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ORIGINAL ARTICLE

Natural killer cells and alterations in collagen density: signs of periradicular herpesvirus infection?

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Abstract This study evaluated the presence and density of natural killer (NK) cells as well as collagen density in chronic apical periodontitis lesions and tried to find any correlations with concomitant herpesvirus infection or histopathological status of the lesion. Surgical specimens of chronic apical periodontitis lesions were surveyed for the presence and density of NK cells by immunohistochemical analysis. Collagen density in these lesions was quantified by means of histochemistry. All specimens were positive for the presence of CD57-positive cells. Topographically, CD57-positive cells were found singly or forming clusters in the granulomatous tissue, as well as subjacent and within the cystic epithelium. No significant differences in the density of CD57-positive cells were found between non-epithelialized and epithelialized lesions or between herpesvirus-positive and herpesvirus-negative lesions. Significant differences were found in volumetric density of collagen when comparing nonepithelialized and epithelialized lesions, with the latter demonstrating higher values. When no distinction of lesion type was made, there was no significant difference in collagen density between herpesvirus-positive and herpesvirus-negative lesions. When comparing the collagen density in herpesvirus-positive and herpesvirus-negative specimens from the same lesion type, a significant difference was found in nonepithelialized lesions, with herpesvirus-positive lesions showing lower values. The presence of CD57-positive cells in all chronic apical periodontitis specimens may indicate that activated NK cells play a role in the pathogenesis of this disease, possibly by participating in innate immunity events involved in the control of virus infection. Collagen density may vary in function of the type of lesion and presence of herpesvirus infection.

Keywords Apical periodontitis · Endodontic infection · Immunohistochemical analysis · Histochemical analysis · CD57

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Introduction

In response to microbial aggression egressing from the root canal, the periradicular tissues mount inflammatory and immunological reactions that represent an attempt to

confine pathogens to the root canal and impede spread of the infection to the bone. Chronic apical periodontitis lesions, such as granulomas and cysts, are proliferative inflammatory processes characterized by a dense accumulation of defense cells, including polymorphonuclear neutrophils, T and B lymphocytes, plasma cells, mast cells, and monocytes–macrophages, as well as fibroblasts and epithelial cells [1, 10–15, 17, 26, 32].

Although several studies have extensively explored the participation of diverse immune cells in apical periodontitis lesions [1, 10–15, 17, 26, 32], there are only a few reports on the occurrence of natural killer (NK) cells in these lesions [10–12, 16, 25]. The apparent lack of interest in endodontic research involving NK cells may be related to the fact that the main function of these cells is to combat and destroy neoplastic and virus-infected cells, which are largely uncommon in apical periodontitis lesions. Therefore, the presence of NK cells in apical periodontitis has never been well explained. However, recent reports on herpesvirus involvement in apical periodontitis have emerged in the literature, suggesting that human cytomegalovirus (HCMV) and Epstein–Barr virus (EBV) can participate in the pathogenesis of these diseases [18–22]. NK cells serve a critical function in host defense against viral infections, especially those caused by members of the herpesvirus family [3, 24]. Low NK cell cytotoxic activity is linked with increased human sensitivity to severe disseminating herpesvirus infections, including those caused by EBV and HCMV [2]. Reports of the occurrence of herpesviruses in apical periodontitis lesions raise the question as to whether NK cells may be involved in the innate immune defense against these viruses in the periradicular tissues.

NK cells are a group of large lymphocytes originally characterized as cells lacking receptors, such as T-cell receptors or surface immunoglobulins, for specific antigens. NK cells are found in the peripheral circulation, where they comprise up to 15% of the recirculating lymphocytes, as well as in the spleen and bone marrow [3]. Like many other leukocytes, they can be recruited to sites of inflammation by chemokines and other chemoattractants. NK cells are able to recognize and destroy virus-infected cells. They kill infected cells through the release of perforin and granzymes from granular storage compartments and through binding of the death receptors Fas and TRAIL-R on target cells through their respective NK cell ligands [24, 27]. Perforin and one or more of granzymes associate with the cell membranes of target cells. One or more of the granzymes appears to activate intracellular pathways leading to target apoptosis through pathways that involve mitochondria, caspases, or both [27]. Separately, binding of the death receptors also activates caspases, causing target cell apoptosis [24]. Additionally, FcγRIII (CD16) can contribute to NK cell cytotoxic activity through mechanisms that include antibody-dependent cell cytotoxicity. NK cells can also release cytokines with antiviral functions, including gamma interferon (IFN-γ) and tumor necrosis factor (TNF) [2].

NK cells can accumulate and interact with other cells in inflamed tissues. They have been found in samples from chronic marginal periodontitis in intimate contact with fibroblasts that exhibited morphologic changes consistent with cellular damage and degeneration [4]. The damage to fibroblasts and impairment of their activities can interfere not only with tissue repair but also with host tissue potential to contain the spread of the infection. Fibroblasts produce many matrix macromolecules to form a fibrous capsule that separates the inflammatory lesion from the bone. Fibroblasts can be infected by herpesviruses [23] and this may exert deleterious effects on tissue reparative and defensive responses, including formation of a defective collagen capsule.

This study aimed to evaluate the presence and density of NK cells in chronic apical periodontitis lesions using an immunohistochemical approach. Also, the density of collagen in these lesions was quantified by means of histochemistry. Findings were examined for any correlations between the density of NK cells or collagen, herpesvirus infection, and histopathological lesion type.

Material and methods

Collection of periradicular specimens

Paraffin-embedded sections of surgical specimens collected for a previous experiment [22] were available for reanalysis in this study. Procedures for specimen collection were as outlined earlier [22]. Briefly, biopsy samples of asymptomatic apical periodontitis lesions were obtained from 35 patients after tooth extraction. All the selected teeth had extensive carious lesions, attached apical periodontitis lesions after extraction, and absence of periodontal pockets deeper than 3 mm. The size of the apical periodontitis lesions ranged from 3×3 to 12×13 mm. Teeth were extracted for prosthetic reasons. Before extraction, patients rinsed with 0.12% chlorhexidine mouthwash for 30 s. Afterwards, the tooth, gingiva, and mucosa of the sampled area were scrubbed with 0.12% chlorhexidine. Immediately after extraction, the teeth were washed with sterile saline solution and the lesions detached using a sterile #15 scalpel blade. Lesions were fixed by immersion in 10% formalin solution and then paraffin embedded. Approval for the study protocol was obtained from the Ethics Committee of the Estácio de Sá University.

Histopathological classification

Serial histological sections of 6 μm from the lesions were obtained. Some sections from each lesion were stained with

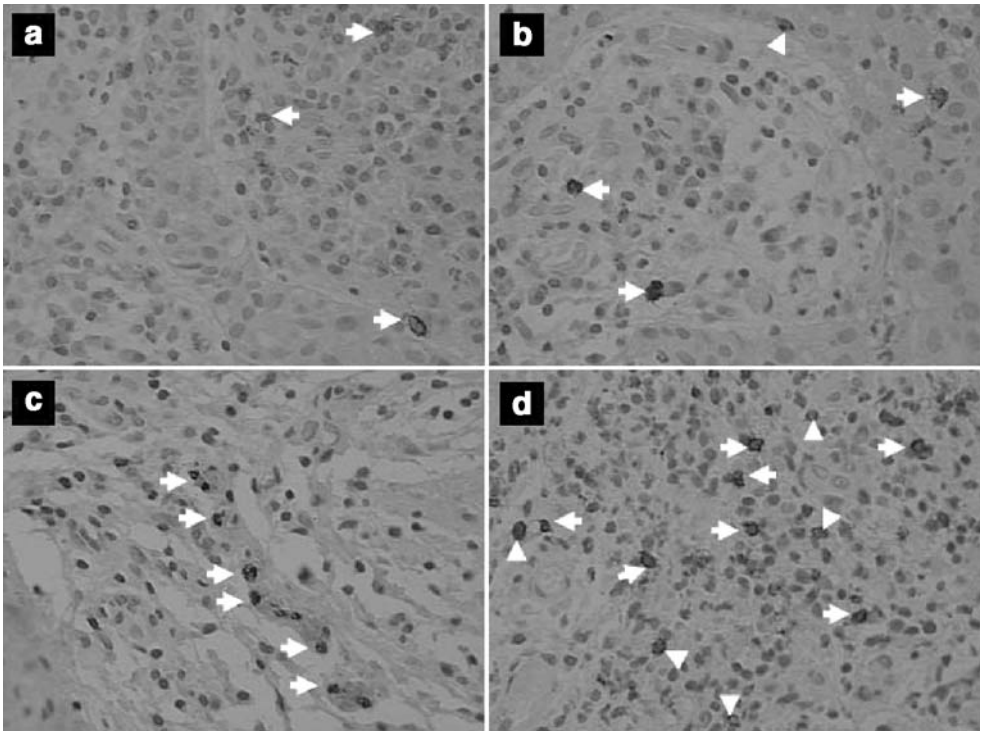

Fig. 1 Representative specimens showing CD57-positive cells (NK cells) in chronic apical periodontitis lesions. **a** and **b** Epithelialized lesion (cyst) with CD57-positive cells subjacent and within the cystic epithelium; **c** and **d** numerous CD57-positive cells distributed through the connective (**c**) and granulomatous tissue (**d**) in a nonepithelialized lesion (granuloma) (anti-CD57 staining, counterstained with Harris' hematoxylin; original magnification ×400)

hematoxylin–eosin and Gomori's trichrome. Sections were analyzed by means of a light microscope (Olympus-BH2-RFCA, Tokyo, Japan) equipped with a digital camera (Sony-CCDDXC151-A, Tokyo, Japan) coupled to a computerized analysis system (Image-Pro Plus, version 1.2 from Media Cybernetics, Silver Springs, MD, USA). Based on histopathological analysis, lesions (n=35) were classified as granuloma (n=22), epithelialized granuloma (n=7), and cyst (n=6).

Immunohistochemical methods

Immunohistochemical procedures for detection of CD57-positive cells were performed in 18 of the 35 specimens using a standardized protocol based on the streptavidin–biotin complex technique [28]. Previous analysis revealed that some of these chronic apical periodontitis specimens showed cells infected by EBV (five cases) and HCMV (seven cases) [22]. Of these virus-infected lesions, coinfection with EBV–HCMV was found in four specimens.

After dewaxing and rehydration, sections were immersed in 0.01 mM citrate buffer (pH=6.0) for 40 min at 95°C for antigen retrieval. Sections were incubated for 20 min in 3% H_2O_2 in methanol at room temperature to block endogenous peroxidase activity, and then washed in distilled water and phosphate buffered solution (PBS, pH=7.6). Afterwards, the sections were incubated in normal goat serum for 10 min at room temperature and then incubated overnight in a humid chamber with mouse monoclonal antibody (primary antibodies) anti-CD57 (Ab-1, clone NK1, Lab Vision, NeoMarkers, CA, USA, 1:100 final dilution). Subsequently, sections were incubated with a biotinylated secondary antibody for 15 min followed by incubation with streptavidin–biotin–peroxidase complex also for 15 min (LSAB kit, DakoCytomation, Glostrup, Denmark). Peroxidase reaction was carried out using a substrate–chromogen system solution containing diaminobenzidine and hydrogen peroxide (DakoCytomation Liquid DAB + Substrate Chromogen System, Dako-K3468, DakoCytomation). Finally, sections were counterstained with Harris' hematoxylin and mounted with synthetic resin. All washing steps were done in PBS (pH=7.6) at room temperature. Positive controls consisted of histological sections of human lymph nodes, according to the manufacturer's recommendation.

All immunostaining procedures were done in triplicate and reaction intensity was determined by considering the

Table 1 CD57-positive cells in nonepithelialized (granulomas) and epithelialized lesions (epithelialized granulomas and cysts)

Lesion	Mean ± SE	P (Mann–Whitney)
Nonepithelialized	0.164±0.0409	0.2815
Epithelialized	0.237±0.0732	

Table 2 CD57-positive cells in herpesvirus-positive and herpesvirus-negative lesions

Lesion	Mean ± SE	P (Mann–Whitney)
Noninfected	0.162±0.0492	0.5457
Infected	0.216±0.0547	

two most similar results. Positive immunostaining was considered when cytoplasmic reaction was present.

Histochemical methods

Histochemical procedures were performed in all 35 specimens. Previous immunohistochemical analysis for the presence of two herpesviruses in these 35 specimens revealed EBV-infected cells in 11 cases and HCMV in eight cases [22]. EBV-HCMV coinfection occurred in five of these cases [22]. After dewaxing and rehydration, sections were stained with picro-sirius red (PSR) for collagen quantification [8, 9]. Collagen quantification was randomly determined in specimens from each type of lesion and analyzed in sections stained with PSR through a light microscope (Olympus-BH2-RFCA, Olympus, Tokyo, Japan) equipped with a polarization filter and a Sony-digital camera (Sony-CCDDXC151-A, Sony, Tokyo, Japan).

Immunohistochemical and histochemical analysis

Images randomly obtained with a 40× objective lens from each specimen were analyzed using Image Pro® Plus computer assisted image analysis system (Media Cybernetics, Silver Springs, MD, USA). Results were expressed in terms of the relative area occupied by the structure of interest, i.e., CD57-positive cells in the immunohistochemical assay and collagen in PSR stained sections.

Statistical analysis

For all analyses, specimens were classified as either epithelialized lesions (including epithelialized granulomas and cysts) or nonepithelialized lesions (granulomas), as well as herpesvirus-positive and herpesvirus-negative lesions. Statistical analysis was carried out by the independent Mann–Whitney test using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA, USA) to compare the density of either CD57-positive cells or collagen in nonepithelialized and epithelialized lesions as well as in herpesvirus-positive and herpesvirus-negative lesions. The significance level for all comparisons was set at $p<0.05$.

Results

Immunohistochemical analysis

All of the 18 specimens analyzed demonstrated the presence of CD57-positive cells with a characteristic cytoplasmic staining. Topographically, these cells were found frequently inside granulomatous and connective tissues, as well as subjacent or within the epithelium, showing a sparse distribution through the lesions (Fig. 1). No significant differences in the density of CD57-positive cells were found between nonepithelialized

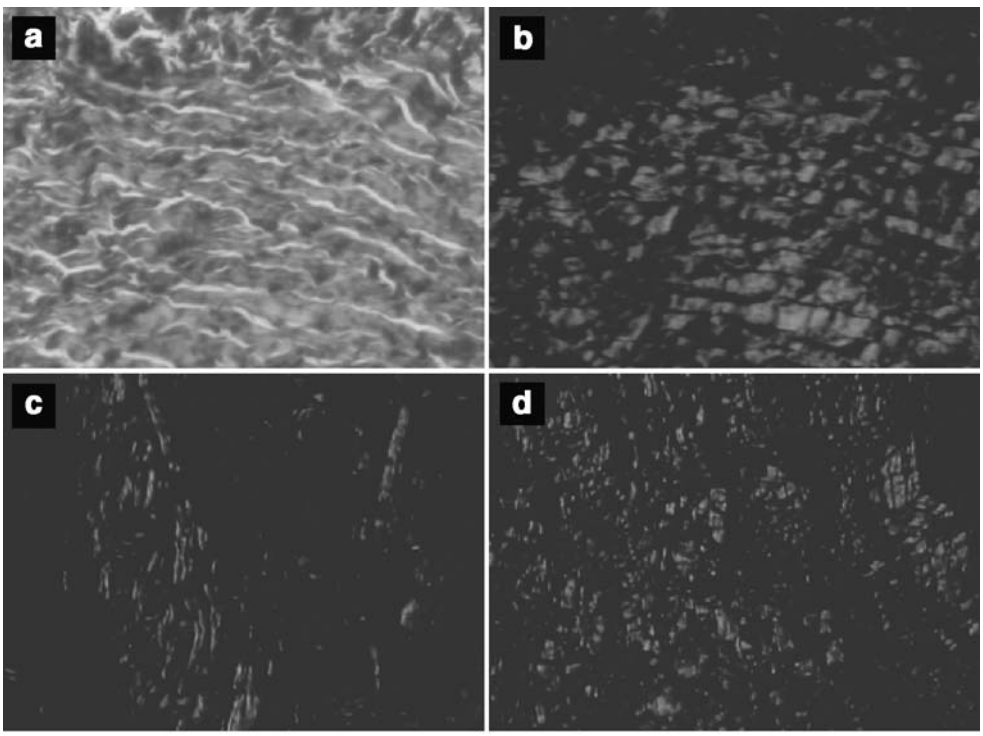

Fig. 2 Histochemical staining with picro-sirius red. **a** Collagen capsule of an apical periodontitis lesion (cyst) viewed with no polarization. Note the sinuous collagen fibers with intermingled fibroblasts; **b** the same field under polarization, evidencing most of the collagen fiber-bundles arranged in a dense array. **c** Representative section of a herpesvirus-positive nonepithelialized lesion. Note a low density of capsular collagen, with fibers in a loose array. **d** Representative section of a herpesvirus-negative nonepithelialized lesion. Note the higher density of capsular collagen, with fibers in a dense array (picro-sirius red; original magnification ×400)

Table 3 Collagen density in nonepithelialized (granulomas) and epithelialized lesions (epithelialized granulomas and cysts)

Lesion	Mean ± SE	*P* (Mann–Whitney)
Epithelialized	2.750±0.9678	0.0233
Nonepithelialized	1.009±0.1988	

Table 5 Collagen density in herpesvirus-positive and herpesvirus-negative nonepithelialized lesions

Lesion	Mean ± SE	*P* (Mann–Whitney)
Noninfected	2.305±0.5265	0.0031
Infected	0.487±0.2074	

(granulomas) and epithelialized lesions (Table 1) or between herpesvirus-positive and herpesvirus-negative lesions (Table 2).

Histochemical analysis

Capsular collagen distribution and arrangement were different between nonepithelialized and epithelialized lesions as well as between herpesvirus-positive and herpesvirus-negative lesions. In epithelialized lesions, collagen fiber bundles showed a dense array and a few fibroblasts and inflammatory cells were encountered interspersed with the fibers (Fig. 2). In nonepithelialized lesions, collagen fiber bundles presented a sparse arrangement and a large number of cells, mostly inflammatory cells, which were distributed among the collagen fibers. A looser pattern of collagen organization could be seen in the collagen capsules of herpesvirus-positive nonepithelialized lesions (Fig. 2).

Significant differences were found in volumetric density (Dv) of collagen when comparing nonepithelialized and epithelialized lesions (Table 3), with the latter demonstrating higher values of collagen Dv. When no distinction of lesion type was made, there was no significant difference in collagen Dv when herpesvirus-positive and herpesvirus-negative lesions were compared (Table 4). When comparing herpesvirus-positive and herpesvirus-negative specimens from the same lesion type, a significant difference was found in nonepithelialized lesions (Table 5), with herpesvirus-positive lesions showing lower values for collagen Dv. No significant differences were found for collagen Dv in herpesvirus-positive and herpesvirus-negative epithelialized lesions (Table 6).

Discussion

T and B lymphocytes and macrophages have been found to make up the majority of the inflammatory infiltrate in apical periodontitis lesions [13, 14, 26]. A few reports have evidenced NK cells in these lesions [10–12, 16, 25]. Presence of NK cells may be a response to virus infection in apical periodontitis lesions, but it had not yet been investigated. In this study, all of the specimens examined demonstrated CD57-positive cells distributed through epithelialized and nonepithelialized lesions. This finding is in accordance with the study by Kettering and Torabinejad [10], which reported the presence of NK cells in all samples of chronic apical periodontitis, but are in disagreement with a recent study [12], which encountered NK cells in a few chronic apical periodontitis lesions. The reasons for such differences are not apparent, but may be related to methodological issues. It is salient to point out that, in the present study, CD57-positive cells were usually found as a few scattered cells in the lesions, which is congruent with NK cell distribution reported by Liapatas et al. [12] in their few positive specimens.

CD57 was chosen in the present study, as it is a marker of the presence of NK cells. Several immunohistochemical studies have used the monoclonal antibody anti-CD57 to consistently detect NK cells in clinical specimens, including paraffin embedded tissues [5, 7, 12, 29, 30]. The use of the anti-CD57 antibody permits not only the detection of classical NK cells but also of some T cells which are normally expanded in the adult population. Although the exact role of these T cells expressing the CD57 NK marker is not yet well established, it appears that they play an important role in host resistance against viruses [6].

Although NK cells may participate in the host defense against many viruses, it seems that they are of particular relevance in protection against herpesviruses [3]. Primary infection with HCMV is persistent and widespread, but the symptoms are usually subclinical. Immunity to HCMV is mediated in the early stages of infection by NK cells. HCMV downregulates host MHC class I and this effect can impair CD8+ T-cell effector functions against HCMV-infected target cells. However, virus-infected cells that fail

Table 4 Collagen density in herpesvirus-positive and herpesvirus-negative lesions

Lesion	Mean ± SE	*P* (Mann–Whitney)
Noninfected	1.457±0.3658	0.8698
Infected	1.353±0.3710	

Table 6 Collagen density in herpesvirus-positive and herpesvirus-negative epithelialized lesions

Lesion	Mean ± SE	*P* (Mann–Whitney)
Non-Infected	1.293±0.6282	0.5273
Infected	1.319±0.3274	

to express MHC class I can be rendered susceptible to attack by NK cells because the inhibitory NK receptors for MHC class I molecules would no longer be engaged [3, 31]. The recent reports of the presence of HCMV and EBV in apical periodontitis lesions suggest that NK cells found in these lesions are there to combat these viruses.

Because our previous analysis showed the presence of EBV- and HCMV-infected cells in some of the specimens examined herein [22], a larger number of NK cells was expected in these specimens, when compared with noninfected patients. Although apparently in elevated numbers in virus-associated lesion, statistical analyses indicated no significant differences between groups. The lack of difference in the number of CD57-positive cells between lesions from herpesvirus-infected and noninfected groups may reflect the asymptomatic state of the lesions and presence of latent viral infection instead of active viral infection. Also, results were available only for EBV- and HCMV-infected cells in these lesions and the possibility exists that infection by other virus could have been present. This might explain the presence of NK cells in all specimens investigated.

Topographically, CD57-positive cells were found singly or forming clusters in the granulomatous tissue, as well as subjacent and within the cystic epithelium. The CD57-positive cells distribution in granulomatous and connective tissue resembled that showed by herpesvirus-infected cells in the same specimens [22]. The tendency of NK cells to occur singly or in small clusters (3–12 cells) has already been reported in samples from chronic adult marginal periodontitis [4]. In these lesions, 3–7% of the total monocytic infiltrated cells were NK cells showing intimate contact with fibroblasts that exhibited morphologic changes consistent with cellular damage or degeneration [4].

Fibroblasts are the most important component of connective tissue and are involved in synthesis and secretion of the major macromolecules presents in the extracellular matrix. These cells exert a pivotal role in turn over and remodeling of the extracellular matrix. In chronic apical periodontitis lesions, fibroblast infection by herpesviruses might theoretically lead to defective collagen capsules, which allegedly form a fibrous barrier to help contain the spread of the disease process.

We investigated the volumetric density of collagen in the capsule of chronic apical periodontitis lesions and expected that viral-infected lesions would demonstrate low values for collagen Dv, assuming that these findings could indicate an interference by NK cells, virus or both on fibroblast physiology. In fact, the capsular collagen distribution and arrangement were different between nonepithelialized and epithelialized lesions, the latter showing Dv of collagen significantly higher than the former. Whereas in epithelialized lesions the collagen fiber bundles showed a dense array and a few fibroblasts and inflammatory cells were encountered interspersed with the fibers, in nonepithelialized lesions, the collagen fiber bundles presented a sparse arrangement and a higher number of cells (mostly inflammatory cells) distributed among the collagen fibers. A looser pattern of collagen organization could be seen in the capsules of herpesvirus-infected granulomas and the Dv of collagen was significantly lower in these lesions than in noninfected granulomas. Some of the inflammatory cells encountered in the capsule were NK cells. Thus, alterations in collagen density in apical periodontitis lesions may be a result of direct action of virus on fibroblasts (HCMV infection), direct cytolytic action exerted by NK cells, or an indirect action caused by many viral influences on host cells involved in tissue defense and repair, especially with regard to cytokines release and balance.

In conclusion, the presence of CD57-positive cells in all chronic apical periodontitis specimens may indicate that activate NK cells play a role in the pathogenesis of this disease, possibly by participating in innate immunity events involved in the control of virus infection. Collagen density may vary in function of the type of lesion and presence of virus infection, and in some cases it may be a result of the interaction of NK cells, herpesvirus infection, and fibroblasts.

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