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RAPID COMMUNICATION

1,25-dihydroxyvitamin D_3 regulates LPS-induced cytokine production and reduces mortality in rats

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Abstract

AIM: To study the immunoregulatory effect of 1,25-dihydroxyvitamin-D_3 Von dominant Th1 response in rats.

METHODS: Sixty adult Lewis rats were randomized into three groups. Rats in group 1 (n=25) were treated with 1,25-$(OH)_2D_3$ first and then challenged with LPS, rats in group 2 (n=25) were treated with vehicle first and then challenged with LPS. Ten animals in groups 1 and 2 were preserved for mortality observation. The remaining animals were injected (i.p) with endotoxin, 24 h after the last administration of 1,25-$(OH)_2D_3$ and vehicle. Rats in group 3 (n=10) were treated with 1,25-$(OH)_2D_3$ only. Serum IL-12, IFN-γ, IL-2 and IL-4 levels were measured and target gene of 1,25-$(OH)_2D_3$ on Th cells was studied after 6 h. Gene abundance was verified by real-time quantitative PCR.

RESULTS: No death occurred in rats pretreated with 1,25-$(OH)_2D_3$ after LPS injection. Death occurred 9 h after LPS injection in rats pretreated with the vehicle, and the number of deaths was 5 within 24 h, with a mortality rate of 50%. There was no change in the number of deaths within 96 h. Six hours after endotoxin stimulation, serum IL-12 and IFN-γ levels decreased significantly in rats pretreated with 1,25-$(OH)_2D_3$ as compared with those in rats pretreated with the vehicle. The serum content of these two cytokines was very low in rats not challenged by endotoxin, and there was a significant difference as compared with the previous two groups.

CONCLUSION: 1,25-$(OH)_2D_3$ attenuates injury induced by the lethal dose of LPS, regulates Th1 and Th2 cells at the transcription level, and dominantly responds to cytokine production in rats.

Key words: Endotoxin; Cytokine; 1,25-dihydroxy-vitamin-D_3; Immunoregulation; Mortality

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INTRODUCTION

1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] is an active form of vitamin D, which not only regulates the dynamic balance of calcium and phosphorus metabolism but also participates in differentiation and regulation of the immune system[1,2]. *In vitro* study[3] showed that both antigen-presenting cells (APCs) and activated lymphocytes express vitamin D receptor (VDR), and that 1,25-$(OH)_2D_3$ acts on APCs (mainly dendritic cells) and helper T cells (Th) through VDR mediation[4], inhibits proliferation and differentiation of Th1 and cytokine production, and induces differentiation of Th2. The status of Th1/Th2 differentiation determines the type of immune response and the final outcome of body response[5]. Cytokine environment is the key factor for initiating Th1/Th2 differentiation[6,7].

Th1 immune response is not only associated with a variety of acute inflammatory responses but also plays a leading role in the development and progression of many autoimmune diseases and transplantation rejection[1,8-12]. Few *in vivo* studies reporting the influence of 1,25-$(OH)_2D_3$ on Th1 immune response are available, and the experimental results about cytokine regulation are conflicting or completely different[5,13-17]. The target gene in Th cells

remains almost unknown[10,17]. *E.coli* endotoxin is a potent bacterial mitogen, able to promote maturity of immature dendritic cells (DC), directly activates T cells and induces Th1 immune response[18]. We established a Th1 dominant response animal model and pre-treated it with 1,25-$(OH)_2D_3$. The results of our study showed that 1,25-$(OH)_2D_3$ was able to regulate the production of IL-12, IFN-γ and IL-4 in dendritic, Th1 and Th2 cells. The effector target point of regulation was at the gene transcription level. It is the regulation of 1,25-$(OH)_2D_3$ on T cell polarization that attenuates injury induced by the lethal dose of LPS in rats and significantly reduces the mortality of rats.

MATERIALS AND METHODS

Animals

Inbreed line Lewis rats (at the age of 3.5-4.5 mo, weighing 242 ± 14 g) were provided by Experimental Animal Center of the Chinese Academy of Medical Sciences (Beijing, China) and fed with normal chow containing 1.6% calcium, 0.9% phosphorus and 0.3% vitamin D (Nanjing Animal Technology Co., Ltd, Nanjing, China) with free access to water. The experiment protocol followed the institutional regulations of the Ministry of Health of the People's Republic of China concerning animal experimentation.

Experiment protocol

Sixty rats were randomized into three groups. Rats in group 1 (n = 25) as the study group, were administered 1,25-$(OH)_2D_3$ by gavage (GmbHcd&Go, Swiss) at 1 μg/animal for 14 d[19], rats in group 2 (n = 25) as the positive control group were administered the same dose of the vehicle for 14 d by gavage. Animals in groups 1 and 2 were injected intraperitoneally with *E.coli* 0111, B4 (Sigma, USA). Rats in group 3 (n =10) as the negative control group were administered 1,25-$(OH)_2D_3$ only by gavage at the dose of 1 μg/animal for 14 d, and injected (i.p) with the same volume of normal saline (Sigma Chemical CO., St Louis, MO, USA).

Ten animals in groups 1 and 2 were preserved for mortality observation. The remaining animals were injected (i.p) with endotoxin (10 mg/kg), 24 h after the last administration of 1,25-$(OH)_2D_3$ and vehicle. Six hours after the injection, they were anesthetized with 50 mg/kg (i.p) pentobarbital (Sigma-Aldrich, USA) and used for drawing 5mL blood from the abdominal major artery. The blood was centrifuged at 4℃ for 15 min, and the serum was stored at -80℃ for test. The spleen was removed aseptically, washed with PBS and stored in liquid nitrogen.

Enzyme-linked immunosorbent assay (ELISA)

Serum IL-12, IL-2, IFN-γ and IL-4 levels were measured with commercially available ELISA kits (Biosource CO., Camarillo, CA, USA) according to the manufacturer's instructions, and the quality control serum values were calculated.

Ca^{2+}/NF-AT signaling pathway gene array

Three spleen tissue samples were chosen randomly from rats in groups 1 and 2 for RNA extraction. UV absorption precipitation method and denaturing gel electrophoresis were used to test the quantity, quality and completion of RNA. The probe was synthesized by RT-PCR. Five μg RNA was used to prepare annealing solution and mixed with RT solution to undergo reverse transcription reaction under the action of reverse transcriptase (M1701, Promega, USA).

Chip hybridization was conducted by using Ca^{2+}/NF-AT signaling pathway gene array chip (Super Array Bioscience CO., Cat.NO.HS-022 USA) and chemiluminescent assay kit (Super Array Bioscience CO., NO.D-01) according to the manufacturer's instructions. The chip was scanned with the ArtixScan 120tf scanner (Micro TEK CO., USA) and the original data were analyzed using the attached software GEArrary analyzer. Each chip had 10 positive controls (2 for GAPDH, 4 for Ppia, 2 for RP113 and 2 for Actinb), three negative controls (PUC18DNA) and 3 blank controls. The original data were deduced by the background minimum value and then corrected by the content of home-keeping gene. The corrected data were analyzed for abundance of gene transcription between the two groups. The ratio $\geqslant 2$ was considered up-regulation of the gene and $\leqslant 0.5$ down-regulation[20].

Verification of IL-2 gene expression by RT-PCR

RNA extraction was done as previously described. The sample was RNA reverse transcripted to synthesized cDNA. The target gene and home-keeping gene of the sample were reacted by RT-PCR. A standard cure was plotted by measurement of the standard sample gradient to calculate the content of gene in the sample, which was corrected by the content of home-keeping gene to obtain the content of the related gene. All reagents used in the experiment were provided by Promega CO., USA. The sequences of β-actin (211 bp) and IL-2 (190 bp) are 5'-CCTGTACGCCAACACAGTGC-3' and 5'-ATACTCC TGCTTGCTGATCC-3', and 5'-CACTGACGCTTGTC CTCCTT-3' and 5'-TTCAATTCTGTGGCCTGCTT-3', respectively.

Statistical analysis

Data were represented as mean ± SD. SSPS 10.0 was used to perform t-test and F-test. $P < 0.05$ was considered statistically significant.

RESULTS

Mortality of rats after LPS injection and protective effect of 1,25-$(OH)_2D_3$

No death occurred in rats pretreated with 1,25-$(OH)_2D_3$ after LPS injection. Death occurred 9 h after LPS injection in rats pretreated with the vehicle, and the number of deaths was 5 within 24 h, with a mortality rate of 50%. There was no change in the number of deaths within 96 h.

Table 1 1,25-$(OH)_2D_3$-regulated LPS-induced cytokine production in rats (pg/mL, mean ± SD)

	1,25-$(OH)_2D_3$ + LPS (n = 15)	Vehicle + LPS (n = 15)	1,25-$(OH)_2D_3$ (n = 10)
IL-12	3986 ± 328[a]	4160 ± 289	69.99 ± 3.99[b]
IFN-γ	4840 ± 802[a]	5264 ± 524	5.42 ± 0.12[b]
IL-4	5.57 ± 1.75[a]	3.72 ± 1.60	

[a]$P < 0.05$ *vs* vehicle + LPS; [b]$P < 0.01$ *vs* 1,25-$(OH)_2D_3$ + LPS and vehicle + LPS.

1,25-$(OH)_2D_3$ inhibited LPS-stimulated production of IL-12 and IFN-γ in rats

Six hours after endotoxin stimulation, serum IL-12 and IFN-γ levels decreased significantly in rats pretreated with 1,25-$(OH)_2D_3$ as compared with those in rats pretreated with the vehicle. The serum level of these two cytokines was very low in rats not challenged by endotoxin, and there was a significant difference as compared with the previous two groups. As the serum IL-2 was below the limit of measurement in most rats 3 and 6 h after LPS attack, measurement was not done.

1,25-$(OH)_2D_3$ promoted IL-4 production in LPS-challenged rats

Six hours after endotoxin stimulation, serum IL-4 level elevated significantly in rats pretreated with 1,25-$(OH)_2D_3$ as compared with that in rats pre-treated with the vehicle. As the serum IL-4 was below the limit of measurement in most rats that are not attacked by LPS, measurement was not done (Table 1).

Quality control of RNA extraction

Electrophoresis showed that RNA extracted from the rat spleen displayed two clear bands (18S and 28S), and the absorbance at 260 nm and 280 nm was between 1.8 and 2.0, indicating that no RNA degradation occurred and the extract outcome was good.

1,25-$(OH)_2D_3$ regulated expression of Th1 and Th2 cytokines and related transcription factors

The gene chip used in the present experiment contains 95 target genes and other positive and negative controls. Expression difference was found in 39 genes between groups 1 and 2, accounting for 41% of the total number of the chip genes. These 39 genes include 10 up-regulated genes and 29 down-regulated genes (Table 2). The chip results showed that 1,25-$(OH)_2D_3$ down-regulated gene expression of Th1 and up-regulated gene expression of Th2, and the gene expression level in related transcription factors (Table 3).

Verification of down-regulation of IL-2 gene expression by RT-PCR

The results of the experiment showed that gene expression level in rats pretreated with 1,25-$(OH)_2D_3$ was significantly lower than that in rats pre-treated with the vehicle (0.476 ± 0.023 *vs* 0.678 ± 0.038, $P < 0.01$).

DISCUSSION

The purpose of the present experiment was to clarify the immune regulatory effect of 1,25-$(OH)_2D_3$ on Th1 dominant response *in vivo*. The results showed that 1,25-$(OH)_2D_3$ inhibited IFN-γ production of IL-12 and Th1 cytokines, suggesting that this inhibitory effect occurs at the transcription level. What implies in the results of the present experiment is the therapeutic effect of 1,25-$(OH)_2D_3$ on diseases mainly characterized by Th1 immune response (including autoimmune diseases) and transplantation rejection[16]. At the same time, as 1,25-$(OH)_2D_3$ affects the secretary profile of Th1 and Th2 cytokines[21,22], it inhibited the acute inflammatory reaction in the rats of group 1, indicating that 1,25-$(OH)_2D_3$ attenuates LPS lethal dose-induced injury in rats. The fact that all rats survived in group 1 suggests that 1,25-$(OH)_2D_3$ may also play a role in inhibiting the development and progression of acute inflammatory reaction.

IL-12 is a cytokine secreted by APCs and plays a central role in the growth of Th1 cells[23]. IL-12 has a potent biological function of inducing T cells to secrete IFN-γ[24]. IFN-γ is a pleiotropic cytokine, promoting inflammatory reaction and inducing expression of main tissue surface compatible complex of multiple cells[25]. Most recent studies found that this cytokine promotes vascular disease of the transplanted organ at the late stage of transplantation[26]. The present experiment confirmed that 1,25-$(OH)_2D_3$ could inhibited IL-12 production in rats, suggesting that it is able to inhibit strong Th1 immune response *via* its action on APCs, thus reducing IFN-γ production. At the same time, 1,25-$(OH)_2D_3$ may also directly inhibit the differentiation and proliferation of Th1 cells, as the cytokines mainly secreted by Th1 cells are reduced, especially transcription of NF-κB is inhibited. NF-κB is a key mediator of gene expression in immune and inflammatory responses. We also found that 1,25-$(OH)_2D_3$ inhibited proliferation of splenic lymphocytes in rats challenged with LPS. We, therefore, think that the results of the above experiment suggest that differentiation and proliferation of 1,25-$(OH)_2D_3$ on Th1 may also have an inhibitory effect on proliferation of splenic lymphocytes and is able to selectively inhibit Th1 immune response.

IL-4 is a main factor influencing the development of T cells into Th2 cells[13,27]. Once IL-4 level is able to resist activation of IL-12 on Th cells and IFN-γ on IL-4, it promotes differentiation of juvenile T cells to Th2 cells[28]. It is controversial over the regulatory effect of 1,25-$(OH)_2D_3$ on IL-4. It was reported that the effect of 1,25-$(OH)_2D_3$ is mediated through IL-4[5], and that it is the up-regulation of IL-4 and TGF-β by 1,25-$(OH)_2D_3$that inhibits the inflammatory reaction rather than by the reduction of Th1 cytokines IFN-γ and TNF-α[29]. It was also reported that 1,25-$(OH)_2D_3$ has no influence on the production of IL-4, or down-regulates IL-4[16,17]. We detected serum IL-4 levels in four batches of rats pretreated with 1,25-$(OH)_2D_3$ and

Table 2 Genes down-regulated by 1,25-$(OH)_2D_3$ in rat spleens

GenBank	Description	Gene name	Gene expression	Abundance	(Exp/vehicle)
NM007595	Calcium/calmodulin-dependent protein kinase Ⅱ, beta	CamK Ⅱ	0.00E + 00	0.00E + 00	0.00E + 00
NM009793	Calcium/calmodulin-dependent protein kinase Ⅳ	CamK Ⅳ	0.00E + 00	0.00E + 00	0.00E + 00
NM009843	Cytotoxic T-lymphocyte-associated protein 4	Cd152	9.20E - 02	0.00E + 00	0.00E + 00
NM031162	CD_3 antigen, zeta polypeptide	CD3Z antigen	5.00E - 02	0.00E + 00	0.00E + 00
NM007726	Cannabinoid receptor 1	cb1	0.00E + 00	3.12E - 02	0.00E + 00
NM009969	Colony stimulating factor	GM-CSF	1.88E - 01	0.00E + 00	1.38E - 01
NM022413	Nuclear factor of activated Epithelial calcium channel 2	Ecac 2	0.00E + 00	0.00E + 00	N/A
NM010118	Early growth response 2	Krox-20	2.99E - 02	0.00E + 00	N/A
NM010184	Fc receptor, IgE, high affinity 1, alpha polypeptide	Fcelα, Fcr-5	1.36E - 01	0.00E + 00	0.00E + 00
NM016863	FK506 binding protein 1b	FKBP 1B/FKBP	0.00E + 00	0.00E + 00	0.00E + 00
NM019827	Glycogen synthase kinase3 beta	Gsk-3	2.31E - 01	4.74E - 02	1.05E + 00
NM008284	Sarcoma virus oncogene 1	H-ras	0.00E + 00	0.00E + 00	1.03E - 02
NM008337	Interferon gamma	IFN-γ	3.44E - 01	1.24E - 02	1.02E + 00
NM008366	Interleukin 2	IL-2	3.98E - 01	2.79E - 01	2.26E - 01
NM008367	Interleukin 2 receptor, alpha chain	CD25	1.99E - 01	3.47E - 01	2.11E - 01
NM010591	Jun oncogene	c-JUN	0.00E + 00	0.00E + 00	0.00E + 00
NM019686	Kinase interacting protein 2	KIP 2	1.84E - 01	0.00E + 00	8.28E - 03
NM007746	Mitogen activated protein kinase 8	Cot	1.50E - 02	0.00E + 00	0.00E + 00
NM011951	Mitogen activated protein Mus musculus Harvey rat	P38MAPK	3.65E - 03	0.00E + 00	1.88E - 02
NM016700	Mitogen activated protein	JNK1	4.21E - 01	4.87E - 01	3.59E - 01
NM008656	Myogenic factor 5	Myf 5	1.83E - 02	0.00E + 00	6.37E - 01
NM016791	Nuclear factor of activated T-cell, cytoplasmic 1	NF-ATc	3.59E - 01	0.00E + 00	1.38E - 01
NM008915	Protein phosphatase 3, catalytic subunit, gamma isoform	Calcineurin A gamma	6.12E - 02	1.06E - 02	3.54E - 01
NM013693	Colony stimulating factor	GM-CSF	1.88E - 01	0.00E + 00	0.00E + 00
NM010188	Fc receptor, IgG, Low affinity Ⅲ	CD16	6.62E - 01	5.25E - 03	1.24E - 01
NM24684	Fos-like antigen 2	fra-2	5.59E - 01	0.00E + 00	0.00E + 00
NM41840	Protein phosphatase 3, regulatory subunit B, alpha isoform	Calcineurin B	2.23E + 00	4.01E - 01	4.73E - 01
NM010177	Tumor necrosis factor (ligand) superfamily, member 6	Fasl	1.02E + 00	0.00E + 00	1.88E - 01
NM019408	Nuclear factor of kappa light polyeptide gene enhancer in B-cell	NF-κB	4.58E - 01	3.50E - 01	4.25E - 01

N/A: Gene expression level, 1,25-$(OH)_2D_3$ = 0 and vehicle ⩾ 2.

Table 3 Genes up-regulated by 1,25-$(OH)_2D_3$ in rat spleens

GenBank	Description	Gene name	Gene expression	Abundance	(Exp/vehicle)
NM010548	Interleukin 10	IL-10	2.30E+00	8.25E-01	1.69E+00
NM010899	Nuclear factor of activated T-cell, cytoplasmic 2	NFAT1 (NFATP)	N/A	N/A	N/A
NM009192	Src-like adaptor	SLA	2.57E + 00	4.21E + 01	8.97E - 01
NM013672	Trans-acting transcription factor 1	Sp1	2.03E + 00	1.49E + 01	4.87E - 01
NM009505	Vascular endothelial growth factor A	VEGF/ VEGI	2.15E + 00	N/A	N/A
NM010234	FBJ osteosarcoma oncogene	c-fos	N/A	N/A	N/A
NM010510	Interferon beta, fibroblast	IFNb-1	N/A	N/A	N/A
NM010583	Mus musculus IL 2-inducible T-cell kinase	Tsk	6.77E - 01	N/A	5.29E + 00
NM021283	Interleukin 4	IL-4	N/A	N/A	N/A
NM010558	Interleukin 5	IL-5	N/A	N/A	N/A

N/A: Gene expression level, 1,25-$(OH)_2D_3$ ⩾ 2 and vehicle = 0.

those pretreated with the vehicle. Although we used inbreed line Lewis rats with little individual variance in establishing the model, we still found a significant individual difference in serum IL-4 level of the same experiment group, where the IL-4 level was lower than the test baseline in some rats. Only when we expanded the sample capacity, were the statistically significant results obtained. The results of gene chip test also showed that there was a great difference in IL-4 expression level between the rats 6 h after LPS stimulation. Only in one of the three rats in the study group, was IL-4 mRNA expression up-regulated by more than two times. However, as the capacity of the samples tested by gene chips was relatively small, and as there was still a tendency to up-regulate the gene expression of IL-4, IL-5 and IL-10 mainly secreted by Th2 cells, we performed another experiment, which confirmed again that 1,25-$(OH)_2D_3$ was able to up-regulate serum IL-10 level in rats challenged with LPS suggesting that 1,25-$(OH)_2D_3$ is able to promote the production of Th2 type cytokines[5,30], and at the same time inhibit the extent and progression of Th1 type immune response, forming the so-called "immune deviation" phenomenon[16], which is believed to help establish peripheral tolerance and is of significance in inhibiting transplantation rejection[4,31].

IL-12 is an allodiploid consisting of two subunits (P_{35} and P_{40}) encoded by two genes independently[32].

It is know that the P_{40} gene initiator region contains a NF-κB combining site[33]. The finding in the present experiment that 1,25-$(OH)_2D_3$ down-regulated the important transcription factor NF-κB, suggests that 1,25-$(OH)_2D_3$ reduces the expression of IL-12P40 subunit by inhibiting NF-κB, thus down-regulating assembly and secretion of IL-12 protein[33]. After activation of T cells, VDR is induced within 6 h, where IL-2 is the first expression-producing gene[34], and 1,25-$(OH)_2D_3$ inhibits the expression of IL-2 and IFN-γ mRNA, reaching the peak in 6-12 h[35]. It has been recognized that NF-κB and NF-ATp/c are specific transcription factors on IL-2 initiators[36,37]. It is also known that IFN-γ, GM-CSF, TNF-α, IL-4 and IL-5 initiators contain NF-AT element[34] and IL-4 enhancer contains 5 independent NF-AT sites, of which NF-ATp is a combining site of high affinity[34,38]. In the present experiment, increased IL-4 secretion by 1,25-$(OH)_2D_3$ might be related to up-regulation of NF-ATp. Although we were unable to identify the respective action of individual members of the NF-AT family on the expression of the cytokines in this study, we may still draw the conclusion that 1,25-$(OH)_2D_3$ influences the activity of NF-κB and NT-AT, two important transcription factors associated with cytokine regulation, by up-regulating NF-ATp gene expression and down-regulating NF-ATc gene expression. Both MAPK P38 and TNK pathways are mitocin-activated protein kinase pathways, not only closely associated with inflammatory reaction but also with cell growth, differentiation and apoptosis. 1,25-$(OH)_2D_3$ down regulates gene transcription of these important regulatory proteins in the MAPK pathways, suggesting that its influence on T help cell differentiation is the result of regulation on multiple signal pathways, and that the effector target of 1,25-$(OH)_2D_3$ regulating cytokines is at the gene transcription level.

COMMENTS

Background

1,25-dihydroxyvitamin D_3(1,25-$(OH)_2D_3$) has many effects on the production of cytokines, the gene expression maps of related cytokines and the mortality of rats. 1,25-$(OH)_2D_3$, the activated form of vitamin D, has, in addition to its central function in calcium and bone metabolism, important effects on the growth and differentiation of many cell types, and pronouced immunoregulatory properties. In the present study, the immunoregulatory effect of 1,25-$(OH)_2D_3$ in dominant Th1 response rats was investigated.

Research frontiers

Th1-biased immune response is associated with acute inflammatory conditions, and also plays a major role in a variety of human autoimmune diseases and graft rejection. However, few studies on the selective immunosuppression *in vivo* of 1,25-$(OH)_2D_3$ are available and the effect of 1,25-$(OH)_2D_3$ on gene expression profiles in Th cells is still unclear.

Innovations and breakthroughs

This is the first study to address the immunoregulatory effects of 1,25-$(OH)_2D_3$ in a dominant Th1 response model. The results show that 1,25-$(OH)_2D_3$ could regulate Th1-derived and Th2-derived cytokine production and protect rats from attacking of the LPS lethal dose.

Applications

The immunoregulatory properties of 1,25-$(OH)_2D_3$ were explored clinically for the topical treatment of psoriasis, a Th1 cell-mediated autoimmune disease of the skin. Our findings suggest that 1,25-$(OH)_2D_3$ may play an important role in Th1-inflammatory, autoimmune diseases and graft transplantation rejection.

Peer review

The manuscript "1,25-$(OH)_2D_3$ regulates LPS-induced cytokine production and reduces mortality in rats" by Qi XP *et al* presents experimental data from rats. The authors claim by pretreating rats with 1,25-$(OH)_2D_3$ that the LPS response is shifted towards a Th2-associated cytokine response with reduced Th1-associated cytokine response, so ensuring increased survival. The topic is of high interest.

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