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Immunohistochemical analysis of Th1/Th2 cytokine profiles and androgen receptor expression in the pathogenesis of nifedipine-induced gingival overgrowth

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Background: Numerous studies have demonstrated that gingival overgrowth may be associated with androgen and cytokine expression in tissues.

Objectives: The aim of this study was to compare the expression of androgen receptor-presenting cells (AR+ cells) and Th1/Th2 cytokine [Th1: interleukin (IL)-2, interferon-γ (IFN-γ); Th2: IL-4, IL-10, IL-13] expression cells in tissue sections of patients with gingival overgrowth.

Materials and methods: Tissue samples were collected from patients with healthy periodontium (H group), adult periodontitis (P group), surgically extracted teeth (S group), and nifedipine-induced gingival overgrowth (NIGO group). The clinical periodontal parameters of pocket depth (PD), bleeding on probing (BOP), and plaque control record (PCR) were measured around selected sample teeth. Gingival biopsies were further processed by immunohistochemical staining method. The expressions of cells positive for AR, IL-2, IFN-γ, IL-4, IL-10, and IL-13 were counted by predetermined semiquantitative methods.

Results: Our results indicated that AR, IL-2, IFN-γ, IL-4, IL-10, and IL-13 were intensively expressed in the nuclei of inflammatory cells and fibroblasts of gingival connective tissue. Stronger expressions of AR, IL-2, and IFN-γ were found in the NIGO group. The AR+ cells/0.01 mm^2 in gingival fibroblasts were significantly higher in the NIGO group (80.2 ± 10.7) than those of the periodontitis group (52.5 ± 11.8) and control group (37.4 ± 11.3) ($P < 0.05$). The cytokine expression of the NIGO group showed a trend towards Th1-type expression (IL-2; $P = 0.0001$). In the surgically extracted tooth group, a stronger expression of Th2-type cytokine (IL-4, Il-10, IL-13; $P < 0.05$) was found in inflammatory cells. In a comparison of the IL-2/IL-4-labeled cell ratio of the four groups, a descending sequence was discovered as NIGO group (0.92 ± 0.97) > H group (0.81 ± 0.61) > P group (0.77 ± 0.82) > S group (0.58 ± 1.77).

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Conclusions: Our data support the following: (i) taking nifedipine may elevate the expression of AR in susceptible oral tissue, e.g. gingiva; (ii) the cytokine profile of T-cells in NIGO tissue indicates a trend preferentially towards Th1 activity; and (iii) elevation of AR expression cells and prominent Th1 cytokine-labeled cells are two significant factors in the pathogenesis of NIGO.

Numerous studies have demonstrated an increase in gingival inflammation during puberty, menstrual cycles, and pregnancy. It was suggested that the aggravation of gingival inflammation and the specific tissue changes seen in these entities may be caused by increased concentrations of circulating sex hormones (1).

The first evidence that gingiva may function as a target organ for androgens was depicted by Southren *et al.* (2). Ojanotko *et al.* (3) also investigated the metabolism of testosterone by human healthy and inflamed gingiva *in vitro*. They also suggested that gingiva may be a target tissue for androgens. Sooriyamoorthy *et al.* studied testosterone metabolism in human gingival fibroblasts in culture (4, 5). Significant levels of androgen metabolism also occur in inflamed tissue. This implies that in the presence of gingival inflammation, variations in circulating androgen levels may be another significant factor in the etiology of drug induced gingival overgrowth (DIGO).

Cytokines in mice model were initially categorized into three broad categories (6): pro-inflammatory [such as tumour necrosis factor-α, interleukin (IL)-1, and IL-6], type 1 [such as IL-2 and interferon-γ (IFN-γ)], which promote the development of antigen-specific cell-mediated immunity, and type 2 (such as IL-4 and IL-10) which facilitate the development of antibody-mediated immunity. T helper cells can be divided into the reciprocally suppressive T helper type 1 (Th1) and Th2 subsets. Th1 cells produce IL-2, IFN-γ, and tumor necrosis factor, and stimulate cell-mediated immune responses, whereas Th2 cells produce IL-4, IL-5, IL-10, and IL-13, which provide help for humoral immune responses (7–9).

Both gonadal [testosterone and dihydrotestosterone DHT)] and adrenal androgens [dehydroepiandrosterone (DHEA)] showed a depressive influence on the production of some cytokines by activated human macrophages and murine T lymphocytes. Rook *et al.* found exposure of isolated murine T cells to DHEA resulted in enhanced potential of activated T cells (Th1) to produce IL-2 and IFN-γ after activation (10, 11). Interestingly, a further study indicated that the addition of testosterone inhibited the production of IL-4, IL-5, and IFN-γ by activated murine T cells without affecting their capacity to produce IL-2 (12).

In the present study, we localized the distribution of Th1/Th2 cytokine secreting cells and the extent of androgen receptor (AR) expressive cells using an immunohistochemical staining method in order to explain the possible relationship of the expression of AR, cytokine profile, and the pathogenesis of nifedipine-induced gingival overgrowth (NIGO).

Materials and methods

Patient selection

Patients treated at the Taipei Medical University's Periodontal Clinic were divided into four groups. The first group included five patients who had taken nifedipine for controlling hypertension (NIGO group) for a period of 5–24 months. The dosage and duration of nifedipine administration are shown in Table 1. In the second group, 15 patients with moderate adult periodontitis (P group, age range 28–55 years) were selected for surgical treatment after scaling and root planning (Sc/Rp). Ten patients with severe periodontitis (S group) and 10 patients with healthy dentition (H group) were selected as the third group and fourth group, respectively.

The clinical data, including probing depth (PD), bleeding on probing (BOP), and plaque control record (PCR) (13), were collected according to the timetable designed for the present study (Fig. 1). Additionally, the tissue overgrowth status of the NIGO group was classified and recorded by using Ingle's gingival overgrowth index (Table 1) (14).

Sample collection

Biopsies from NIGO cases ($n = 5$) were taken 3 months after Sc/Rp was performed. It was taken under local anesthesia from the interdental papilla of maxillary anterior or posterior sextants where the status of gingival was classified as or over grade 2 in Ingle's gingival overgrowth index. Tissue samples of P group ($n = 15$) were harvested from interdental areas (PD > 5.0 mm) during surgical intervention for pocket reduction. Ten biopsies of granulomatous tissues of gingiva around extracted hopeless teeth were chosen from S group patients ($n = 10$). Gingival samples were gathered from the wedge tissues of H group patients ($n = 10$) who accepted crown lengthening procedure.

Harvested tissues were fixed in 3.5% buffered formalin and washed with 0.05 M phosphate-buffered saline and double-distilled (dd) water. Samples were then embedded in paraffin wax.

Table 1. Biographical data of NIGO patients (n = 5)

Patient	A	B	C	D	E
Gender	M	F	F	M	M
Age (year)	62	51	55	63	60
Nifedipine dosage	30	30	60	30	30
Duration of dosage (mo)	24	10	5	12	8
Overgrowth Index	2	3	2	2	2

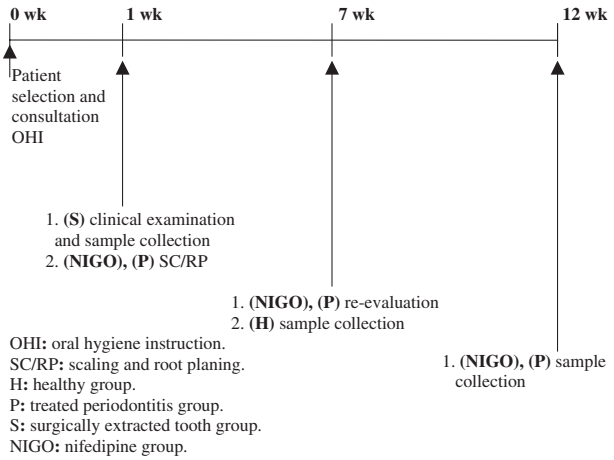

Fig. 1. Clinical protocol for the collection of the clinical data for the present study, including probing depth (PD), bleeding on probing (BOP), and plaque control record (PCR).

Five-micrometer serial sections were collected on silane-coated glass slides and used for subsequent immunohistochemical staining.

Immunohistochemical staining

Tissue sections were deparaffinized by immersion in xylene and immersed in ethanol for rehydration. Tris-HCL buffer was used to wash the deparaffinized sections three times for 5 min each. Entire sections were placed in a plastic box filled with 0.001 M citrate buffer and heated in a microwave oven for 10 min for antigen retrieval procedure. Three per cent H_2O_2 was used to block the remaining activity of endoperoxidase. Tissue sections were washed with double-distilled water for 5 min again and Tris-HCL buffer three times for 5 min each. Slides were further conditioned with 5% skim milk/dd H_2O for 30 min and then incubated with mouse antihuman primary monoclonal antibodies (IL-2, IL-4, IL-10, IL-13, IFN-γ, AR; Santa Cruz Biotechnology, CA, USA) overnight at 4°C. After rinsing in Tris-HCL buffer three times (5 min/time), sections were subsequently incubated with biotinylated linked antimouse and antirabbit IgG antibody for 10 min. Following washing with Tris-HCL buffer 5 min each for three times, sections were treated with streptoavidin–HRP complex (Dako Japan Co. Ltd, Kyoto, Japan) for 10 min, Tris-HCL buffer three times (5 min/time), and 3,3′-diaminobenzidine tetrahydrochloride for 7 min at room temperature. Technical control using mouse antihuman monoclonal PCNA antibody (Santa Cruz Biotechnology, CA, USA) was simultaneously conducted. Negative controls were also included, in which the primary antibodies were replaced by phosphate-buffered saline for all tissues investigated. Counterstaining was performed with hematoxylin for 15 s and then washing for another 5 min. Slides were finally dehydrated with ethanol and subsequently mounted with xylene for further microscopic observation and cell counting.

Semiquantitative evaluation of stained cells

The immunostained cells that appeared to be AR positive were counted in five randomly selected fields of every 100 cells of infiltrated inflammatory cells (positive cells/100 cells) in gingival connective tissue (GCT) magnified 400×. Because we found the widespread character of gingival fibroblasts in tissue samples, AR+ cells of gingival fibroblasts were counted in five randomly selected fields in a $0.1 \times 0.1\text{-mm}^2$ area defined by the grid scale mounted in an optic lens under 400× magnification, instead of the counting methods used for the infiltrated inflammatory zone of GCT.

The mean numbers of stained cells of the four groups were compared, and differences between groups were statically analyzed using the Kruskal–Wallis test and Mann–Whitney *U*-test.

Numbers of cells labeled with anti-IL-2, IFN-γ, IL-4, IL-10, and IL-13 were determined in the inflammatory zone of GCT. The same counting method was used as the modality for every 100 cells count of infiltrated inflammatory cells for AR+ cells. The Kruskal–Wallis test was first used for statistical analysis for the four groups. Mann–Whitney *U*-test was further used to analyze the difference between any two groups.

Th1/Th2 ratio

The ratio of IL2/IL4-positive cells was calculated as the mean ± SD for each sample of four groups. Wilcoxon 2-sample test with continuity correction of 0.5 was used to analyze results between four groups. Statistical significance was determined at 0.05 level.

Results

Clinical parameters

Our study intends to verify that inflammatory factor is not the main issue in the pathogenesis of NIGO. NIGO and P groups received Sc/Rp in order to eliminate the superimposed factor – chronic inflammation. All the selected patients in the four groups showed a similar level of PCR after clinical OHI was conducted. The PD and BOP of P and NIGO groups achieved a similar clinical level after Sc/Rp (Table 2). However, the PD and BOP data of non-treated S group showed a significant difference to the other three groups ($P < 0.01$).

Androgen receptors

The results of immunohistochemical staining picture showed that AR+ cells were distributed in GCT. AR+ cells were localized and scattered in both the infiltrated inflammatory area and the non-inflamed area.

By comparing the distribution of AR+ cells/100 cells in inflammatory tissue zone, no statistical difference was seen between the NIGO group and the

Table 2. Biographical and periodontal characteristics of subjects with healthy, treated periodontitis, a surgically extracted tooth, and the nifedipine group

	H group	P group	S group	NIGO group
Biographical data				
n	10	15	10	5
Mean age (year)	39.3	42.4	48.4	58.2
Age range (year)	(30–47)	(28–55)	(24–79)	(51–63)
Male/female	5/5	7/8	6/4	3/2
Periodontal data				
PPD (mm) (mean ± SE)	2.2 ± 0.7	5.3 ± 0.6	9.9 ± 1[b]	6 ± 0.7
BOP (%) (mean ± SE)	4.8 ± 7.7	72.8 ± 11	93.2 ± 12[b]	72.8 ± 15.2
PCR (%) (mean ± SE)[a]	15.9 ± 2.5	17.9 ± 5.5	20.1 ± 3.3	17.2 ± 3.1

PD = probing pocket depth; BOP = bleeding on probing; PCR = plaque control record.
[a]O'Leary plaque control record; Kruskal–Wallis test: $P < 0.05$.
[b]Mann–Whitney *U*-test: $P < 0.05$. Surgically extracted tooth group vs. nifedipine, periodontitis, and healthy groups.

Table 3. Distribution of AR-positive cells in inflammatory tissue zones and connective tissues of gingiva

	H group	P group	S group	NIGO group
n	10	15	10	5
Inflammatory cell (+ cells/100 cells)	66.1 ± 11.8	69.6 ± 12.8	68.6 ± 23.6	81.3 ± 22
Gingival fibroblasts (+ cells/0.01 mm^2)	37.4 ± 11.3[b]	52.5 ± 11.8[b]	50.8 ± 15.1[b]	80.2 ± 10.7[ab]

[a]Kruskal–Wallis test: $P < 0.05$.
[b]Mann–Whitney *U*-test: $P < 0.05$. Gingival fibroblast: nifedipine group vs. healthy, treated periodnotits group, and surgically extracted tooth group.

Table 4. Distribution of cytokine-positive inflammatory cells/100 cells in the inflammatory GCT of the four groups

Cytokine	H group	P group	S group	NIGO group
n	10	15	10	5
IL-2	38.8 ± 3.6[b]	58.0 ± 9.4[b]	53.8 ± 9.9	77.6 ± 8.0[ab]
IFN-γ	46.5 ± 14.2[b]	76.7 ± 18.9	76.2 ± 5.5[b]	87.4 ± 5.6[b]
IL-4	48.1 ± 5.9[b]	75.7 ± 11.5[b]	92.1 ± 5.6[b]	84.4 ± 8.3
IL-10	29.0 ± 8.3[b]	51.4 ± 8.8[b]	82.6 ± 5.6[b]	64.2 ± 5.3[b]
IL-13	36.4 ± 11.5[b]	66.7 ± 13.2	67.4 ± 9.7[b]	49.8 ± 9.5[b]

[a]Kruskal–Wallis test: $P < 0.05$.
[b]Mann–Whitney *U*-test: $P < 0.05$. IL-2: nifedipine vs. treated periodontitis and healthy group; IFN-γ: nifedipine vs. surgically extracted tooth and healthy group; IL-4: surgically extracted tooth group vs. treated periodontitis and healthy group; IL-10: surgically extracted tooth group vs. nifedipine, treated periodontitis, and healthy groups; IL-13: surgically extracted tooth group vs. nifedipine and healthy groups.

other groups (Table 3). However, the data for AR+ cells/0.01 mm^2 in GCT fibroblastic zone of the NIGO group, as tested with the Kruskall–Wallis test, showed a significant difference to the other three groups ($P = 0.001$). In a comparison of AR+ cells/0.01 mm^2 in GCT of the S and NIGO groups, the extent of the AR+ cell count in the S group (50.8 ± 1.15), was less than that of the NIGO group (80.2 ± 10.7) ($P < 0.05$, Mann–Whitney *U*-test).

Th1/Th2 cytokine profile

Data on the variable of Th1/Th2 cytokine-positive cells in GCT of all groups are presented in Table 4. When the Kruskal–Wallis test was used to analyze statistical differences among these four groups for each cytokine, the only significant difference was seen in IL-2-labeled cells of the NIGO group over those of the other three groups ($P = 0.0001$). By using the Mann–Whitney *U*-test, IL-2-postive cells in NIGO group (77.6 ± 8.0) significantly differed ($P < 0.05$) from those of the P group (58.0 ± 9.4) and H group (38.8 ± 3.6). IFN-γ-positive cells in the NIGO group (87.4 ± 5.6) significantly differed ($P < 0.05$) from those of the S group (76.2 ± 5.5) and H group (46.5 ± 14.2). For IL-4, the S group (92.1 ± 5.6) significantly differed from the P group (75.7 ± 11.5) and H group (48.1 ± 5.9) ($P < 0.05$). As for IL-10, the S group (82.6 ± 5.6) also significantly differed from the other three groups (NIGO: 64.2 ± 5.3; P: 51.4 ± 8.8; H: 29.0 ± 8.3) ($P < 0.05$). The mean cell count of IL-13-labeled cells of the S group (67.4 ± 9.7) also markedly differed from those of the NIGO group (49.8 ± 9.5) and H group (36.4 ± 11.5) ($P < 0.05$). According to the magnitude of the results, we arranged the sequence of the four groups in a descending cascade for each cytokine (IL-2: NIGO > P > H; IFN-γ: NIGO > S > H; IL-4: S > P > H; IL-10: S > NIGO > P > H; IL-13: S > NIGO > H).

IL-2/IL-4 ratio

IL-2/IL-4-positive cell ratios were calculated and tabulated for each group (Table 5). The Wilcoxon rank sum test revealed no significant difference of the H group with the NIGO group ($P = 0.0563$). However, a significant difference was discovered between the H, P, and S groups ($P < 0.05$). Comparing the NIGO group to the P and S groups, biostatistical differences were also found for both groups ($P < 0.05$). According to the magnitude of IL-2/IL-4 positive-staining cell ratio in Table 5, a descending order can be

Table 5. IL-2/IL-4-positive staining cell ratio

	NIGO group		H group		P group		S group	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
IL-2/IL-4	0.92[b]	0.97	0.81[a]	0.61	0.77[ab]	0.82	0.58[ab]	1.77

[a]Wilcoxon rank sum test: $P < 0.05$; H group > P and S groups.
[b]Wilcoxon rank sum test: $P < 0.05$; NIGO group > P and S groups.

arranged as NIGO group > P group > S group.

Discussion

Androgen receptors are ligand-activated transcription factors that are regulated by androgens via complex tissue-specific processes. Androgen sensitivity is related to the concentration of AR (15), and the correlation also exists between AR levels and sensitivity of target tissues to androgen (16). Therefore, we intended to quantify AR+ cells to reflect the feedback control of androgen changes in various tissues.

In our study, we found that the percentages per 100 cells count of AR+ cells were significantly higher in non-inflammatory GCT, but not in the infiltrated inflammatory zone, of the NIGO group compared to those in the P and H groups ($P < 0.05$). AR+ cells/0.01 mm^2 of GCT of the NIGO group was approximately twofold greater than values for both the S and P groups as examined by the Kruskal–Wallis test ($P < 0.001$). It was about sixfold greater than that of the H group. Southren *et al.* (2) provided evidence for specific cytosolic receptors for 5α-DHT in human gingival tissue. They also showed a result with highly significant increase (9–12-fold) in the number of antigen receptors in patients with gingival overgrowth. According to studies of Sooriyamoorthy *et al.* (17, 18), it was speculated that androgen metabolism can be a significant factor in the pathogenesis of NIGO. Free androgen may passively diffuse into all target cells, e.g, gingival fibroblasts, and be preferentially retained in target cells through the formation of a high-affinity complex with the steroid-specific receptor and cause an increase in collagen synthesis and/or a decrease in collagenase activity. A link between elevated levels of adrenal androgen (DHT) and increased repair potential in fibroblasts was strongly suggested. Androgen exerts a marked anabolic effect in both non-drug-induced and drug-induced gingival overgrowth (19, 20).

In a comprehensive review and analysis of nifedipine-induced gingival hyperplasia, a combination of deficient intracellular folic acid uptake, an increase in 5α-DHT receptors, and suppression of both IL-2 production and T-cell proliferation were postulated as the 'three models' for drug-induced gingival hyperplasia (21). However, looking at the distribution of cytokine-positive inflammatory cells (%) in GCT of present study, the NIGO group showed more significant labeling of IL-2 cells as indicated by the Kruskal–Wallis test ($P < 0.001$). The NIGO group also showed significant labeling of IFN-γ-immunostaining cells as compared to the S and H groups. It may be concluded that the cytokine profile of tissue samples of the NIGO group showed a trend towards greater Th1-type cytokine expression. This is contradictory to Harel-Raviv's model that dictates the down-regulation of the Th1 profile in tissue with drug-induced gingival hyperplasia (21). Moreover, a study of exposure of isolated murine T cells to DHEA resulted in an enhanced potential of activated T cells (Th1) to produce IL-2 and IFN-γ after activation with no effect on their production of IL-4 (Th2) (11). Suzuki *et al.* (22) also found that DHEA may enhance the production of IL-2 and the cytotoxic function of human T cells. Another investigation evaluated the influence of androgens on cytokine production by activated murine T cells co-cultured with macrophages. The addition of androgen was found to inhibit the production of IL-4, IL-5, and IFN-γ by activated T-cells without affecting their capacity to produce IL-2 (12). Our results (Table 3) show a general aspect of the distribution of AR+ cells in GCT of the four groups as NIGO group > P group > S group > H group. As compared to the results of the IL-2/IL-4 ratio (NIGO group > P group > S group), it implied that androgens are regarded as natural immunosuppresors and exert their modulatory activities through both a direct influence on cytokine production by activated macrophages and an indirect influence on cytokine production by activated Th1 cells.

A number of studies have reported on the presence of cytokines in periodontal disease (23–25). A cell dot-blot analysis of cytokine-producing gingival mononuclear cells showed a significantly lower IL-2/IL-4 ratio for cells derived from periodontitis tissues compared with cells from gingivitis tissues (26). Seymour *et al.* raised a hypothesis that Th1 cells are associated with stable lesions in periodontal disease, whereas a Th2 response may lead to non-protective destruction of the periodontium (27). Our study showed that the IL-2/IL-4-positive cell ratio decreases gradually from the NIGO group (0.92 ± 0.97), H group (0.81 ± 0.61), and P group (0.77 ± 0.82), to the S group (0.58 ± 1.77). The IL-2/IL-4-labeled cell ratio of the NIGO group was higher than that of the inflammatory S group. Overgrowth status of NIGO did not subside after gingival inflammation was controlled by Sc/Rp. This indicates that the Th1/Th2 cytokine ratio may play certain role in the pathogenesis of NIGO.

Several studies have demonstrated that good oral hygiene and control of gingival inflammation may reduce the chance of drug induced gingival hyperplasia (DIGH) (28–30). A clinical and *in vitro* study also indicated that both local inflammatory factors and long-term administration of nifedipine are responsible for the changes in gingival overgrowth (31). However, the effect of nifedipine variables and hygiene control on the gingival overgrowth score was examined using univariate and multivariate regression analyses in a recent study (32). Ultimately, there were no significant differences between the group with a

gingival overgrowth score < 30% and the group with clinically significant overgrowth (> 30%) for the parameters of sex distribution, plaque score, or any of the nifedipine variables. In the comparison of PD, BOP, and PCR between the P and NIGO groups in our study, all patients of both groups had accepted Sc/Rp and controlled their clinical status to a same clinical level. However, the extent of AR+ cells in gingival fibroblasts of the NIGO group still remained higher than that of the P group ($P < 0.05$, Mann–Whitney *U*-test). On the contrary, PD and BOP of the S group were larger than those of the NIGO group, but the extent of AR+ cells/0.01 mm^2 (50.8 ± 15.1) in GCT was lower than that of the NIGO group (80.2 ± 10.7) ($P < 0.05$). It suggests that after ruling out inflammatory factors from this cohort study by conducting Sc/Rp, the significant amount of AR+ cells presented in NIGO tissues seem to be the other major differences in the pathogenesis of NIGO. This is in concert with part of Harel-Raviv's model that under the condition of increasing calcium accumulation in gingival fibroblasts by taking nifedipine, androgen receptor would also be up regulated after nifedipine therapy (21).

In conclusion, our data support the administration of nifedipine for controlling blood pressure possibly elevates the expression of AR in susceptible gingiva of patients. After controlling the inflammatory factors, the significant amount of AR+ cells and the predominant expression of Th1 cytokine profile presented in NIGO tissues seem to be the major factors in the pathogenesis of NIGO. The significant AR expression can alter the development of effecter T cells in NIGO and govern more predominant Th1 activity.

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