ACTIONS OF RECOMBINANT INTERLEUKIN-1β, TUMOR NECROSIS FACTOR-α AND INTERLEUKIN-6 ON BONE RESORPTION IN VITRO

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I. INTRODUCTION

Bone resorption is the typical feature of chronic periodontal disease. Thus many researches focused on the mechanism of bone resorption and many different mediators which have implicated in the stimulation of bone loss in periodontal disease, based on mainly upon the ability to stimulate bone resorption in vitro and their presence in periodontal tissues. In recent, of those mediators, several cytokines are introduced that mediate the inflammatory process and play a major role in its amplification and perpetuation, and the ensuing tissue destruction.

In connection with the pathogenesis of periodontitis, the presence of several cytokines and their associations with disease status have been studied through gingival crevicular analysis[1-6], gingival biopsy[7-10] and investigation of the cultured peripheral blood mononeuclear cells from patients with periodontitis[11]. However because progress in our understanding of production and action of the cytokines is more recent, relative less is known of their likely role in periodontal disease. Those cytokines which have been showen to stimulate bone resorption in vitro and may be of relevance include interleukin-1[IL-1](α and β)[12-21], tumor necrosis factor [TNF](α and β)[17,22-24], transforming growth factor [TGF][25] and platelet drived growth factor[PDGF][26]. In addition, interleukin-6[IL-6] produced by fibroblasts and endothelial cells may stimulate the formation of osteoclast[27]. Of above mentioned cytokines, IL-1(α and β), TNF(α and β) and IL-6 are very interested in the mechanism of bone resorption in chronic periodontal disease.

IL-1 and TNF are potent stimulators of bone resorption and have been implicated in bone loss in periodontal disease. The bone resorbing factor referred to in the earlier literature as OAF(osteoclast activating factor) is no longer considered to exist as such but represents a family of cytokines including IL-1 and TNF. IL-1 is a particularly potent stimulator of bone resorption and its effects on calcium release in vitro bone resorption studies can be demonstrated at picomolar concentration. Hönig et al. detected IL-1β assay in extracts of granulation tissues from periodontitis patients[6]. Masada et al. showed that both IL-1α and IL-1β were produced and released into crevicular fluid locally in periodontal disease at concentration sufficient to mediate tissue inflammation and bone resorption[3]. TNF(α and β), although less potent than IL-1, can still

induce bone resorption. Rossomando et al detected TNF(α and β) in gingival crevicular fluid[5]. IL-6 increased multinucleated cell formation in a long-term human marrow culture, and it was suggested that the activity of IL-6 was mediated by IL-1β[26]. In any case, IL-6 may also be responsible for bone resorption. Therefore IL-1, TNF and IL-6 would be involved in alveolar bone loss in the pathogenesis of periodontal disease.

The purpose of present study is to investigate the effects of recombinant IL-1β, TNF-α and IL-6 on bone resorption and modulations of their activities by cyclooxygenase inhibitor and recombinant IFN-γ in neonatal mouse calvariae in organ culture.

II. MATERIALS AND METHODS

1. Cytokines and Cyclooxygenase Inhibitor

Recombinant human interleukin-1β [rHuIL-1β] (specific activity 2×10^8 U/mg), recombinant human tumor necrosis factor-α [rHuTNF-α] (specific activity 2×10^7 U/mg), recombinant human interleukin-6 [rHuIL-6] (specific activity 4×10^6 U/mg) and recombinant murine interferon-γ [rMurIFN-γ] (specific activity 10^7 U/mg) were purchased from Genzyme Corp.(U.S.A.).

Indomethacin which was used as a cyclooxygenase inhibitor was purchased from Sigma Chemical Corp.(U.S.A.).

2. Bone Resorption Assay

The bone resorbing activity of cytokine was assessed using a ^{45}Ca-labeled mouse calvaria system[28]. The calvariae of mice aged 1-2 days were prelabeled with ^{45}Ca by the subcutaneous injection of 1μCi $^{45}CaCl_2$. After 4 days, the mice were sacrificed and half calvariae were removed aseptically by microscopic dissection. Bones were precultured 24h in BGJb medium (Gibco, U.S.A.) supplemented with 5% fetal calf serum (FCS : Gibco, U.S.A.) and 1% antibiotic-antimycotic solution (Gibco, U.S.A.) to remove noncoporated ^{45}Ca. The explants were then transferred to 0.5ml fresh medium containing each cytokine(rHuIL-1β, rHuTNF-α and rHuIL-6) and any other additions and cultured for 48h without medium exchange in 24 well-culture plate (Nunc, U.S.A.). Controls were the paired half calvarias without any cytokine. Incubations were carried out at 37℃ in anaerobic incubator in a 100% humidified atmosphere of 5% CO_2 : 20% O_2 : 75% N_2. At the end of culture period the explants were dried and dissolved in 0.5ml of 90% formic acid at 60℃. The complete sample was dissolved in a cocktail solution and counted in a liquid scincillation counter (LS5000TA : Beckman, U.S.A.). Samples of culture medium were also dissolved in cocktail solutions and counted. Similar experiment were carried out with addition of indomethacin and rMurIFN-γ.

Bone resorption was expressed as the percentage of the total bone isotope release into culture medium, and as the ratio of ^{45}Ca released into medium from treated as compared to control bones (ratio T/C). Differences between groups were analyzed by using Student's t-test.

III. RESULTS

1. Bone Resorbing Activities of rHuIL-1β, rHuTNF-α and rHuIL-6

At first, activity of each cytokine on bone resorption was evaluated. Treatment of calvariae with rHuIL-1β resulted in a dose dependent stimulation of bone resorption from 10^{-12} M to 10^{-9} M. The minimal concentration to elicit a significant bone resorption was observed at 10^{-11} M. (Table-1, Fig.-1)

Bone resorbing activity of rHuTNF-α was also observed in a dose dependent manner from 10^{-10} M to 10^{-8} M. Compared with activity of rHuIL-1β, much lesser degree of resorption was observed. The minimal concentration to elicit a significant bone resorption was observed at 10^{-9} M. (Table-2, Fig.-2)

However, rHuIL-6 was found to have no signifi-

cant resorptive effect on calvarial explant at any concentration from 10^{-10} M to 10^{-8} M for 48 h culture period of present study. (Table-3, Fig.-3)

2. The Effects of Indomethacin on Bone Resorbing Activities of rHuIL-1β and rHuTNF-α

In order to investigate whether prostaglandin are involved in mechanism of each cytokine, the effects of cyclooxygenase inhibitor, indomethacin (10^{-6} M) on bone resorbing activities of rHuIL-1β and rHuTNF-α were evaluated at each concentration (at 10^{-9}M of rHuIL-1β and 10^{-8}M of rHuTNF-α) which had a maximal resorbing activity in present experiment. Indomethacin (10^{-6} M) slightly decreased bone resorbing activity of rHuIL-1β, however statiastical significance was not observed. In case with rHuTNF-α, indomethacin decreased its resorbing activity but statistical significance was not observed, too. (Table-4, Fig.-4)

Therefore, within the limitation of present study, it is not convinced that these two cytokines induced bone resorption is prostaglandin-dependent.

3. Inhibitory Effects of rMurIFN-γ on Bone Resorbing Activities of rHuIL-1β and rHuTNF-α

The effects of rMurIFN-γ on bone resorbing activity of rHuIL-1β and rHuTNF-α were evaluated at each concentration (at 10^{-9}M of rHuIL-1β and 10^{-8}M of rHuTNF-α) which had a maximal resorptive effect in present experiment. In case with rHuIL-1β as well as rHuTNF-α, rMurIFN-γ (1000 U/ml) decreased the bone resorbing activities of both cytokines. (Table-5, Fig.-5) Especially, inhibitory effect of rMurIFN-γ on rHuIL-1β induced bone resorption was evident. Inhibitory influence of rMurIFN-γ on rHuTNF-α induced bone resorption was also significant.

Table−1 Effect of rHuIL-1β on bone resorption

rHuIL-1β conc.(M)	^{45}Ca release(% total bone radioactivity)		^{45}Ca release (T/C ratio)
	treated	paired control	
10^{-12}	12.47±0.99	11.67±1.01	1.07±0.04
10^{-11}	12.91±1.56*	9.56±0.62	1.25±0.14
10^{-10}	14.44±2.28*	10.83±1.82	1.34±0.12
10^{-9}	20.40±1.62**	11.52±1.09	1.78±0.20

*P<0.05 vs. paired control

**P<0.01 vs. paired control

Table−2 Effect of rHuTNF-α on bone resorption

rHuTNF-α conc.(M)	^{45}Ca release(% total bone radioactivity)		^{45}Ca release (T/C ratio)
	treated	paired control	
10^{-10}	11.90±1.17	11.75±1.48	1.02±0.08
10^{-9}	11.94±0.41*	10.13±0.26	1.17±0.03
10^{-8}	14.04±1.29*	10.49±1.07	1.34±0.03

*P<0.05 vs. paired control

Table－3 Effect of rHuIL-6 on bone resorption

rHuIL-6 conc.(M)	^{45}Ca release(% total bone radioactivity)		^{45}Ca release (T/C ratio)
	treated	paired control	
10^{-10}	10.86±0.89^{ns}	10.72±0.49	1.03±0.09
10^{-9}	11.75±0.95^{ns}	11.83±1.04	0.94±0.04
10^{-8}	12.23±0.97^{ns}	11.96±0.89	1.01±0.03

ns : no significant difference, vs. paired control at $P<0.05$

Table－4 The effects of indomethacin on bone resorbing activities of rHuIL-1β and rHuTNF-α

Group	^{45}Ca release (% total bone radioactivity)
Control	11.10±1.56
＋rHuIL-1β(10^{-9}M)	18.81±2.49*
＋rHuTNF-α(10^{-8}M)	14.05±1.05*
Indomethacin(10^{-6}M)	9.45±0.36*
＋rHuIL-1β(10^{-9}M)	15.90±1.31^{ns}
＋rHuTNF-α(10^{-8}M)	12.70±1.10^{ns}

*$P<0.05$ vs. control

ns : no significant effect of indomethacin at $p<0.05$

Table－5 Inhibitory effects of rMurIFN-γ on bone resorbing activities of rHuIL-1β and rHuTNF-α

Group	^{45}Ca release (% total bone radioactivity)
Control	11.34±0.95
＋rHuIL-1β(10^{-9}M)	19.05±0.30**
＋rHuTNF-α(10^{-8}M)	14.37±0.79*
rMurIFN-γ(10^{-3}U/ml)	7.83±0.41*
＋rHuIL-1β(10^{-9}M)	13.56±0.93^{++}
＋rHuTNF-α(10^{-8}M)	11.29±0.91^{+}

*$P<0.05$ vs. control

**$P<0.01$ vs. control

$^{+}P<0.05$, effect of rMurIFN-γ

$^{++}P<0.01$, effect of rMurIFN-γ

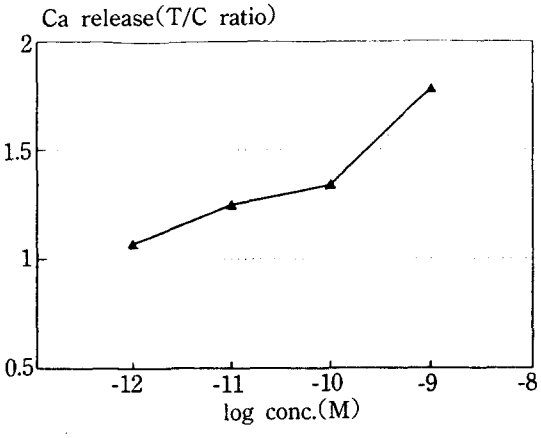

Fig.－1 Effect of rHuIL-1β on bone resorption

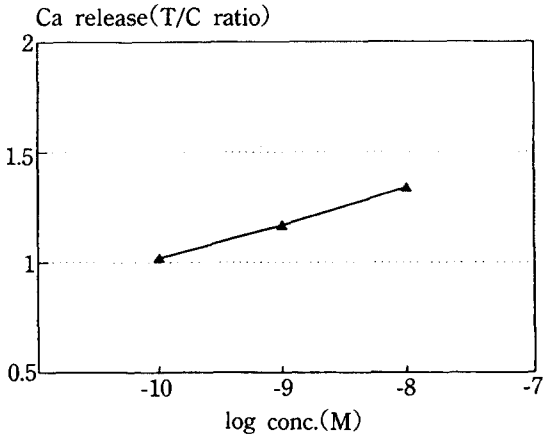

Fig.－2 Effect of rHuTNF-α on bone resorption

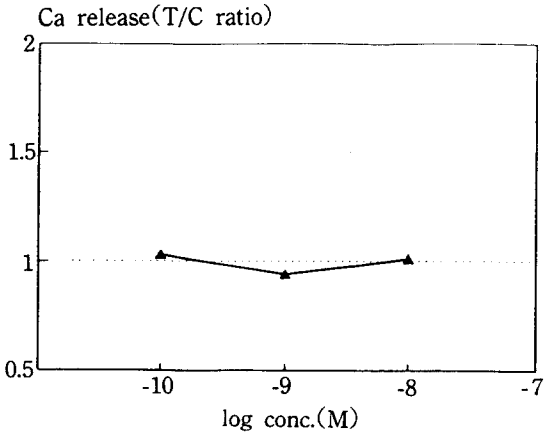

Fig.－3 Effect of rHuIL-6 on bone resorption

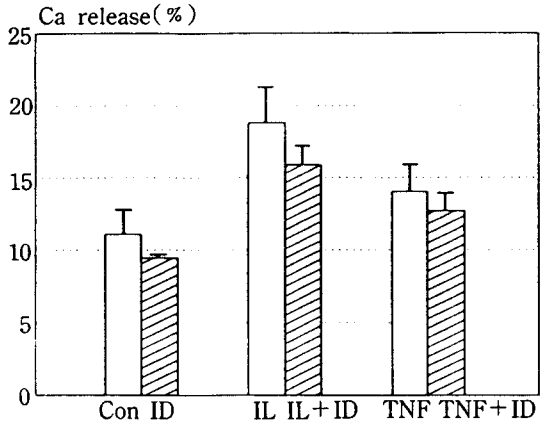

Fig.－4 The effects of indomethacin on bone resorbing activities of rHuIL-1β and rHuTNF-α

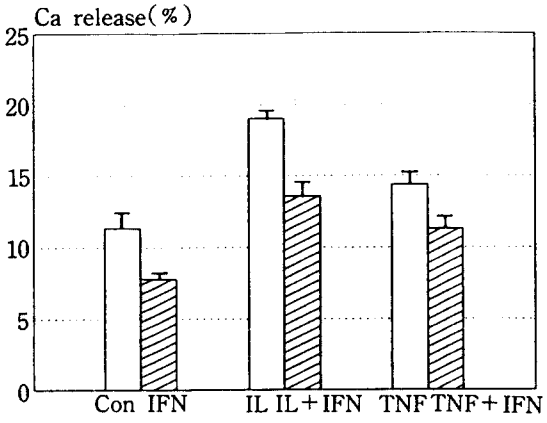

Fig.－5 Inhibitory effects of rMurIFN-γ on bone resorbing activities of rHuIL-1β and rHuTNF-α

Ⅳ. DISCUSSION

In connection with the pathogenesis of periodontitis, the presence of several cytokines and their associations with disease status have been studied. Since Charon et al. in 1982[1], many reports demonstrate the elevated level of IL-1 (especially IL-1β) in gingival crevicular fluid (GCF)[2-4] and gingival tissue extracts[7-10] from periodontally-diseased sites. It was also reported that TNF can be detected in GCF and gingival tissue from site of periodontitis [5,9]. Besides IL-1 and TNF, IL-6 also detected in GCF of patients with periodontitis[6]. In addition to above mentioned evidence, in recent, these cytokines are very interested in the association with periodontal disease activity[32-35].

In present study, it is demonstrated that rHuIL-1β and rHuTNF-α are potent stimulators of bone resorption in vitro. Particulary, bone resorbing activity of rHuIL-1β is evident. RHuIL-1β used in this experiment is about 100 times more potent than rHuTNF-α in resorbing activity was assessed by measuring the release of ^{45}Ca from prelabeled neonatal mouse calvariae. That is similar with the result of Stashenko et al. that used in vitro resorption assay by fetal rat long bone[17]. It is known in many reports that IL-1 is active at picomolar or nanomolar concentrations in vitro bone resorption assay. It is consistent with other similar in vitro studies[14,17,18] that rHuIL-1β is active at 10^{-11} to 10^{-9} M in present report, although its potency was more or less lower than other results. Besides this study, in many in vitro studies, bone resorbing activity of IL-1 is some conflictings in potency and concentration range at which IL-1 is active. They are probably due to purity of material, species and assay differences. In this experiment, rHuTNF-α was active in bone resorption at 10^{-9} to 10^{-8} M. This result also consists with other similar studies[17,22,24].

According to present study and other reports, on the basis of the reports that crevicular fluid for disease sites contain nanomolar amount of IL-

1β[3,4], these levels are sufficient to stimulate bone resorption. It is known that TNF although less potent than IL-1, can stimulate bone resorption at concentration between 10^{-9} to 10^{-7} M[17,22,24]. Also in this study, it was observed that rHuTNF-α have potential resorptive effect at concentration more over than 10^{-9}M. It is less likely however that it play a major role in periodontal bone loss based on the amount of TNF-α detected in crevicular fluid by Rossomando et al[5]. At any rate, it is confirmed that rHuIL-1β and rHuTNF-α have potential activities of bone resorption through present in vitro study and these findings indicate that IL-1β and TNF-α would be involved in alveolar bone loss in the pathogenesis of periodontal disease.

However, rHuIL-6 was found to have no significant resorptive effect on calvarial explants at any concentration from 10^{-10} M to 10^{-8} M for 48 h culture period of present study. Kurihara et al. showed that IL-6 increased multinucleated cell formation in long-term human marrow culture, and they suggested that the activity of rHuIL-6 was mediated by induction of IL-1β[27]. But Mundy suggested that IL-6 may be a central factor involved in cytokine mediated osteoclastic bone resorption, because his group have found that antibody to IL-6 blocks bone resorption stimulated by IL-1 and TNF[36]. These reports show the complex interactions which occur between all of these cytokines in the bone resorption process. Thus, the precise mechanisms by which these complex interactions between cytokines are mediated will require further detailed study.

The role of prostaglandin (PG) in IL-1 action in bone is a matter of controversy. It varies according to the assay systems employed. In this study, cyclooxygenase inhibitor, indomethacin(10^{-6} M) slightly decreased the bone resorbing activities of rHuIL-1β and rHuTNF-α, however statistical significance was not observed. Therefore, within the limitation of present study, there is no evidence that these two cytokines induced bone resorption is prostaglandin-dependent. The possible role of PG in IL-1 stimulated bone resorption has been extensively studied, with conflicting results. In some reports, the effects of IL-1 are described as PG-independent[12,14,16], but in some they appear PG-dependent [13,15,19]. Garret and Mundy addressed this question using mouse calvaria, and their in vitro findings suggest that, although PGs contribute to the resorptive process by enhancing the IL-1 effect, IL-1 does not require PGs for its resorptive effect[37]. The results of an in vivo study by Boyce et al. indicate that IL-1 stimulates bone turnover systemically, independent of PG production, and that it has profound long-term local effects on bone turn over that are mediated through PG[31]. The current evidence, therefore, suggested that IL-1 has both PG-dependent and PG-independent effects on resorption. About PG-mediation in action of TNF on bone resorption, Tashjian et al. reported that rHuTNF-α and rMurTNF-α stimulate bone resorption in neonatal mouse calvariae via a PG mediated mechanism[24]. But Thomson et al. reported that PG do not mediate the actions of TNF(α and β) on osteoclastic bone resorption[23]. Therefore, it will be also required further detailed study whether PG is involved in mechanism of each cytokine.

Another cytokine, IFN-γ has been showen to inhibit the cytokine induced bone resorption and the present results consist with that. These effects of IFN-γ have been showen for resorption stimulated by a variety of agents, but appear to be most sensitive for resorption stimulated by IL-1 and TNF [14,38-40]. Addition to above report, it have been reported that IFN-γ inhibits prostaglandin synthesis[41] : both IFN-γ and IFN-α inhibit IL-1-stimulated osteoclastic cell formation[42], suggesting possible mechanisms for inhibitory effect of rMurIFN-γ on bone resorbing activities of rHuIL-1β and rHuTNF-α in present study. Besides IFN-γ, IL-4 has been recently showen to be a potent inhibitor of bone resorption stimulated by IL-1 and other bone resorbing factors[43]. Probably, these interactions between

these cytokines each other and with other osteotropic factors may represent the extraordinary complexity of bone metabolism.

V. CONCLUSION

This study was performed to investigate the effects of recombinant IL-1β, TNF-α and IL-6 on bone resorption and modulations of their activities by cyclooxygenase inhibitor and recombinant IFN-γ in neonatal mouse calvariae in organ culture.

The results of this study are as follow :

1. Treatments of calvariae with rHuIL-1β(10^{-12}M to 10^{-9}M) and rHuTNF-α(10^{-10}M to 10^{-8}M) resulted in a dose dependent stimulation of bone resorption. However rHuIL-6(10^{-10}M to 10^{-8}M) was found to have no significant resorptive effect.
2. Cyclooxygenase inhibitor, indomethacin(10^{-6}M) was found to have no significant inhibitory effects on the bone resorbing activities of rHuIL-1β and rHuTNF-α.
3. RMurIFN-γ(1000 U/ml) significantly decreased the bone resorbing activities of rHuIL-1β and rHuTNF-α.

These findings indicate that IL-1β and TNF-α would be involved in alveolar bone loss in the pathogenesis of periodontal disease. But the precise mechanisms of these cytokines in bone resorption and interactions with other osteotropic factors will require further detailed study.

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국문초록

RECOMBINANT INTERLEUKIN-1β, TUMOR NECROSIS FACTOR-α 및 INTERLEUKIN-6의 골흡수 유도 효과에 관한 연구

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치조골흡수는 만성치주질환의 전형적인 증상이다. 골흡수에 작용하는 여러 요인들 중에서도, 특히 최근에 들어서 몇몇 cytokine들에 대한 관심이 높아지고 있는데, interleukin-1(IL-1), tumor necrosis factor(TNF) 및 interleukin-6(IL-6)등이 치주질환의 진행과정에서 중요한 치조골흡수요인으로 제안되고 있다. 본 연구의 목적은 신생쥐의 골조직 배양실험을 통해서 recombinant human interleukin-1β(rHuIL-1β), recombinant human tumor necrosis factor-α(rHuTNF-α)및 recombinant human interleukin-6(rHuIL-6)의 골흡수 유도효과를 알아보고, cyclooxygenase 억제제인 indomethacin과 recombinant murine interferon-γ(rMurIFN-γ)가 이들 cytokine의 골흡수 유도능력에 미치는 영향을 알아봄으로써 이들 cytokine의 작용기구에 대해서 알아보고자 하는데 있다. 생후 1-2일된 쥐에게 1μCi $^{45}CaCl_2$를 피하주사하고 4일 후에 쥐를 희생시켜 ^{45}Ca 로 표지된 두개골을 얻어 24시간 전배양 후, 각 cytokine (rHuIL-1β, rHuTNF-α및 rHuIL-6)과 cytokine 및 첨가약제(indomethacin 및 rMurIFN-γ)가 함유된 배지로 교환하여 48시간 배양한다. 골흡수 유도효과는 두개골에서 48시간의 배양 중 유리되는 ^{45}Ca의 방사능 정도로 평가하였다. 본 연구를 통해 다음과 같은 결과를 얻었다.

1. rHuIL-1β ($10^{-12}-10^{-9}$M) 및 rHuTNF-α ($10^{-10}-10^{-8}$M)는 농도변화에 따르는 골흡수 유도효과를 보였으나, rHuIL-6 ($10^{-10}-10^{-8}$M)는 유의할 만한 효과를 보이지 않았다.
2. Indomethacin (10^{-6}M)은 rHuIL-1β 및 rHuTNF-α의 골흡수 유도작용에 유의할 만한 억제효과를 나타내지 않았다.
3. rMurIFN-γ (1000 U/ml) 은 rHuIL-1β 및 rHuTNF-α의 골흡수 유도작용에 유의한 억제효과를 나타내었다.

본연구를 통해 치주질환 환자의 치주조직에서 검출되는 IL-1β 및 TNF-α가 치조골 흡수에 중요한 역할을 할 것으로 생각된다.

주요어 : 골흡수, 치주질환, interleukin-1β, tumor necrosis factor-α, interleukin-6, cyclooxygenase 억제제, interferon-γ.

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