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Identification of γδT lymphocytes in human periapical lesions

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Endodontic (root canal) therapy is required when the pulp of a tooth becomes necrotic due to a bacterial infection or trauma. A proportion of patients who receive endodontic therapy subsequently have periapical (around the tooth root) lesions detected by radiolucency. Currently, there are no means to identify susceptible patients. Although tissue from periapical lesions has been described as inflammatory, inflammatory cell types and their functions have been poorly characterized. For example, T lymphocytes were identified using pan specific anti-CD3 mAb, which recognizes both αβ and γδT cells. Using the current model of γδT cells as immunoregulatory cells; γδT cells can mediate protective or destructive milieus. We postulated that patients who have a periapical lesion, as identified by radiographic bone loss, mount a γδT cell response. We collected specimens removed by surgery from both periapical lesions and other oral tissues, generated total RNA and performed reverse-transcriptase polymerase chain reaction to identify rearranged δ genes. Results were confirmed with semi-nested polymerase chain reaction. In addition, we demonstrate that these lesions contain a population of $CD3^+$ cells that are αβT cell receptor negative, implying that these cells are γδT cells. Here we show that 36/37 of periapical lesions and only 2/11 of other lesions contain γδT cells ($P < 0.0001$). $V\delta2^+$ T cells were the most common subtype identified (30/36) in these samples. This is the first report in the literature of the presence of γδT cells in human periapical lesions.

Key words: inflammation; granuloma; endodontics; non-vital teeth; radicular cyst

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A chronic inflammatory response to bacterial challenge in the root canals of compromised teeth is believed to be responsible for periapical lesion development (25). Histologically, non-cystic periapical lesions vary from the classic periapical granuloma consisting of granulation tissue and fibrous connective tissue infiltrated by a variety of inflammatory cells to periapical lesions composed of dense fibrous connective tissue containing few chronic inflammatory cells. The inflammatory cells infiltrating periapical lesions include macrophages, neutrophils and lymphocytes, including plasma cells and T lymphocytes (9, 25, 26). The T lymphocytes in periapical lesions were identified using antibodies against CD3, CD4 and CD8 (9, 25). CD3 is found on all αβ and γδ T cells, and CD4 and CD8 may be expressed by both αβ and γδT cells (5). Initial studies did not examine large numbers of lesions or serial sections with dual staining, so it is unclear how many $CD3^+$ cells also stained with CD4 or CD8 (25, 26). Thus, earlier studies using anti-CD3, anti-CD4 and anti-CD8 antibodies do not accurately identify T cells as either αβ or γδ.

There are conflicting reports as to whether γδT cells are found in normal and diseased oral tissues (4, 10, 11). Lundqvist et al. report that γδT cells are found in normal and diseased gingival tissues (10, 11), but Freysdottir et al. did not find γδT cells in normal oral tissues (4). However, Freysdottir et al. did find γδT cells in patients with autoimmune diseases with oral complications such as Behçet's disease. In Behçet's disease, the presence of γδT cells correlates with chronic disease (4). Thus, γδT cells can be detected in diseases affecting the oral cavity.

Emerging evidence suggests that γδT cells are immunoregulatory (2, 5); γδT cells can mediate either protective or destructive milieus. As γδT cells are involved in many different chronic inflammatory diseases, we hypothesized that γδT cells would be present in periapical tissues from patients with a periapical radiolucency. Here, using polymerase chain reaction (PCR), we show that the vast majority (97%) of periapical lesions in this study contain γδT cells. This result is supported by immunohistochemical results showing that there are more $CD3^+$ T cells than αβ TCR^+ cells. In contrast, only a small proportion of healthy and inflamed oral tissues (18%) examined in this study were

infiltrated by γδT cells, consistent with some published observations (4). The majority of γδT cells present in the periapical lesions we studied are Vδ2$^+$. This is the first report showing that γδT cells are present in human periapical lesions.

Material and methods

Patient and tissue selection

Patients were selected for this study based on their need for surgical removal of oral tissues. Periapical lesions were taken from adults of the following ethnic groups: Caucasian (14), African-American (7), Hispanic (8), Middle-eastern (2), Asian (2) and ethnically unidentified (4), and both sexes (21 male, 16 female). Periapical lesions are extremely rare in children, making it difficult to obtain sufficient data to be statistically relevant; therefore, children were excluded from this study. As no patient identifiers were connected to the tissue collected, Internal Review Board exempt status was granted and no patient consent was necessary. Periapical lesions were identified by the presence of a radiolucency and tissues were obtained by apicoectomy. For purposes of comparison we collected a variety of other oral tissues. Other oral surgical procedures were performed as appropriate and included retromolar pad and palate from impacted third molar extraction, gingiva from gingivectomies and crown lengthening procedures. A portion of the tissues were formalin fixed for routine histopathologic examination and the remaining tissue samples were placed in phosphate buffered saline (PBS) containing penicillin/streptomycin and stored on ice until RNA collection.

Total RNA production

Total RNA was extracted using RNAzol™ (Teltest, Friendswood, TX) according to the manufacturer's instructions and as previously described (13).

PCR

RT-PCR

The strategy and primers for detecting rearranged δ genes have been described (16). Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using Super-Script™ (Gibco-BRL, Grand Island, NY) according to the manufacturer's instructions. A reaction containing all ingredients except RNA was included as a negative control. The expected band size for a rearranged Vδ2 gene is 589 base pairs (bp).

Semi-nested PCR

Samples were prepared for semi-nested PCR by removing primers and nucleotides using a Qiaquick nucleotide removal kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Semi-nested PCR was performed using previously described primers (16). Samples were amplified using stringent conditions; denaturation at 94°C for 30 s, annealing at 60°C for 30 s and amplification at 72°C for 30 s for 20 cycles. All PCR products were analyzed on a 1.5% agarose gel.

Immunohistochemistry

Immunohistochemistry was performed on formalin fixed paraffin embedded tissues with appropriate positive and negative tissues (human tonsil and colon). Tissue sections 7 μm thick were prepared onto charged glass slides, baked at 40°C overnight and de-paraffinized through washes of xylene (2×) and graded alcohols (90–70%) to phosphate-buffered saline. Tissue antigen retrieval was performed using a microwave oven and boiling in a solution of 10 mM citrate buffer, pH 6, for 10 min. Slides were stained on an automated immunostainer, NexES, (Ventana Medical Systems, Tucson, AZ) using a prediluted polyclonal anti-CD3 antibody (Ventana Medical Systems, Gibco BRL, Grand Island, NY) or a 1 : 10 dilution of anti-αβ T cell receptor antibody (Becton-Dickenson, CA) and using 3,3 diaminobenzidine as the chromogen. Positive staining was detected by observation of a membranous deposition of 3,3 diaminobenzidine in lymphocytes. The anti-αβ antibody is normally used for flow cytometry, not paraffin sections, which accounts for the higher backgrounds.

Statistical analysis

The test of significance between two proportions was adopted. In this case, the proportion of samples containing γδT cells from periapical lesions was compared with that of non-periapical lesions. It has been shown that the proportion difference follows a nearly normal distribution. Consequently, a Z score test statistic was computed and its associated P value was obtained.

Results

Detection of γδT cells

The goal of this study was to determine the presence of γδT cells in periapical lesions requiring surgery. RNA generated from patient samples was tested for the presence of rearranged δ genes using RT-PCR (16). As a positive control, total RNA was generated from a Vδ2$^+$ γδT cell clone, HF2. Our data show that 30 of 37 (81%) periapical samples contain Vδ2$^+$ γδT cells (representative samples are shown in Fig. 1). Periapical lesions from 5 of 37 (14%) patients contained Vδ1 γδT cells (representative samples are shown in Fig. 2). A single periapical lesion (3%) contained Vδ3 γδT cells (Fig. 2), and one periapical sample (3%) contained no detectable γδT cells (data not shown). Combined, these data show that 36 of 37 (97%) of periapical lesions examined in this study contained γδT cells.

In contrast to the prevalence of γδT cells in periapical lesions, only two of 11 samples (18%) taken from patients undergoing surgery at other sites in the oral cavity contained Vδ2$^+$ γδT cells. We tested lesions from other sites in the oral cavity for the presence of Vδ1$^+$ γδT cells. No Vδ1$^+$ γδT cells were detected. Furthermore, no other specificity of γδT cell was detected in lesions from other sites in the oral cavity. These data show that the vast majority of periapical lesions examined in this study, but few non-periapical lesions, contained γδT cells.

To confirm that samples contained rearranged δ genes, all samples were re-amplified as described. Vδ1$^+$ and Vδ2$^+$ and Vδ3$^+$ amplification products were confirmed using semi-nested PCR with an expected band size of 171 bp (data not shown). These data show that the RT-PCR conditions used specifically amplified Vδ1, Vδ2 and Vδ3 gene products.

Periapical lesions contain CD3$^+$ αβTCR$^-$ lymphocytes

Ideally, the presence of γδT cells should be demonstrated by staining with anti-γδT cell receptor antibodies. However, none of the available antibodies reacts on paraffin-embedded samples (data not shown), and for this study frozen samples were not available. To overcome this obstacle, we have stained serial sections with either anti-CD3 or anti-αβT cell receptor antibodies (Fig. 3) to demonstrate that there is a population of CD3$^+$ αβTCR$^-$ lymphocytes (Fig. 3c, white arrows). All of the lymphocytes will not be present in all of the 7-μm-thick sections, so we have labeled (with white arrows) only those lymphocytes that appear in both the CD3 and αβTCR stained sections. A comparison of the number of CD3$^+$ lymphocytes and αβTCR$^+$ lymphocytes shows that there are more CD3$^+$ lymphocytes present.

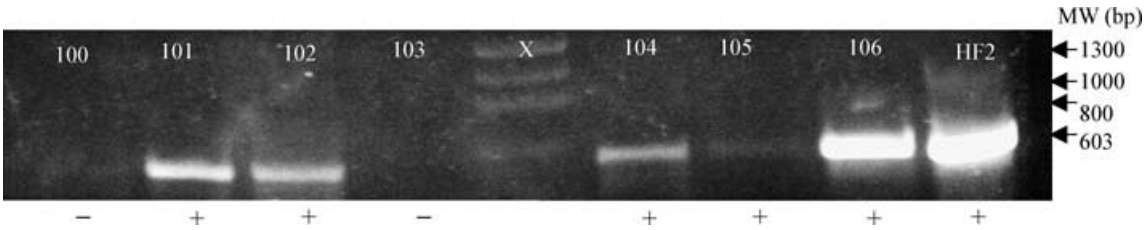

Fig. 1. Identification of rearranged Vδ2 γδT cells using RT-PCR. EtBr stained 1.5% agarose gel showing RT-PCR amplification products from patients listed at the top of the gel. The presence or absence of a band is indicated at the bottom of the gel. The MW marker is the ϕX 174 cut with *Hae*III.

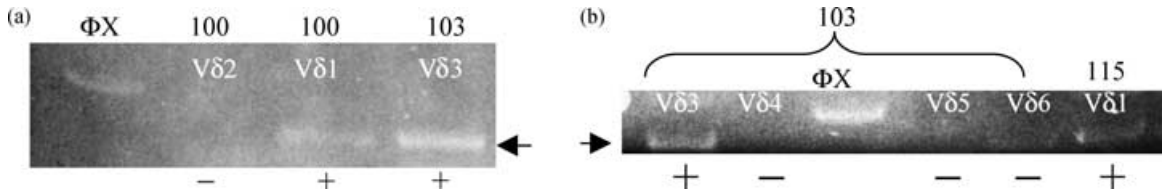

Fig. 2. Identification of alternate Vδ γδT cells using RT-PCR. EtBr stained 1.5% agarose gel, showing RT-PCR amplifications. Patient number is indicated above the gel. The presence or absence of a band is indicated at the bottom of the gel. The molecular weight marker ϕX 174 cut with *Hae*III. Vδ primers are indicated at the top of the gel. The detection of the 589 bp band is indicated by the arrow.

As CD3 is found on all T lymphocytes and has been reported only occasionally on non-T cells, the most probable conclusion is that the $CD3^+$ non-αβT cells are γδT cells.

Combined, these data show whereas 36 of 37 tissue samples from periapical lesions contained γδT cells, only two of 11 samples from other oral tissues contained γδT cells. The Z test for proportional differences yielded a Z value of 5.67 that is highly significant ($P < 0.0001$). In this small sample size we detected no correlation between ethnicity or sex and presence of γδT cells or the use of the Vδ2 gene.

Discussion

γδT cells are associated with several chronic inflammatory human diseases (4, 18, 20, 21). Human periapical lesions are caused by chronic inflammation (6), but only a proportion of patients who received endodontic therapy have a periapical radiolucency. The reason these patients have continued to mount an inflammatory response after therapy is not well understood. One possible explanation for the continued inflammatory response could be the presence of immunoregulatory cells such as γδT cells. We postulate that patients who have periapical lesions mounted a γδT cell response. Here we show that 36/37 or 97% of human periapical lesions, obtained by apicoectomy, contained γδT cells. This result was supported by immunohistochemical results showing a population of $CD3^+$ $\alpha\beta TCR^-$ lymphocytes. The most likely conclusion from these data is that many $CD3^+$ cells are γδT cells. However, this study does not demonstrate a causal relationship between the presence of γδT cells and the existence of the periapical lesion. In conclusion, these two different approaches demonstrate the first identification of γδT cells in periapical lesions.

PCR shows that only one Vδ gene subtype was detected in any sample, suggesting that the γδ response was oligoclonal. Analysis of V gene usage for γδT cells found in these periapical lesions shows that 30/37 are $V\delta2^+$, 5/37 are $V\delta1^+$, and 1/37 is $V\delta3^+$. Of the six different Vδ gene segments, only Vδ1 and Vδ2 are normally found in peripheral blood, with occasional reports of Vδ3-6 (19, 17). Furthermore, Vδ2 is more common than Vδ1 (6, 21). The frequency of gene use in periapical lesions mirrors the frequency of γδT cells found in peripheral blood. The frequencies of Vδ1 and Vδ2 γδT cells found in periapical lesions examined in this study are consistent with studies of γδT cells in other tissues (15, 16). The striking result of this study is finding such a high proportion of γδT cells in the periapical lesions in comparison with non-periapical lesions. Thus, our data support the conclusions of Freyersdott et al. (4) that normal oral tissues do not contain γδT cells.

There is limited information about the role played by different subtypes of γδT cells. $V\delta2^+$ γδT cells respond to host derived phosphoantigens (22), there is an upregulation of $V\delta1^+$ γδT cells in rheumatoid arthritis and inflammatory bowel disease (1, 14, 16) and $V\delta3^+$ γδT cells are increased in perennial allergic rhinitis (18). Other studies have not distinguished between subtypes. Here we show that different subtypes are present in individual patients with the prevalence of subtype paralleling that seen in peripheral blood (15, 16). We have an insufficient sample size to draw additional conclusions. Nonetheless, as 97% of the periapical lesions examined in this study contained γδT cells, we have very strong evidence that γδT cells play a role in the inflammatory process that creates lesions requiring apicoectomy. Interestingly, the diseases mentioned above are autoimmune diseases, showing that γδT cells can respond to damaged host tissues. Thus, it may be that γδT cells in periapical lesions can respond to host antigen(s) and may not require bacterial challenge.

Because there is little information about why patients develop chronic inflammatory periapical lesions, and we have demonstrated such a strong correlation (97%) between the presence of γδT cells and periapical lesions, we speculate on a role for γδT cells as involved in the pathogenesis of the chronic inflammatory process. γδT cells secrete a variety of cytokines, including tumor necrosis factor α TNFα) (8). TNFα has been shown to be the major upregulator of interleukin (IL)-1 production

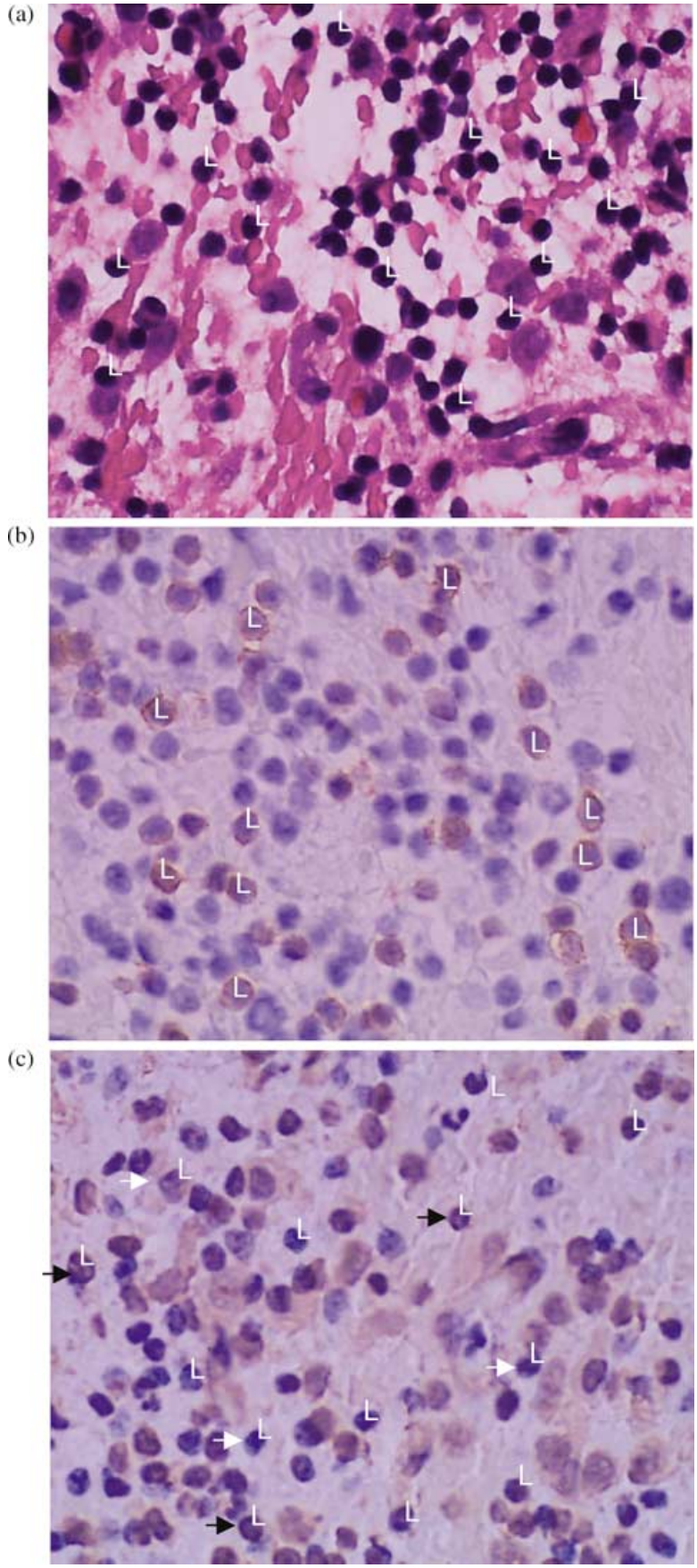

Fig. 3. Periapical lesions contain $CD3^+$ αβT cell receptor negative lymphocytes. Representative section of a periapical lesion. (a) Hematoxylin and eosin staining demonstrating the dense lymphocytic infiltrate (40× magnification). (b) Anti-CD3 staining identifying T lymphocytes (60× magnification). (c) Anti-αβTCR staining identifying αβT cells (60× magnification). Stained cells are dark brown and indicated by black arrows. The white arrows indicate cells that are $CD3^+$ and do not stain with the anti-αβTCR antibody. For all sections, lymphocytes are labeled with an ''L''. There are high backgrounds because the anti-αβTCR antibody is designed for flow cytometry, not paraffin sections.

(3). Periapical lesions in mice and rats contain IL-1 that has been shown to activate osteolasts, causing bone resorption (23, 24, 27) and IL-1 has been identified in periapical lesions in humans (7, 12). TNFα can also activate osteoclasts, although with a much lower affinity than IL-1 (23, 24, 27). Although we do not yet know what cytokines are secreted by γδT cells found in periapical samples, we speculate that they secrete TNFα, that can directly stimulate osteoclast formation and bone resorption and, by causing IL-1 production, further stimulate osteoclast activity.

Finally, we recognize the importance of characterizing both the clonality of γδT cells and clinical and histologic findings of periapical lesions that contain γδT cells. We anticipate that determining the relationship between clinical, histologic and immunologic findings will improve our understanding of how periapical lesions develop. These studies are currently underway. If γδT cells are involved in causing periapical lesions, therapy aimed at eliminating γδT cell activity may permit resolution of this disease without surgical intervention.

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