonly assumed that *all* of the above long list of potential activities of the macrophage take place in the periapical granuloma, it is not essentially true. Certain processes may be active while others may rarely occur in this lesion. Similarly, it is commonly implied that *all* macrophages perform *all* the above tasks, which similarly is erroneous: subsets of these cells, which may exist in relatively small numbers, may be responsible for a specific activity. Emerging evidence indicates that some of these functions, such as active production of IL-1, involve only a few activated macrophages, which in chronic human periapical granulomas do not exceed 2%–3% of the macrophages present in these lesions (9).

Evolvement of the immunobiological concept of periapical lesions

Studies that aim to elucidate the immunobiology of periapical lesions may roughly be divided into three eras. Early studies concentrated on the production and function of immunoglobulins in these lesions. Next, the specific T-lymphocyte function was empha-

sized and their subsets meticulously studied in relation to periapical inflammation. The third and current era has been initiated by the use of immunodeficient animal models such as athymic and "knock-out" animals, which reveal the central role that macrophages have in this complex local host response.

In the long-term perspective the inevitable conclusion is that the availability of new methodologies influenced the type of studies performed and eventually affected the evolvement of the immunobiological understanding of the complex nature of periapical host response.

Initially, the commercial availability of specific antibodies directed against human IgG, IgM, IgA and IgE allowed immunofluorescent or immunohistochemical detection of these molecules in periapical lesions, either in a free form or as a marker of subsets of B-lymphocytes and plasma cells. Later, the combination of these antibodies with those directed against human complement allowed the demonstration of *activity* rather than the simple *presence* of immunoglobulins in periapical lesions: Johannessen et al. (10) have demonstrated intracellular colocalization of IgG and C3b in macrophages in periapical inflammatory lesions, suggesting phagocytosis of bacteria by dual opsonization by both opsonins.

At a later stage, the availability of monoclonal antibodies against T-lymphocyte subsets made it possible to explore the presence of these cells in both human periapical lesions as well as in those experimentally induced in rats. T-cells in human periapical granulomas were studied by Cymerman et al. and others (11–13). It became apparent that both T-helper and T-suppressor lymphocytes are present in these lesions (11). In delayed hypersensitivity in humans, a typical T-helper to T-suppressor relation is about 2:1 (14). It was therefore of interest to define whether T-lymphocytes in periapical lesions follow this trend. Babal et al. (12) have found a T-helper to T-suppressor ratio which was <1.0 in periapical granulomas, while Barkhordar & Desouza (13) report a ratio of ~ 1.0. Therefore, it seems that the predominance of T-helper lymphocytes, which is typical of delayed hypersensitivity, does not exist in the chronic periapical granuloma. Nevertheless, this is not a uniform finding, as Kopp & Schwarting (3) found a T-helper to T-suppressor ratio of 3:2 in periapical granulomas, which diminished to ~ 1.0 in periapical scars.

The rat model allows a further insight into the kinetics of T-lymphocyte subsets in the developing periapical lesion. Stashenko & Yu (15) demonstrated that during the early, active, phase of lesion development T-helper cells predominate while at the later chronic stage T-suppressor cells outnumber the T-helper cell population. The initial T-helper to T-supressor ratio of 1.7 turned at the later stage into <1.0, as compared to a T-helper to T-supressor ratio of 2.0 in peripheral blood. These findings were interpreted as an initial active function of T-lymphocytes, which is later down-regulated and controlled by T-suppressor cells. The balance of their action is expressed in chronic periapical lesions, such as those encountered in humans.

Protective function of T-lymphocytes in periapical lesions

A protective role of T-helper lymphocyte function should eventually be expressed as a better ability of the host to prevent bacteria from spreading from the infected root canal. This may be accomplished by (a) producing antibodies locally and (b) increasing the local availability of phagocytes and enhancing their function (Fig. 1). Local activation of antigen-specific T- helper lymphocytes is a prerequisite for a local production of antibodies specific to the bacteria that periodically emerge from the root canal (16, 17). This in turn will enable the effective opsonization of the bacteria, followed by phagocytosis and killing.

Local macrophage activation is accomplished mainly by γ-interferon produced by the activated T-helper cells (Fig. 1). Even though the activation of the lymphocytes is antigen-specific, once macrophages are activated, the effector result will be nonspecific, and their phagocytic and killing abilities will be greatly enhanced. IL-1 production by activated macrophages will locally elevate CAM-1 molecule ex-

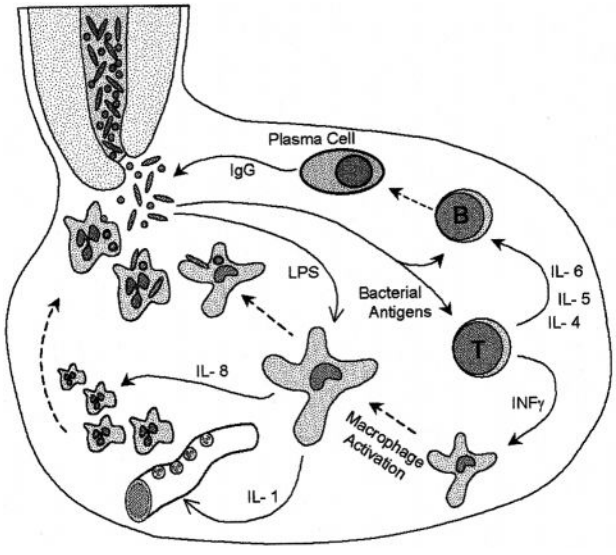

Fig. 1. Protective role of activated macrophages and T-lymphocytes in periapical granulomas. T-lymphocyte activation leads to antigen-specific B-lymphocyte activation and local production of specific antibodies. It also leads to macrophage activation, which will result in enhanced phagocytosis by these cells, as well as in cytokine-mediated enhanced PMN margination, chemotaxis and their activation. Macrophage activation may also be achieved independently of T-lymphocytes by bacterial endotoxin (LPS).

pression by endothelial cells in the capillaries, thus enhancing the local attachment of PMNs and monocytes and enhancing their migration into the area. IL-8 produced by these macrophages will chemotactically attract the PMNs and activate them, making them more available and more competent to engage and kill the bacteria (Fig. 1). Activation of the macrophages has a major role in maintaining the two lines of phagocitic cell defence, typically described in the periapical lesion: an inner area, closer to the apex, in which PMNs predominate; and around it the area in which the phagocytic macrophages are seen (5).

Therefore, the defensive function of T-helper lymphocytes is achieved indirectly through allowing the activation of (a) specific B-lymphocytes to become plasma cells and produce antibodies and (b) nonspecific effector cells: the macrophages. In order to avoid an endless loop of mutual activation of macrophages and T-lymphocytes, the process is actively controlled and down-regulated by T-suppressor lymphocytes.

The essential role of the T-lymphocytes in the process is generally acknowledged. The first studies that used athymic mice and rats to study the formation of periapical inflammatory lesions were accordingly designed to finally "nail down" the critical role of T-lymphocytes in the formation of these lesions (18, 28). As it transpired, these studies are the turning point in proving otherwise. Both demonstrated that periapical lesions can develop *independently* of T-lymphocyte activity, thus leaving the stage to the other key actor: the macrophage (as will be detailed below).

Bone resorption in periapical lesions

Bone resorption in the periapical region is one of the clinical hallmarks of periapical pathosis. Host defense against the spread of bacteria from the infected root canal does not depend directly on bone resorption in the area. The resorption may be viewed as either an undesirable byproduct of the host response, as is the case with periodontal disease, or alternatively as a process by which the bone is removed from a risky area, thus allowing a "buffer zone" to be formed, in which host-response constituents engage the bacteria (20). In either case, it is bone resorption in the periapical area which serves the clinician as a major indicator for either progress of disease or repair of the periapical lesion. As such it has been thoroughly studied in both humans and animal models.

Potential *vs* actual bone-resorbing agents

Bone resorption occurs through the activation of the bone-resorbing cells: the osteoclasts. A wide range of biologically active molecules have been demonstrated to have the capacity to activate osteoclastic bone resorption in *in vitro* models. These include prostaglandins (21), bacterial endotoxin (22), complement activation products, as well as the inflammatory cytokines IL-1α, IL-1β, TNFα, TNFβ, IL-6 and IL-11 which, as a group, were previously referred to as "osteoclast activating factor" (OAF) (23). Among these IL-1β is the most active cytokine and its bone-resorbing capacity is 13 times that of IL-1α and 1000 times that of TNFα or TNFβ (24).

All of these have been mentioned in relation to the periapical bone resorption associated with infected root canals. The question is which of these *potential* bone-resorbing stimuli is *actually involved* in activating the osteoclasts in these lesions?

Two recent studies by Wang & Stashenko (25, 26) provided convincing evidence that among the long list of *potential* mediators that may activate osteoclasts and cause periapical bone resorption, the main and most important in human chronic periapical lesions are IL-1β and TNFβ. In the rat model of active periapical bone resorption, IL-1α and, to a lesser extent IL-1β and TNFβ, are the major bone-resorbing cytokines. Both studies indicate that osteoclast activation by these cytokines is mediated by the formation of cyclooxygenase pathway products such as prostaglandins, as the effect could be significantly blocked by nonsteroid anti-inflammatory drugs (NSAID) (25, 26).

The formation of periapical lesions was studied in the rat model by Kakehashi, Stashenko and others (5, 15, 27–30). Following the exposure and contamination of the pulp and root canal, an inflammatory response is activated in the periapical region. This is associated with a rapid growth of a periapical lesion whose size can be monitored using either radiographs or histologic sections. This rapid growth persists for 15 days and is associated with "bone-resorbing activity" that can be detected in homogenates of the lesions and measured using an *in vitro* bone resorption assay (29). Following the active resorptive phase, the size of the lesions remains stable for up to 30 days (29). During this stationary phase the bone-resorbing activity declines to 10%–30% of that in the active growing stage. This stationary phase is considered an equivalent of an existing, chronic, periapical granuloma in humans which also contains bone-resorptive activity (25).

The cytokines defined in the above studies are found in human periapical lesions in measurable amounts. Lim et al. (31) found significant amounts of IL-1β in homogenates of human periapical lesions, even though none of the patients had detectable serum levels of this cytokine. Noninflamed pulp tissue that served as control was also free of the cytokine. Periapical exudates were studied by Matsuo et al. (32) for their IL1-α and IL-1β content. Exudates, obtained through the root canal, contained an average level of 6.57(±0.73) ng/ml of IL-1-β and 3.23

(±0.66) ng/ml of IL-1α. The cytokine profile changed following root canal treatment with a tendency of IL-1α to increase and of IL-1β to decrease.

Cellular sources of bone-resorbing cytokines

Although IL-1 and TNF may be produced by many cell kinds, the activated macrophage is considered the main source of IL-1α, IL-1β and TNFα (33). On the other hand, TNFβ is commonly considered an activated T-lymphocyte product (34).

In view of the above, two cell types should be considered responsible for bone-resorbing activity in periapical lesions: activated T-cells and activated macrophages. Not all T-lymphocytes or macrophages in the periapical lesion are in a state of activation. Kopp & Schwarting (3) found that only 20% of the T-lymphocytes in human periapical granulomas are activated. Artese et al. (9), who also used human periapical granulomas, demonstrated that while 41% of the mononuclear inflammatory cells are macrophages, only 2%–3% of these cells are activated and produce IL-1β and TNFα, which were used in this study as markers of their activation (9). Therefore, it seems that a rather small part of the cells in the periapical granuloma are of potential importance as the source of bone-resorbing activity in these lesions. It may not be the total number of T-lymphocytes or macrophages in the lesion that is important but rather the number of activated cells of each kind.

The states of activation of these cells are closely related to each other: T-helper lymphocytes may be activated in an antigen-specific manner by antigen-presenting macrophages which also produce the IL-1 required for this process. Macrophage activation, as part of the acquired, specific, immune response, may be achieved by cytokines such as interferon-γ, produced by the activated T-lymphocytes (Fig. 1). Nevertheless, macrophages may also be activated by other routes, such as exposure to bacterial endotoxin (LPS), as part of the innate, nonspecific immunity (35).

Studies in athymic animals

Athymic rats and mice are powerful tools to study and demonstrate the essential role of T-lymphocytes, in immunobiologic processes (36, 37). These animals lack T-cells, and consequently T-cell function is missing in a variety of immune responses which are thus inactive. Such animals were recently used in two studies, and it was assumed that periapical bone resorption and development of periapical lesions will be defective. The results of these studies should be viewed as a turning point in understanding the immunobiology of the host response and bone resorption in periapical lesions. Wallstrom et al. (18) demonstrated that no significant difference exists between periapical tissue responses of conventional and athymic rats (18). A similar result is reported by Tani-Ishii et al. (28) who used athymic mice. They also found that periapical lesions develop in animals lacking T-cells at a rate that precludes the possibility that T-lymphocytes are an essential prerequisite for the development of these lesions. Even though T-lymphocytes may, and most probably do, usually contribute to the process, alternative routes exist that enable the formation of the lesions in their absence.

The activated macrophage may serve as such a route in the formation of periapical lesions. Macrophage activation may occur by a variety of pathways. Cytokines such as γ-interferon, which are produced by antigen-specific activated T-lymphocytes, are the main immune-response-related activators of the macrophage (33, 38). Nevertheless, in their absence, bacterial endotoxin (LPS) may successfully accomplish this task (35, 39, 40). This activation of the macrophage may be viewed as part of the innate immunity, which is independent of specific response to antigens. This may be a mechanism by which the lesions developed in the athymic animals. The bacterial content of the infected root canals in these animals gradually developed to a 46% gram-negative flora (41). LPS derived from these gram-negative bacteria could activate macrophages in the periapical area. These cells, in turn, produce their cytokines IL-1α, IL-1β and TNFα, which activate osteoclastic bone resorption. This does not preclude participation of the T-cell in the process in normal animals, but rather turns the spotlight on *the main effector cell*: the macrophage. This is also in agreement with the finding that in the rat model IL-1α is the major bone-resorbing cytokine while TNFβ, the T-cell product, could not be detected in these lesions, neither in antibody-blocking nor in immunohistochemical studies (26, 42).

Kinetics of macrophage infiltration in periapical lesions

The unique study by Akamine et al. (6) followed rat periapical lesions for as long as 150 days. Analysis of their data reveals that the active growing stage of periapical lesions in the rat, which lasted for the first 60 days, coincides with a peak of macrophage presence in the lesion. When active growth stops and a stationary stage is reached, the presence of macrophages in the lesion gradually declines. This may be a coincidence; nevertheless, it may express a significant correlation. Further support for this notion may be found in a recent study by Kawashima et al. (5) who showed that macrophage infiltration in the periapical lesions is associated with bone resorption in the area. In their study, macrophage infiltration preceded that of lymphocytes and gradually increased throughout the 56 days of the experiment.

IL-1 and macrophages in the periapical granuloma

The presence of IL-1β in association with a subpopulation of the macrophages in periapical lesions has been reported by several investigators (9, 30, 42). Artese et al. (9) reported that in established human periapical granulomas, there are very few cells with immunoreactivity of IL-1β and TNFα and that these cells have a macrophage morphology. Tani-Ishii et al. (42) demonstrated in the rat model that IL-1α and TNFα are associated with macrophages in the periapical lesion as soon as 2 days after exposure of the pulp. They persisted through the 30 days of the experiment. In contrast, TNFβ and IL-1β could not be detected in the sections.

Nevertheless, the presence of cytokines *in association* with these cells does not essentially prove that they are the source of these molecules. For example, IgE is found in specific association with basophils and mast cells, even though it is a plasma cell product that attaches itself to a receptor on the former cells. Similarly, the cytokine may potentially have been attached to, or taken into, these cells, rather than produced by them. Recently, direct proof was provided which clearly demonstrates that the activated macrophages in fact produce IL-1β in periapical lesions. In an *in situ* hybridization study, Hamachi et al. (30) demonstrated the presence of messenger RNA for IL-1β in the macrophages. This proves not only that these cells are capable of producing cytokines in general and that the cytokines are associated with them in the periapical lesion, but also that subpopulations of macrophages are actively engaged in *producing* this cytokine in periapical granulomas.

Future perspective

Endodontic treatment aims to eliminate bacteria from the infected root canals, which will later be sealed, to prevent recontamination. With the bacterial stimuli that evoked the periapical inflammation gone, the periapical lesion should resolve, and repair should take place. Nevertheless, healing of the lesion may take many months. It may be argued that if the lesion eventually heals in 12 months, there is no benefit in rushing the process. Nevertheless, this may have clinical importance, as it may allow earlier decisions to be made in regard to the restorative treatment plan for the treated teeth.

This prolonged healing process raises the possibility that the activated cells in the lesion may maintain their state of activation long after the initial cause of their activation has been eliminated.

Macrophages are known to persist in the tissues for many months and if their state of activation persists, they may inhibit the fibroblasts and maintain osteoclast activity, thus preventing both soft connective tissue repair and bone repair from taking place (19, 35).

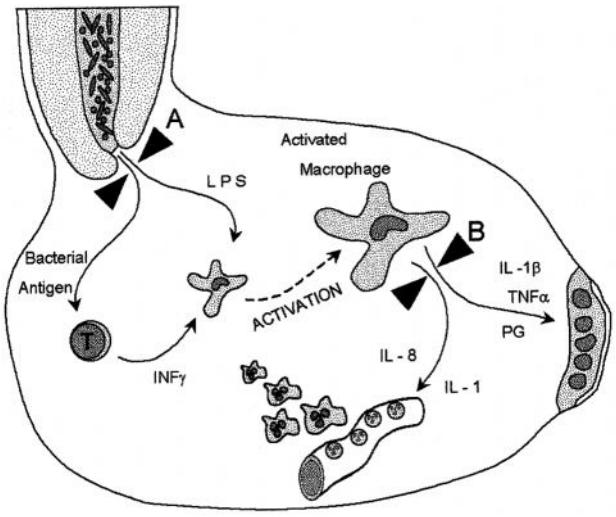

Fig. 2. Elimination of macrophage-derived destructive mediators. (A) Traditional method: by root canal treatment, eliminating activation stimuli such as bacterial antigens and LPS. (B) Proposed method: by pharmacological agents such as steroids, tetracyclines, receptor antagonists or NSAIDs, which interfere with mediators' production or action.

If this is true, it might be important and possible to monitor their state of activation by sampling the interstitial fluid of the lesion through the root canal (32, 43). Recently Kuo et al. (43) were able to measure the IL-1β content of apical exudates and correlate it with clinical and radiological features of the lesions. A longitudinal study to establish a correlation between the diminishing IL-1β content of the lesions and their gradual radiographic repair will be required to prove this point.

Assuming that such inhibitory mechanisms are involved in the prolonged and delayed repair of periapical lesions, pharmacological modulation of the process may be considered (Fig. 2). Stashenko et al. (23) demonstrated that IL-1 receptor-antagonist may be used in animals to reduce bone-resorbing activity and the formation of periapical lesions. Similarly, NSAIDs were successfully used for a similar purpose in experimental and human periodontal diseases, as well as in the cat model for periapical lesion (44, 45). These two approaches are directed at either blocking the *binding* of the already produced cytokine to its target cells or *interfering with its action* on osteoclasts and osteoblasts, which involves prostaglandin production (25, 26).

Tetracyclines may be used to inhibit cytokine *secretion* by activated macrophages (46). Shapira et al. (46) studied tetracycline inhibition of TNF and IL-1 production by LPS-activated macrophages and found its effect to be at a post-transcriptional level: both m-

RNA and the cytokines themselves are produced but are not secreted to the cell surroundings.

An alternative strategy may be to try to "turn off" the activated macrophages, thus lowering the local *production* of IL-1 in the lesion. Modulation of macrophage activation have been attempted both *in vivo* and *in vitro* using glucocorticoids (35, 38, 47, 48). Macrophages, which are activated to become tumoricidal, are turned off *in vivo* by a process involving steroids (47). Recently Metzger et al. (35) reported that suppression of fibroblast proliferation by LPS-activated macrophages is reversed hydrocortisone (35). Such effects on macrophage activation have also been reported by others and are attributed to inhibitory effects of the steroids at the gene transcription level (49), (38, 50).

If and when bacteria are no longer present in the root canal, the state of activation of the macrophages may outlive its useful and beneficial purpose. Attempts to turn off the host response in the lesion may represent a new biological treatment modality that may elevate suppression and enhance repair of these lesions (Fig. 2). Prolonged local delivery of drugs for this purpose may be achieved using biodegradable slow-release devices in the form of a resorbable point that may be inserted through the root canal and deliver the drug locally for a predetermined period of time.

A better understanding of the immunobiology of periapical lesions may eventually result in a different endodontic practice than is encountered today. Chairside diagnostic kits that will allow a periapical lesion to be defined as "active" or "healing", by sampling via the root canal prior to obturation, seems logical and possible. Similarly, pharmacological modulation of the healing process may also not be far off.

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