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**Original Article**

# Inflammatory Mediators in the Regulation of Tooth Organ Development

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## ABSTRACT

Tooth eruption represents a dynamic, multi-step biological process driven by coordinated molecular and cellular interactions that allow the tooth to migrate through surrounding tissues. As development progresses, the stratum intermedium is replaced by the papillary layer within the enamel organ. Earlier work identified intercellular adhesion molecule-1 (ICAM-1) in this papillary region, which later gives rise to ICAM-1–positive junctional epithelium. Since ICAM-1 production can be stimulated by inflammatory cytokines such as interleukin-1 and tumor necrosis factor, both known to mediate tissue breakdown, this study explored whether inflammatory events are part of the eruption mechanism. Reverse transcription–polymerase chain reaction (RT-PCR) results showed sequential activation of hypoxia-inducible factor-1α, interleukin-1β, and chemotactic mediators like keratinocyte-derived chemokine (KC) and macrophage inflammatory protein-2 (MIP-2) during the eruption stage. Immunohistochemistry further demonstrated KC and MIP-2 presence within papillary layer cells of maturing enamel organs. Numerous macrophages and neutrophils were also observed within the connective tissue between the developing tooth and oral epithelium. These patterns indicate that inflammatory pathways contribute to the removal of tissue above erupting teeth, potentially triggered by local hypoxia resulting from reduced vascular supply. The data suggest this process is sterile and governed by innate inflammatory regulation rather than bacterial infection.

**Keywords:** HIF-1, IL-1, KC, Neutrophil, Tooth eruption

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## Introduction

For organs and tissues to mature properly, their growth must be balanced with controlled degradation of adjacent structures [1–3]. Tooth eruption exemplifies this balance, involving remodeling between the reduced enamel epithelium and oral epithelium until they fuse and break down, allowing tooth emergence.

This process depends on a sequence of molecular and cellular events guiding tooth migration. The dental follicle plays a pivotal regulatory role by releasing signaling molecules such as tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interleukin-1 (IL-1), colony-stimulating factor-1 (CSF-1), and receptor activator of nuclear factor kappa-B ligand (RANKL). These factors, produced by follicle cells, attract mononuclear precursors from bone marrow and promote their transformation into osteoclasts [4–6]. At eruption onset, these cells move into the connective tissue surrounding the tooth germ [7–10], where they resorb alveolar bone to open the eruption pathway. Most of these molecules and enzymes, such as matrix metalloproteinases (MMPs), are classically regulated through inflammatory signaling.

Matrix turnover is essential in diverse biological phenomena—from tissue regeneration to pathological invasion—and depends on locally coordinated

cytokine and chemokine activity that can occur without infection.

Noninfectious inflammation, also known as innate sterile inflammation, has been associated with disorders like rheumatoid arthritis and chronic pulmonary disease [11, 12]. Such responses can arise from internal triggers, including degradation products produced during cell death [13].

Our earlier investigations revealed that inflammatory cells influence organ formation and tissue stability [14–16]. We also observed ICAM-1 expression within the enamel organ throughout tooth morphogenesis [17, 18], which is known to be modulated by cytokine signaling [19, 20]. Collectively, these results imply that even sterile inflammatory responses may regulate tooth eruption. During eruption, vascular density in the region declines markedly [21]. Hypoxia-inducible factor-1 (HIF-1) serves as a principal oxygen sensor [22] and a core element in inflammation regulation [23, 24].

Therefore, this study aimed to investigate HIF-1 expression together with associated cytokines and chemokines, and to assess the infiltration of macrophages and neutrophils in tissues overlaying the erupting tooth.

## Materials and Methods

### *Animals*

The Animal Experimentation Committee of Showa University (Shinagawa-ku, Tokyo, Japan) reviewed and authorized all procedures described in this study. Ten BALB/c females at gestational day 16 (E16) were procured from Sankyo Laboratory Service Corporation (Tokyo, Japan). Animals were kept under standard environmental conditions in the Laboratory Animal Center of Showa University until delivery. Mandibular samples were obtained from offspring between postnatal days 7 and 21. Of these, twenty-one pups (seven per group at 7, 14, and 19 dPN) were used for morphological and histological evaluation, while another eighteen (nine each from 10 and 14 dPN) were employed for gene expression studies.

### *Tissue handling and sectioning*

Isoflurane was administered for anesthesia, followed by cervical dislocation to ensure euthanasia. For histological and immunohistochemical processing, mandibles were excised and immersed in 4% paraformaldehyde at 4 °C for six hours. Decalcification was performed using 10% ethylenediaminetetraacetic acid (EDTA) for a two-week period at 4 °C. The tissues were then frozen in optimal cutting temperature (OCT) compound (Sakura, Torrance, CA, USA). Serial sagittal sections (10 μm) aligned with the first molar axis were obtained and mounted on glass slides. Each third section was stained with hematoxylin and eosin for light microscopy to identify the appropriate region for analysis. The remaining sections were kept for subsequent immunohistochemical detection.

### *Immunohistochemical procedures*

For the identification of specific cell types and proteins, primary antibodies were applied at a 1:100 dilution. The following antibodies were used: rat anti-mouse Gr-1 (553126, BD Pharmingen, Tokyo, Japan), F4/80 (565409, BD Pharmingen), goat anti-mouse macrophage inflammatory protein-2 (MIP-2/CXCL2; AF-452-NA, R&D Systems, Minneapolis, MN, USA), goat anti-mouse keratinocyte-derived chemokine (KC/CXCL1; AF-453-NA, R&D Systems), and rat anti-mouse ICAM-1 (550287, BD Pharmingen). After washing with phosphate-buffered saline (PBS), endogenous peroxidase activity was inhibited by treating slides with 1% hydrogen peroxide for 30 minutes. Non-specific binding was reduced by incubating with either normal goat or horse serum for 30 minutes at room temperature. Sections were then exposed to the corresponding primary antibodies for 24 hours at 4 °C, followed by multiple PBS washes. The appropriate secondary antibodies—biotinylated goat anti-rat IgG (BA-9400, Vector Laboratories, Burlingame, CA, USA) or rabbit anti-goat IgG (BA-1000, Vector Laboratories)—were applied for one hour. After that, an avidin–biotin–peroxidase complex (Vectastain Elite ABC Kit, PK-6100, Vector Laboratories) was used for 30 minutes. Color reaction was achieved using a TRIS-buffered 3,3′-diaminobenzidine (DAB) substrate containing hydrogen peroxide (Reagent Set 54-10-00, KPL, Gaithersburg, MD, USA). Counterstaining was carried out using hematoxylin or methyl green. For negative controls, the primary and secondary antibodies were replaced with normal rat or goat serum.



### *Quantitative PCR analysis*

Oral mucosa samples were collected from nine animals each at postnatal days 10 and 14. Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Tokyo, Japan). Reverse transcription was performed with the Primer Script RT Reagent Kit together with gDNA Eraser (Takara Bio Inc., Shiga, Japan) to synthesize complementary DNA (cDNA). Real-time polymerase chain reaction (PCR) was executed on a LightCycler 96 system (Roche Diagnostics, Tokyo, Japan) using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific). The following TaqMan probes were utilized: IL-1β

(Mm01336189_m1), HIF-1α (Mm00468869_m1), MIP-2 (Mm00436450_ml), KC (Mm00433859_m1), and GAPDH (Mm99999915_g1). Expression of GAPDH served as the internal normalization reference. The amplification program consisted of an initial activation step at 95 °C for 20 seconds, followed by 40 cycles of denaturation at 95 °C for 3 seconds and combined annealing/extension at 60 °C for 30 seconds. The ΔΔCt method was applied to determine relative gene expression. Each time point included nine biological replicates analyzed four times per sample for technical consistency.

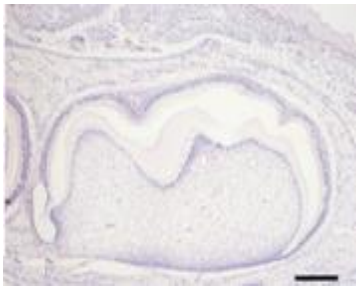
*Statistical processing*

All numerical results are shown as mean ± standard deviation (SD). Statistical differences among groups were evaluated through one-way analysis of variance (ANOVA), and Tukey's multiple comparison test was applied as a post-hoc procedure. A probability value of $p < 0.05$ was considered to denote statistical significance.

**Results and Discussion**

*ICAM-1 expression during tooth eruption*

At 7 days postnatal (dPN), cells of the inner enamel epithelium in the developing mandibular first molar had transformed into secretory-stage ameloblasts, initiating deposition of enamel matrix upon the dentin surface **(Figure 1a)**. A stratum intermedium layer had developed adjacent to the ameloblasts, and no ICAM-1 signal was observed in any enamel organ component **(Figure 1a)**. By 14 dPN, the stratum intermedium transitioned morphologically into the papillary layer, paralleling the shift of ameloblasts toward the maturation phase. Distinct ICAM-1 immunoreactivity was confined to the papillary layer cells (**(Figure 1b)**, arrowheads). At 19 dPN, as the tooth crown penetrated the oral epithelium, ICAM-1 staining intensified within the papillary layer (**(Figures 1c and 1d)**, arrowheads) and was also evident in surrounding vasculature (**(Figure 1d)**, arrows).



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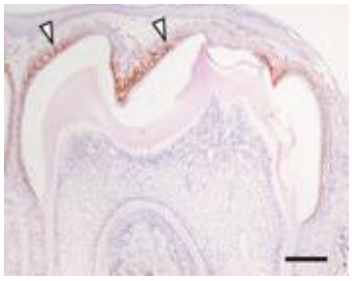

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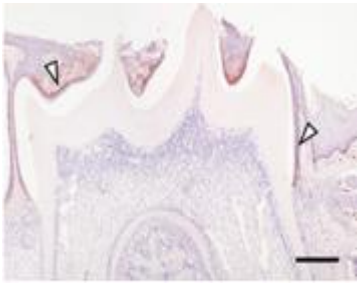

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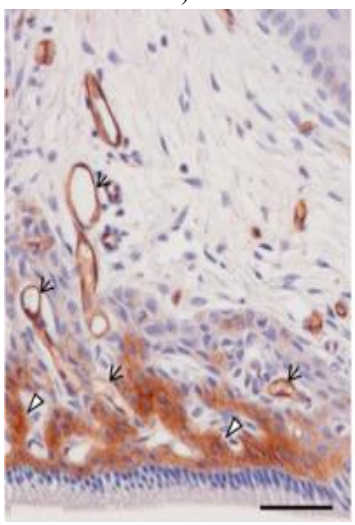


d)

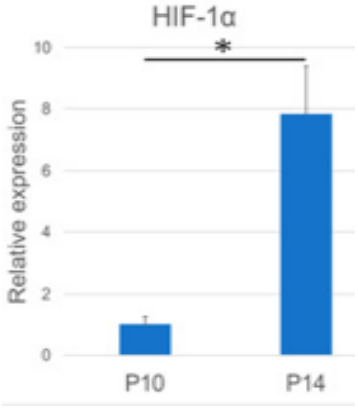
**Figure 1.** Distribution pattern of intercellular adhesion molecule-1 (ICAM-1) during molar formation at 7 dPN (a), 14 dPN (b), and 19 dPN (c,d).
(a) Absence of detectable ICAM-1 within the enamel organ.
(b) Immunopositivity in papillary layer cells (arrowheads).
(c) Enhanced labeling in the papillary layer (arrowheads).
(d) Enlarged view at 19 dPN showing ICAM-1 in blood vessels (arrows) and papillary cells (arrowheads).
Scale = 200 µm (a–c); 50 µm (d).

*Chemokine and cytokine expression during tooth eruption*

Significant tissue remodeling occurred above the erupting mandibular molar. To evaluate inflammatory signaling, quantitative RT-PCR was performed to

determine expression profiles of cytokines and chemokines at each stage.

Expression of HIF-1α, IL-1β, MIP-2, and KC was markedly upregulated at 14 dPN **(Figure 2)**. Immunostaining confirmed the localization of KC and MIP-2 in the papillary layer and in odontogenic epithelial cells located immediately above the enamel organ (**(Figures 3a and 3b)**, arrowheads and asterisks).

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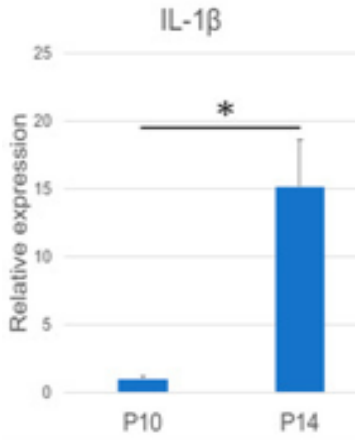


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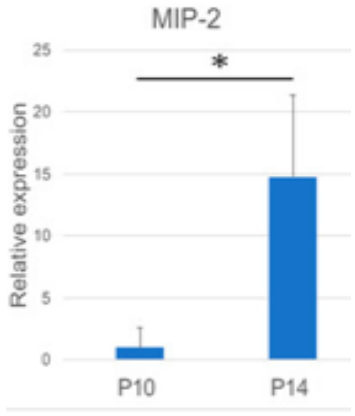


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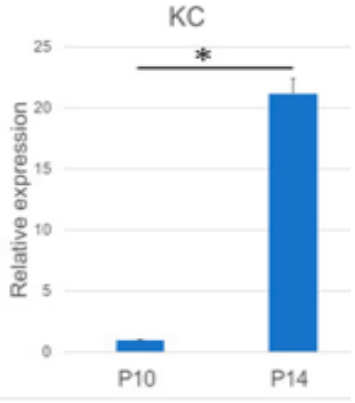
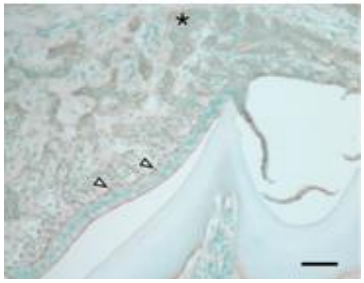



d)

**Figure 2.** Quantitative analysis of Mm00468869_m1 (HIF-1α), Mm01336189_m1 (IL-1β), MIP-2, and KC transcripts by RT-PCR (n = 9 per group). Expression of all four genes showed a significant rise at the eruptive phase ($p < 0.05$).



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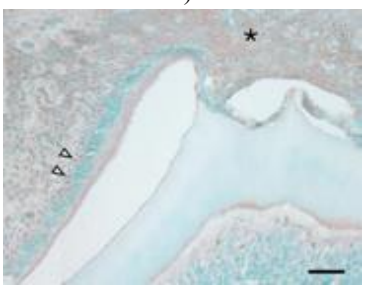


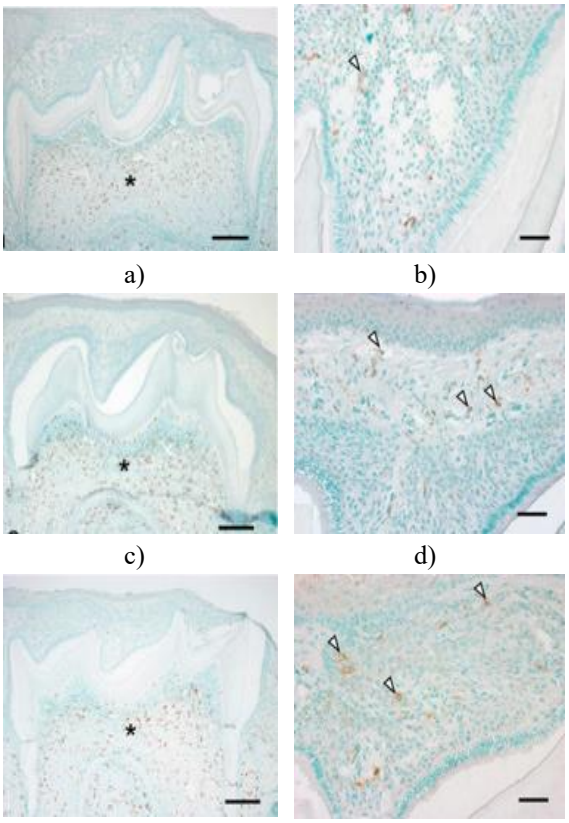
b)

**Figure 3.** Immunolabeling of KC (a) and MIP-2 (b) in molar organs at 14 dPN. Both markers were localized within the papillary layer (arrowheads) and overlying odontogenic epithelium (asterisk). Scale = 50 µm.

*Migration of macrophages and neutrophils during tooth eruption*

Since cytokine and chemokine levels increased during eruption, the recruitment of inflammatory cells—particularly macrophages and neutrophils—was examined. Numerous resident macrophages were evident in the dental papilla at every time point (**(Figures 4a, 4c and 4e)**, asterisks). Additionally, macrophages were identified within the tissue overlying the tooth organ throughout all stages (**(Figures 4b, 4d and 4f)**, arrowheads). At 7 dPN, they

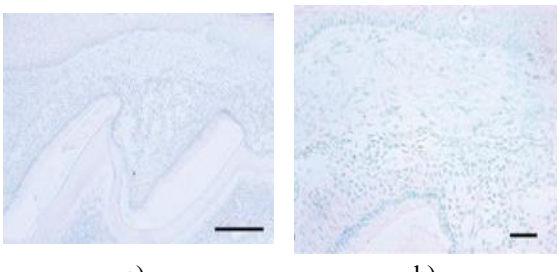
appeared near the enamel organ **(Figure 4b)**, and by 14–19 dPN, they occupied the connective tissue above the developing crown **(Figures 4d and 4f)**.



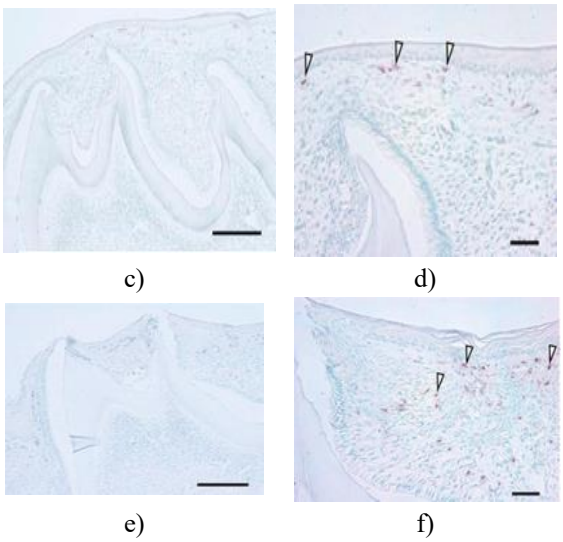
a) b) c) d) e) f)

**Figure 4.** Distribution of macrophages at 7 dPN (a,b), 14 dPN (c,d), and 19 dPN (e,f). Resident macrophages were abundant in the dental pulp at all stages (asterisks), while from 14 dPN onward, they were clearly observed in the connective tissue above the tooth germ (d,f, arrowheads).
Scale = 200 μm (a,c,e); 50 μm (b,d,f).

No neutrophils were visible within the dental papilla at any stage **(Figures 5a, 5c and 5e)**. At 7 dPN, neither the enamel organ nor its overlying tissue showed neutrophil presence **(Figure 5a)**. By 14 dPN, a few neutrophils had appeared in the connective tissue above the enamel organ (**(Figure 5d)**, arrowheads), and at 19 dPN, their infiltration increased notably (**(Figure 5f)**, arrowheads).



a) b)



c) d) e) f)

**Figure 5.** Neutrophil distribution during eruption at 7 dPN (a,b), 14 dPN (c,d), and 19 dPN (e,f). Neutrophils were absent from the dental pulp throughout. By 14 dPN, they emerged within the connective tissue above the tooth organ (d, arrowheads), with greater accumulation at 19 dPN (f, arrowheads).
Scale = 200 μm (a,c,e); 50 μm (b,d,f).

During the differentiation of ameloblasts from the secretory to the maturation phase, the stratum intermedium underwent a morphological transition, giving rise to the papillary layer. Alongside this alteration, ICAM-1 synthesis commenced within papillary layer cells. Earlier investigations by our group demonstrated that the junctional epithelium (JE) originates from these ICAM-1–positive papillary cells [16, 17, 25]. The transcription of ICAM-1 is known to be influenced by pro-inflammatory agents such as IL-1 and TNF-α [26, 27].

Tooth eruption depends on the dental follicle (DF), bone resorption to create the eruption pathway, and bone deposition beneath the forming crown. Inflammatory mediators, particularly IL-1α, are elevated in teeth affected by periapical inflammation [28]. In this investigation, ICAM-1 expression was observed within the papillary layer located beneath the alveolar bone covering the eruptive crown, suggesting that its presence was not directly related to the cytokines governing bone resorptive activity.

RT-PCR results revealed a marked elevation of IL-1β within tissues overlying the developing tooth germ. This observation supports the notion that pro-inflammatory cytokine signaling contributes to ICAM-1 activation within the papillary layer. Notably, the vascular endothelium of the covering tissue also displayed pronounced ICAM-1 reactivity, implying endothelial participation in this mechanism.

According to De Pizzol *et al*. [21], a decrease in vascular density occurs in the connective tissue above erupting teeth, consistent with hypoxic stress in that region. Our data showed that HIF-1α levels rose considerably during this stage, and since this molecule is a known modulator of inflammatory responses [23], it may participate in a cascade in which hypoxia-driven HIF-1α induction enhances IL-1β production, subsequently leading to ICAM-1 upregulation within both papillary and endothelial cells of the overlying tissue.

For successful eruption, the connective tissue barrier above the crown must undergo degradation. Because inflammatory reactions are capable of mediating tissue breakdown, they likely assist this process. Evidence from other biological contexts shows that innate inflammatory activity is involved in the early phases of disorders such as type 1 diabetes and Parkinson's disease [29, 30]. During eruption, an accumulation of apoptotic cells within the lamina propria above the tooth germ has been documented [21]. Signals released from these dying cells, together with oxygen deficiency, may initiate sterile inflammatory pathways facilitating tissue remodeling.

In the current study, the connective tissue above the tooth organ displayed mild leukocyte infiltration, mainly of macrophages and neutrophils. Comparable findings have been described by de França Landim *et al*. [31] in rat models. Additionally, RT-PCR analysis showed a significant rise in MIP-2 and KC expression—chemokines that attract macrophages and neutrophils, respectively. Immunohistochemistry localized both factors to the papillary layer and surface epithelial cells above the tooth germ.

Earlier research by our group reported that KC and MIP-2 are constitutively present in the junctional epithelium, even in germ-free animals, allowing continuous cell migration into this region [16]. Together, the current and previous data imply that the junctional epithelium originates from papillary-derived cells, and that persistent KC/MIP-2 expression ensures immune-cell surveillance at this barrier even without external bacterial stimuli.

Enzymes of the matrix metalloproteinase (MMP) family have been implicated in lamina propria degradation during molar eruption in rodents [32, 33]. These enzymes are produced by macrophages and neutrophils in response to pro-inflammatory cytokine signaling [34, 35]. Hence, the rapid disintegration of the connective tissue above the eruptive crown—driven by these immune cells—appears necessary for proper eruption mechanics.

Clinically, eruption gingivitis is a frequent finding in children [36]. Although it is generally attributed to bacterial exposure, the current results suggest that endogenous, non-infectious inflammation already occurs prior to microbial invasion, and secondary infection merely enhances the existing inflammatory state.

## Conclusion

Our findings indicate that non-infectious inflammatory signaling, initiated through HIF-1α activation under hypoxic conditions, orchestrates the tissue remodeling associated with tooth eruption. This suggests that endogenous inflammation not only supports tooth development but may also contribute to the initiation or intensification of various pathological and developmental processes.

**Acknowledgments:** None

**Conflict of Interest:** None

**Financial Support:** None

**Ethics Statement:** None

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