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# Drug-induced gingival hyperplasia – experimental model

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**Abstract**

Several causes of gingival hyperplasia are known, the most widely accepted being the drug-induced gingival augmentation, a side effect associated mainly with three classes of drugs: anticonvulsants (Phenytoin), immunosuppressants (Cyclosporine A), and various calcium channel blockers (Nifedipine, Verapamil, Diltiazem). We studied the effect of Cyclosporine A (CsA) and Nifedipine on gingival fibroblasts extracted from the rat gum. Gingival fibroblasts were isolated from 6-week-old male rats weighing 150–170 g, from gingival explants, and grown in a specific culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with antibiotic and 10% fetal bovine serum (FBS). The medium was also supplemented with CsA (1 µg/mL) and Nifedipine (3 mM). We also used a control group that received no treatment. The cells were photographed at 7, 14 and 30 days, with a Nikon Eclipse TE300 phase contrast microscope. For cell viability evidence, we used immunofluorescence (flow cytometry) with a FACS (fluorescence-activated cell sorting) Calibur device and its software. We noticed that the proliferation of these cells increased with the period of drug administration, and the subsequent morphological changes that occurred were related to the presence of drug accumulations in the cell cytoplasm. Modern molecular techniques are needed to shed some light upon the pathogenesis of drug induced gingival overgrowth and, thereby, provide novel information for preventative and effective future therapeutic strategies.

***Keywords***: gingival hyperplasia, Cyclosporine A, Nifedipine, gingival fibroblasts.

## Introduction

Gingival hyperplasia occurs mainly as consequence of the treatment with certain antiepileptic, immunosuppressant or antihypertensive drugs. The excess gingival tissue affects oral health as well as esthetics. Oral hygiene is compromised by gingival overgrowth, which can have a negative impact on the systemic health of the patients.

Cyclosporine A (CsA) is an immunosuppressant widely used to prevent transplant rejection, but also for the treatment of certain autoimmune diseases such as bullous pemphigoid, psoriasis and rheumatoid arthritis [1]. CsA induced gingival hyperplasia was first described in 1983 by Rateitschak-Plüss *et al.* [2] and is estimated to occur in about 30% of the patients that receive this medication [3].

In medical practice, calcium ($Ca^{2+}$) channel blockers are frequently used in cardiovascular disease management. Most classes of $Ca^{2+}$ channel blockers were proved to be involved in gum overgrowth [4], but Nifedipine is the most common agent causing $Ca^{2+}$ channel blocker induced gingival hyperplasia.

Clinical observations of the pathogenesis of CsA and Nifedipine-induced gingival hyperplasia indicate a possible role of periodontal bacteria in the onset of this condition. Most studies were performed *in vivo* and the results are not conclusive. It is not clear whether the accumulation of dental plaque is a result of gingival alterations induced by these drugs or is an essential factor for the onset of the condition [5, 6]. This difficulty in determining the exact role of gingival inflammation is probably a result of the *in vivo* complexity. Moreover, a short-term treatment with Azithromycin, a macrolide antibiotic that acts against both Gram-positive and Gram-negative bacteria, was shown to improve the gingival status of patients with CsA-induced gingival hyperplasia [7].

Seymour & Smith [8] showed that a proper oral hygiene significantly reduces the severity of this hyperplasia, but does not prevent it. Animal studies proved that the presence of dental plaque contributes to the development of gingival hyperplasia and that local administration of Chlorhexidine reduces the development of the plaque [9]. McGaw *et al.* [10] suggested that the dental plaque acts as a local reservoir of CsA and Nifedipine, which results in a constant exposure to these compounds of the juxtaposed gingival epithelium.

Thus, an *in vitro* assessment of the role of the pre-existent periodontal condition in the onset of the pathogenesis of the CsA- and Nifedipine-induced gingival hyperplasia is required. Furthermore, a study investigating whether exposure to a combination of CsA and bacteria or Nifedipine and bacteria can alter the cellular response is necessary.

The interactions between CsA, Nifedipine and gingival fibroblasts may have an essential contribution to gingival hyperplasia. Variable direct behavioral responses of fibroblasts in the presence of CsA and Nifedipine include a modified metabolic activity [11].

## Materials and Methods

The main objectives of the research were to obtain gingival fibroblast from rats and to study the effects of CsA and Nifedipine on these cultivated cells. To achieve these goals, we performed a series of experiments meant to determine the effects of CsA and Nifedipine on the morphology and viability of normal gingival fibroblasts from rats.

### Normal and CsA-/Nifedipine-treated fibroblast cultures achievement

Gingival fibroblasts were obtained from the gingivae of untreated male rats by using the explant technique. Gingival samples were minced, washed six times with phosphate-buffered saline (PBS) and then placed into Petri dishes containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 584 mg/L L-Glutamine, 4500 mg/L Glucose, 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin antibiotic mix, and 1% Amphotericin B (Figure 1).

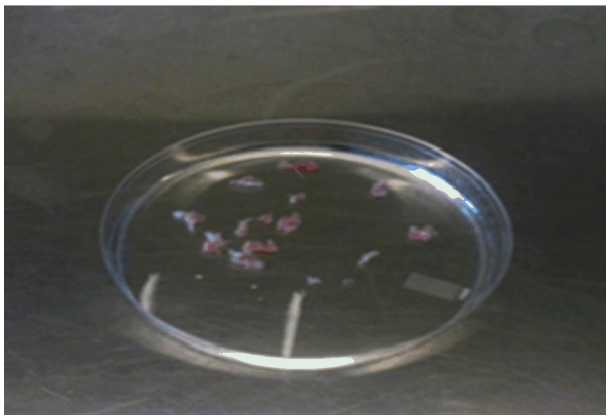

**Figure 1 –** ***Minced rat gingival samples.***

The samples were incubated for 2–7 days at 37°C and 5% $CO_2$. The gingiva was subsequently removed and the fibroblasts that were attached to the walls were left to further multiply in fresh medium at 37°C and 5% $CO_2$ for another 48–72 hours. After two more passages, the cells were divided into three groups and placed in the same culture medium mentioned above.

The study was conducted in accordance with the rules and principles of the Ethics Committee of the "Grigore T. Popa" University of Medicine and Pharmacy, Iași, Romania, complied with all the rules of international forums governing scientific research for animals' tissue sampling.

### Assessment of CsA and Nifedipine effects

The first group was assigned to be the control group and was, therefore, left untreated. The second group was treated with 1 μg/mL (1 μM) CsA, while the third group was treated with 5 μg/mL (3 mM) Nifedipine. All three groups were then incubated at 37°C and 5% $CO_2$, until they became fully confluent. During the incubation, the cells were photographed at 7, 14 and 30 days, with a phase contrast Nikon Eclipse TE300 microscope and its software. The photographs were taken at 40×, 100× and 200× magnification.

The effect of the drugs on gingival fibroblast viability was assessed by immunofluorescence (flow cytometry) with a FACS (fluorescence-activated cell sorting) Calibur device and its software. The used acquisition settings were FL1 623 V, FL2 505 V, 10 000 events and 488 nm laser. Data analysis was performed by using FlowJo® 7.6.1 software.

The preparation of drug-treated cells for flow cytometry involved trypsinization with Trypsin ethylenediaminetetraacetic acid (EDTA), washing by repeated suspension in PBS and centrifugation at 300×g for 5 minutes, and, finally, resuspension in 1 mL of culture medium. The cells were then counted (approximately $10^6$/mL) and equally divided into several test tubes. One test tube was assigned to be the control while in the others 5 μL/mL (2 μM) of Calcein and 5 μL/mL (80 mM) of $CoCl_2$ were added. The test tubes were then incubated at 37°C and 5% $CO_2$ for 20 minutes.

## Results

### Morphological modifications in CsA- and Nifedipine-treated gingival fibroblasts

Fibroblasts are a type of cell that produces an extracellular matrix (ECM) and synthesizes collagen, and they have an important role in the healing process. From a morphological point of view, fibroblasts have a heterogeneous appearance, which varies depending on their location and activity.

By continuously secreting ECM precursors, the fibroblasts have as their main function the maintenance of the structural integrity of connective tissue. These cells secrete precursors of all ECM components, *i.e.*, the fundamental substance and a variety of fibers.

Fibroblasts are the main active cells of the connective tissue. Their function is to secrete tropocollagen (the precursor of collagen) and ground substance constituents, and to preserve these extracellular tissue components. Fibroblasts appear to play an important role in wound healing. Following tissue injury, fibroblasts migrate to the site of damage, where they deposit new collagen and facilitate the healing process.

During our experiments, we did not notice any major morphological modifications of gingival fibroblasts following their isolation from rat gingiva. The CsA and Nifedipine treatments, however, did induce morphological alterations of gingival fibroblasts. We observed that cell proliferation increased with drug exposure time. The morphological changes included altered shapes and the presence of drug accumulations in the cytoplasm (Figures 2–4).

Hematoxylin and Eosin staining (Figure 4) showed the loss of the star shape in fibroblasts treated with CsA and Nifedipine for more than 14 days.

### Fibroblast viability

The effects of CsA and Nifedipine on the viability of gingival fibroblasts were assessed by using Calcein AM (ester of Calcein with acetoxymethyl), a non-fluorescent molecule that passively diffuses through the membrane of living cells and accumulates in cytosol compartments including the mitochondria (Figure 5). Owing to this property, Calcein AM is a very useful tool for the study of cell membrane integrity, while its very low toxicity makes it the fluorophore of choice for long-term experiments.

Inside the cells, the acetoxymethyl esters are hydrolyzed by the endogenous esterases. This generates negatively polarized Calcein, which is intensely fluorescent (green emission) and is retained in the cytoplasmic compartments of living cells. To reduce the intensity of the cytoplasmic fluorescence, the cells were incubated with $CoCl_2$. This, however, does not affect mitochondrial fluorescence.

The analysis of fluorescence histograms showed a decreased viability of Nifedipine (1 mM)-treated cells (Figure 6). The CsA (1 μM)-treated cells, however, showed a slightly increased viability (Figure 7). This is most probably due to a mechanism of blocking the mitochondrial permeability transition pore, which is known to be involved in triggering the first step of apoptosis.

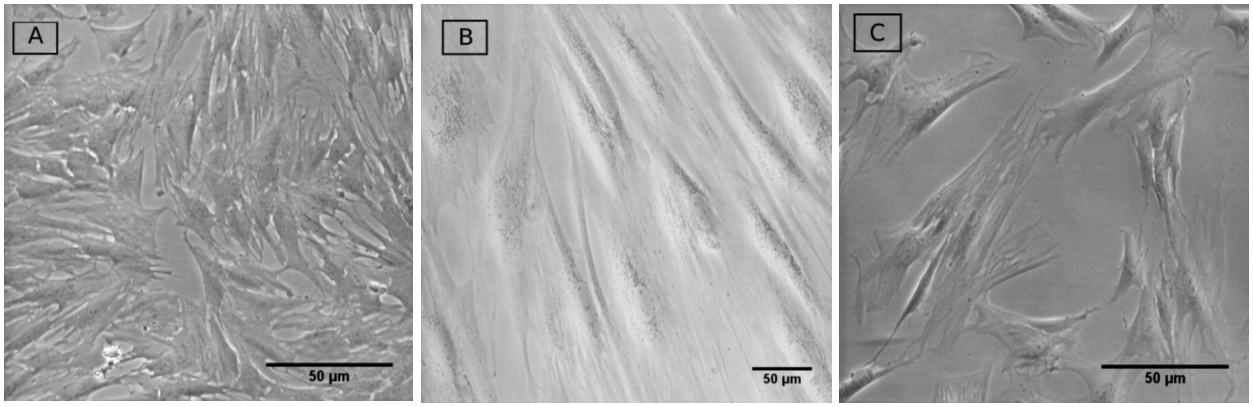


**Figure 2** – ***Gingival fibroblasts after seven days of incubation (×100; Scale bar = 50 μm): (A) Normal; (B) CsA treated (1 μM); (C) Nifedipine treated (1 mM). CsA: Cyclosporine A.***

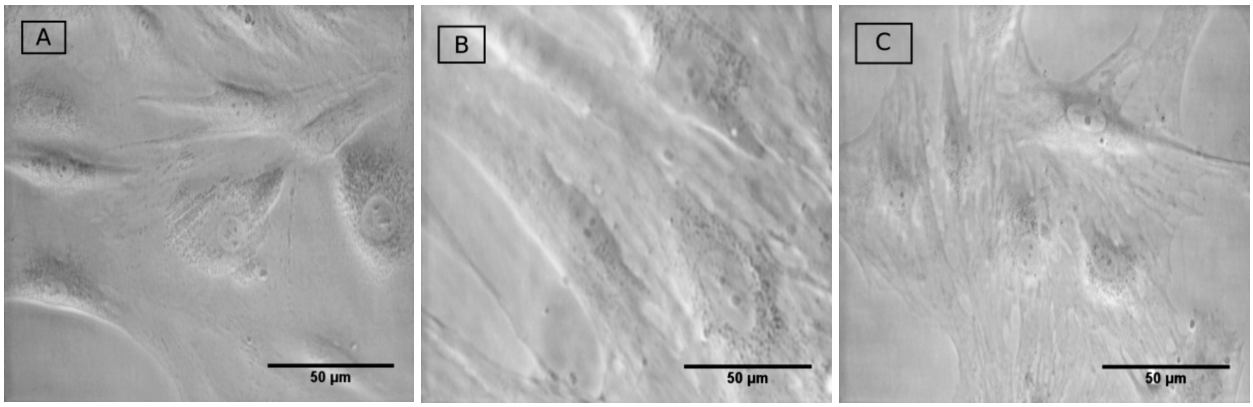


**Figure 3** – ***Gingival fibroblasts after 30 days of incubation (×100; Scale bar = 50 μm): (A) Normal; (B) CsA treated (1 μM); (C) Nifedipine treated (1 mM). CsA: Cyclosporine A.***

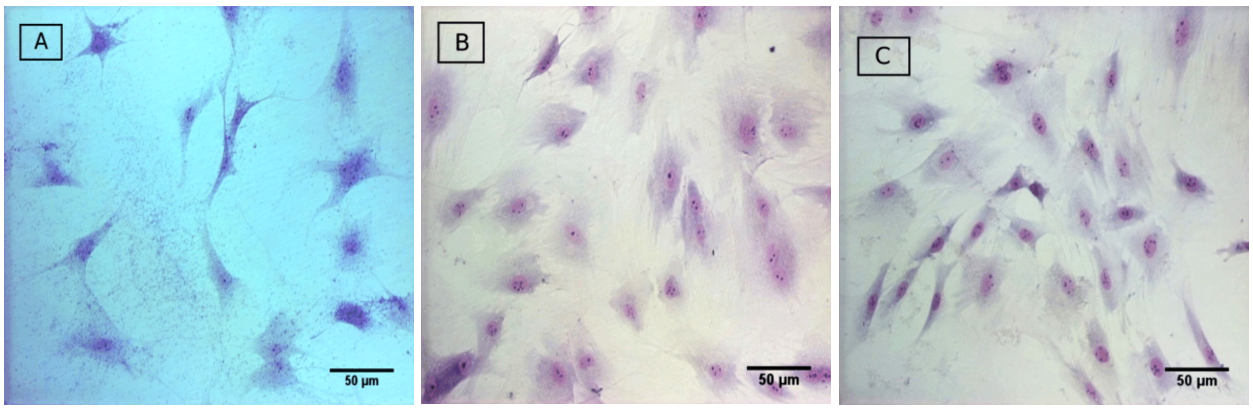


**Figure 4** – ***Hematoxylin and Eosin staining of gingival fibroblasts (×100; Scale bar = 50 μm): (A) Normal; (B) CsA treated (1 μM); (C) Nifedipine treated (1 mM). CsA: Cyclosporine A.***

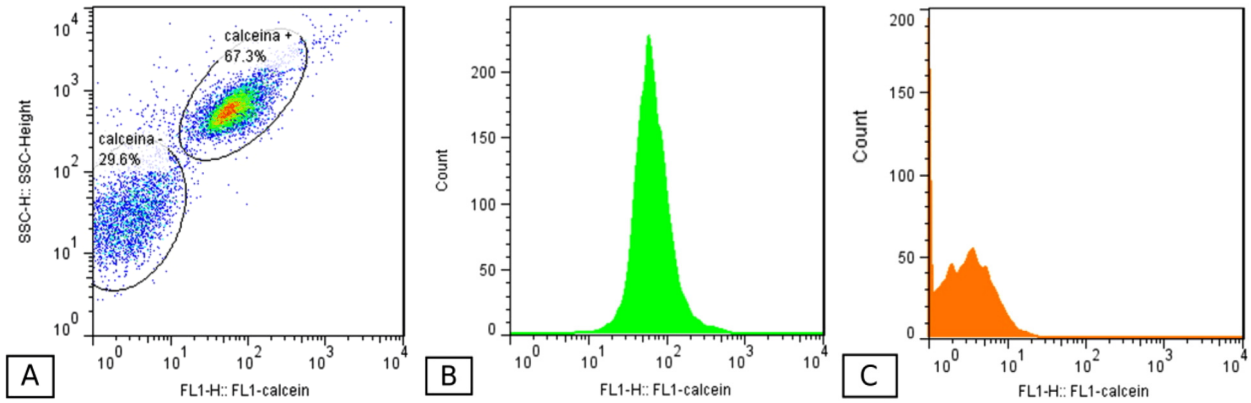


**Figure 5** – ***Representative FACS image for the Calcein loading of normal gingival fibroblasts: (A) FACS image; (B and C) Fluorescence histograms. FACS: Fluorescence-activated cell sorting.***

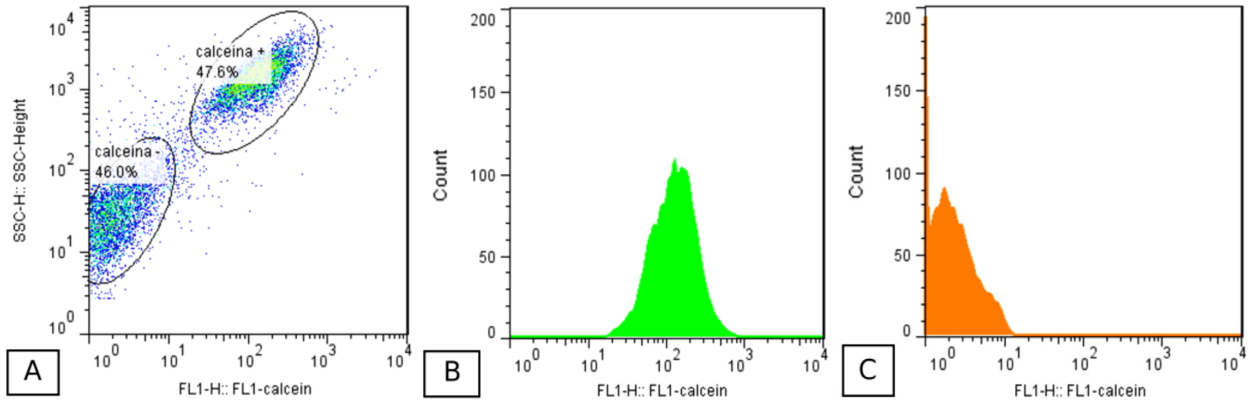


**Figure 6** – ***Representative FACS image for the Calcein loading of Nifedipine-treated gingival fibroblasts: (A) FACS image; (B and C) Fluorescence histograms. FACS: Fluorescence-activated cell sorting.***

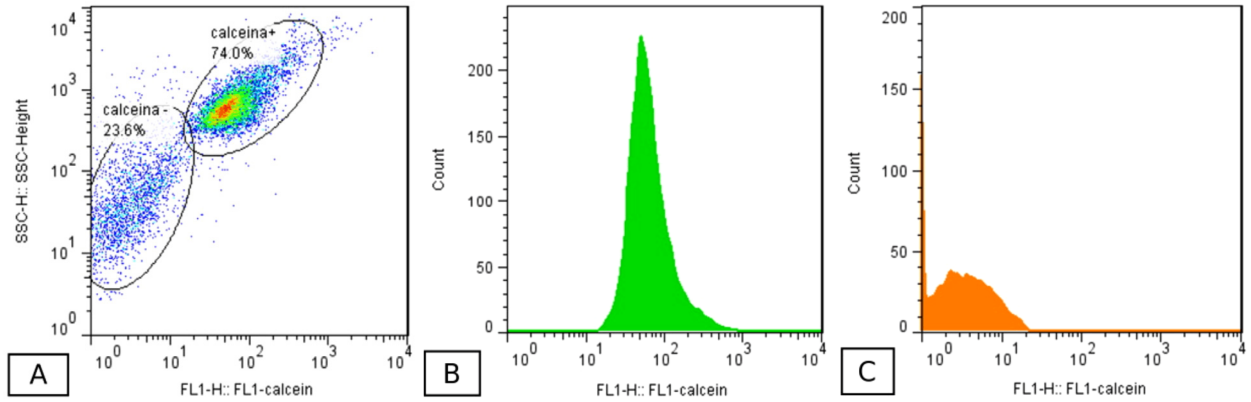


**Figure 7** – ***Representative FACS image for the Calcein loading of CsA-treated gingival fibroblasts: (A) FACS image; (B and C) Fluorescence histograms. FACS: Fluorescence-activated cell sorting; CsA: Cyclosporine A.***

## Discussion

Gingival hyperplasia has been currently associated to CsA and Nifedipine treatment. A gingival hyperplasia that is similar to the one occurring in human patients can be induced by these drugs in experimental models of rats as well [12].

Whether a relationship exists between the dose and the incidence or the severity of the induced gingival overgrowth remains controversial.

It was also proposed that susceptibility or resistance to drug-induced gingival overgrowth may be controlled by the existence in each individual of different proportions of fibroblast subsets, which exhibit different fibrogenic responses to the medication [13].

Several studies demonstrate that CsA and Nifedipine inhibit the production of ECM by the gingival fibroblasts and/or *in vitro* cell proliferation. These findings are inconsistent with the *in vivo* characteristics of drug-induced gingival overgrowth. Together, these conflicting studies seem to support the conclusion that direct regulation of ECM metabolism or of gingival fibroblast proliferation may not the primary mechanism responsible for the gingival overgrowth induced by these drugs.

Thus far, proof of gingival inflammation as an

important factor for severity of CsA- and Nifedipine-induced gingival hyperplasia is insufficient. It can be concluded that an optimal oral hygiene can minimize the severity of drug induced gingival hyperplasia, because it eliminates the inflammatory component [14].

Several factors, such as age, genetic predisposition, the presence of bacterial plaque and gingival inflammation, influence the occurrence of this condition. The response of patients to drugs, including immunosuppressive and antihypertensive medication, is variable [15]. The terms "response" and "non-response" can be found in the literature, depending on the occurrence of this adverse effect in patients. In addition, a variability of the extent and severity of the gingival alterations appears to exist within the group of patients that develop this unwanted condition. On the other hand, the awareness of this effect of the drugs on gingival tissues is minimal within the medical community [16].

In addition to being unaesthetic and uncomfortable for the affected individuals, moderate to severe forms of gingival overgrowth can damage oral hygiene and may lead to increased accumulation of microorganisms [17]. Oral infections caused by these microorganisms could potentially compromise the general health of patients [18].

Understanding the various mechanisms underlying the pathogenesis of drug-induced gingival overgrowth may help the clinician develop better prevention strategies for patients susceptible to gingival overgrowth. Hence, it would be useful to identify and explore possible risk factors relating to both prevalence and severity of drug-induced gingival overgrowth [6, 19]. An effective management of these patients, which should minimize the possibility of complications, clearly requires the active involvement of both dental and medical professionals [20]. Newer molecular approaches are needed to clearly establish the pathogenesis of drug-induced gingival overgrowth and to provide novel information for the design of future preventive and therapeutic strategies [21, 22]. Recent studies indicate salivary markers, such as interleukin-1$\beta$ (IL-1$\beta$)-type cytokines, as reliable predictors of periodontal inflammation along with drugs such as Cyclosporine A, Nifedipine and Phenytoin [23].

## Conclusions

Our research did not reveal any major impact of the explant technique on the isolated gingival fibroblasts. The investigated drug treatments, however, resulted in morphological changes and an exacerbated proliferation that enhanced with drug exposure time. As the understanding of gingival proliferation pathogenesis is, to date, incomplete, the simplest treatment of drug-induced gingival hyperplasia would be the use of an alternative medication that does not have this side effect, drugs of this type being currently under consideration.

**Conflict of interests**

The authors declare no conflict of interests.

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