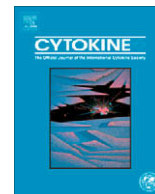




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Analysis of eotaxin 1/CCL11, eotaxin 2/CCL24 and eotaxin 3/CCL26 expression in lesional and non-lesional skin of patients with atopic dermatitis

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ABSTRACT

Eotaxins are the chemokines which are highly selective chemotactic agents for eosinophils. The aim of our study was the evaluation of the gene expression level for eotaxin 1/CCL11, eotaxin 2/CCL24, and eotaxin 3/CCL26, both in skin changes and in uninvolved skin of atopic dermatitis (AD) patients. The study comprised 19 patients with AD and 10 healthy controls. The gene expression level for eotaxins in the skin biopsies was evaluated by the real-time quantitative PCR. The change of the gene expression level, calculated as log10 skin lesions/non-lesional skin, was 0.635 for CCL11, 0.172 for CCL24 and 0.291 for CCL26. The change of the gene expression level, calculated as log10 non-lesional skin of AD patients/healthy control, was 0.394 for CCL11, −0.216 for CCL24, and 0.229 for CCL26, while skin lesions of AD patients/healthy control, was: 0.788, −0.046, and 0.483, respectively. **Conclusion:** The mean gene expression level for CCL11, CCL24, CCL26 was higher in skin changes of AD patients than in uninvolved skin. The higher level of CCL26 in skin changes, indicates its role in their aetiology in AD. The gene expression level for CCL24 in AD patients was lower, both in involved and uninvolved skin vs. the healthy control.

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1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory disease of the skin, characterized by intensive itching and typical for the age localization of skin changes [1]. A considerable amount of controversy prevails in literature reports, regarding the pathogenesis of the disease and the role of numerous immunological processes, as observed in AD-affected patients [2–5].

Inflammatory infiltrations consisting of T lymphocytes, macrophages and eosinophils are present in histological pictures of AD [6,7], while eosinophilic granulations and associated major basic protein predominate in chronic skin changes [8,9]. Peripheral eosinophilia, often identified in AD, is also observed as a medical condition, accompanying, among others, asthma or allergic rhinitis [10]. The recruitment of eosinophils to inflamed tissues is associated with the local production of chemokines, i.e., chemotactic cytokines [11]. Depending on their structure, chemokines are divided into four different families: CXC, CC, CX3C, and C, which control the intensity and duration of immunological processes [12].

Many authors have postulated that CC chemokines play an important role in the induction process of allergic inflammatory reaction. Eotaxins family known as the strongest chemotactic factors for eosinophils, include: eotaxin 1/CCL 11, eotaxin 2/CCL24 and eotaxin 3/CCL26 [11]. CCR3 (chemokine cysteine–cysteine receptor 3), a membranous receptor, present mainly on eosinophils (30,000 receptors per cell) but also found on T lymphocytes, basophils (19,000/cell), macrophages, mastocytes, epithelial cells and cells of airway smooth muscles, is a specific, highly selective receptor for eotaxins [13,14].

The production of eotaxins is stimulated by interleukin 4 (IL-4), secreted by fibroblasts, and by inflowing Th2 lymphocytes [15,16]. The mechanism of eotaxin gene expression is associated with the activity of transcription factors, such as STAT6 and NFκB [17–19]. Eotaxins are responsible for taxis of eosinophils from peripheral blood to tissues [20–22]. Eotaxins and IL-5 induce accumulation of eosinophils in the skin [20], while the cytotoxic proteins, secreted from their granules, enhance inflammatory reaction in AD [8,9,23–25]. The expression of eotaxin mRNA has been shown to affect endothelial cells, lung epithelial cells, macrophages, corneal keratocytes and dermal fibroblasts [26]. It has also been demonstrated that levels of some eotaxins positively correlate with the disease severity of AD patients [27,28].

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The unveiling of the mechanisms, which induce eosinophil accumulation in tissue, may be of the key importance for the understanding of lesion development processes in AD, therefore, the goal of the study was to evaluate the gene expression levels for eotaxin 1/CCL11, eotaxin 2/CCL22 and eotaxin 3/CCL26, both in skin changes and in uninvolved skin of adult patients with AD.

2. Materials and methods

The study included 19 adult patients with atopic dermatitis (10 women and 9 men), their age varying from 20 to 38 (the mean age: 26.6) and 10 volunteers in good health as a control panel. The diagnosis was obtained by meeting Hanifin and Rajka's criteria [6]. The range of severity scoring of atopic dermatitis patients (SCORAD index) was between 36.0 and 74.5 (average at 49.78). The patients had no allergic diseases, other than atopic asthma, allergic rhinitis or allergic conjunctivitis. The patients did not receive any drugs which – by the mode and schedule of their administration – could affect the results of performed studies. The study protocol was approved by the Bioethical Commission of the Military Institute of the Health Services.

Skin specimens were collected by a 4 mm disposable biopsy punch (Miltex Inc., York, USA), following local anaesthesia with ethyl chloride (Aethylum chloratum, FILOFARM, Bydgoszcz, Poland). In patients with AD, biopsy specimens were collected from skin lesions as well as from non-lesional skin areas. One biopsy was also collected from health volunteers of the control group. The collected specimens were immediately immersed in RNAlater solution.

2.1. RNA isolation and cDNA synthesis

The specimens were placed in RNAlater solution (Qiagen) and stored at -70°C until use. Total RNA was extracted, using an RNeasy Lipid Tissue Mini Kit (Qiagen). DNA contamination was removed with a RNase-free DNase Set (Qiagen), according to the manufacturer's indications. RNA concentration was determined by measuring absorbancy at 260 nm and calculating optical density. The amount of 500 ng RNA of each sample was then used to perform the first-strand cDNA synthesis, using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen).

2.2. Real-time quantitative PCR

Real-time quantitative PCR evaluation was performed with an ABI-Prism 7500 Sequence Detector System (Applied Biosystems). For real-time PCR, 2 μl of cDNA was amplified in a 50 μl PCR volume, containing a Power SYBR green PCR master mix (Applied Biosystems, USA) with 150 nM of specific primers. Each sample was measured in duplicate. The sequences of primers were as follows: Eotaxin 1: forward, 5'-CTCGCTGGGCCAGTTCTGTC-3'; reverse, 5'-GGCTTTGGAGTTGGAGATTTTGG-3' (227 bp). Eotaxin 2: forward, 5'-CACATCATCCCTACGGGCTCT-3'; reverse, 5'-GGTGGCAGGATATCTCTGGACAGG-3' (88 bp). Eotaxin 3: forward, 5'-GGAAGTCCACACGTGGGAGTGAC-3'; reverse, 5'-CTCTGGGAGGAAACACCCTCTCC-3' (354 bp). GAPDH: forward, 5'-GAAGGTGAAGGTCCGAGTC-3'; reverse, 5'-GAAGATGGTGATGGGATTTC-3' (903 bp).

For cDNA amplification, a 10-min incubation at 95°C was performed to activate AmpliTaqGold DNA polymerase; followed by 45 cycles, each of 15 s and at 95°C and 1 min at 60°C . Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was applied for each sample as an internal control in order to normalize eotaxin 1, eotaxin 2, and eotaxin 3 expression levels. The results were expressed as relative quantification units (fold change).

Relative quantification values were calculated by the $2^{-\Delta\Delta\text{CT}}$ method. The cycle threshold (CT) for the target amplicon and CT for endogenous control (GAPDH) were determined for each sample. Differences were calculated between these two CTs (for target amplicon and for the internal control) and called ΔCT , in order to account for the difference in the amount of total nucleic acid added to each reaction. The values of ΔCT for healthy skin (calibrator) was subtracted from the ΔCT of each sample and termed $\Delta\Delta\text{CT}$. The target normalized to endogenous control ratio, relative to the calibrator, was then calculated by the formula $2^{-\Delta\Delta\text{CT}}$. Two different calculations were made, where in the first one, CT of atopic skin was calibrated with CT of healthy skin from that same patient, while in the other, CT of the skin specimens from patients with atopy was calibrated with the average CT of skin specimens from healthy subjects.

2.3. Statistical analysis

Data were analysed, using the Mann–Whitney *U*-test. *P*-value <0.05 was considered to be statistically significant.

3. Results

It was demonstrated in the studied group of 19 patients with AD that the change of gene expression level for the investigated eotaxins was higher in lesional skin vs. non-lesional skin, especially for eotaxin 1/CCL11 and eotaxin 3/CCL26. The analysis addressed skin biopsates from eczematous locations, calibrated with the biopate of non-lesional skin from patients with AD. The mean change of gene expression level vs. control, calculated as $\log_{10}$ skin lesions/non-lesional skin in patient with AD was 0.635 (95%CI: 0.275–1.041) for eotaxin 1/CCL11, 0.172 (95%CI: 0.007–0.336) for eotaxin 2/CCL24, and 0.291 (95%CI: 0.082–0.449) for eotaxin 3/CCL26. Fig. 1 presents expression level values for particular patients.

Further analysis comprised the change of gene expression level for eotaxins between the patients with AD and the control group of health subjects. The analysis concerned the biopsates of involved and uninvolved skin from the patients with AD, calibrated with a skin biopate from the subjects of healthy control.

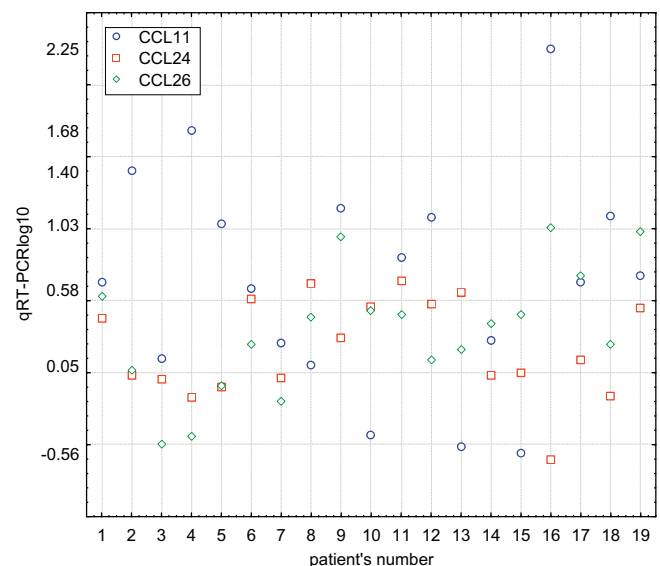


Fig. 1. The change of gene expression level of the investigated eotaxins of particular AD patients, calculated as $\log_{10}$ skin lesions/non-lesional skin.

The mean change of gene expression level vs. control, calculated as $\log_{10}$ non-lesional skin of AD patients/healthy control was 0.394 (95%CI: 0.074–0.714) for eotaxin 1/CCL11, -0.216 (95%CI: -0.415 to -0.017) for eotaxin 2/CCL24 and 0.229 (95%CI: 0.049–0.410) for eotaxin 3/CCL26. The change of gene expression level for eotaxin 1/CCL11 and eotaxin 3/CCL26 in non-lesional skin of the patients with AD was higher, while that for eotaxin 2/CCL24 was lower in comparison with healthy control. Fig. 2 presents the results of particular patients.

The mean change of gene expression level vs. control, calculated as $\log_{10}$ skin lesions of AD patients/healthy control was 0.788 (95%CI: 0.457–1.119) for eotaxin 1/CCL11, -0.046 (95%CI: -0.248 to 0.155) for eotaxin 2/CCL24 and 0.483 (95%CI: 0.321–0.645) for eotaxin 3/CCL26. The gene expression level for eotaxin 1/CCL11 and 3/CCL26 in skin changes of the patients with AD was higher, while that for eotaxin 2/CCL24 was lower in comparison to the figures obtained in the subjects of healthy control. Fig. 3 presents the results of particular patients.

A comparative analysis of the mean changes of gene expression level for eotaxins 1, 2, 3, when referred to the control, calculated as $\log_{10}$ skin lesions of patients with AD/healthy control and non-lesional skin of patients with AD/healthy control, demonstrated higher gene expression levels in skin changes. In case of eotaxin 3/CCL26, that difference was statistically significant ($P = 0.04$) (Fig. 4).

4. Discussion

Eosinophils play an important role in the pathogenesis of allergic diseases [11,29]. The clarification of factors responsible for eosinophil recruitment in tissues may be of key importance for unveiling the mechanisms which control the development of all allergic diseases, including AD. This relationship was, in particular, confirmed in studies on atopic asthma [30–32]. In this disease, the recruitment of eosinophils to the inflammation site is a both multifactor and multistage process. Eosinophils enhance inflammatory reaction by degranulation and release of cytotoxic proteins, contained in their granules [33,34]. The levels of eosinophil granule proteins (e.g., major basic protein – MBP) often positively correlate with disease intensity [22]. There are cytokines which are responsible for the recruitment of eosinophils to inflammatory sites

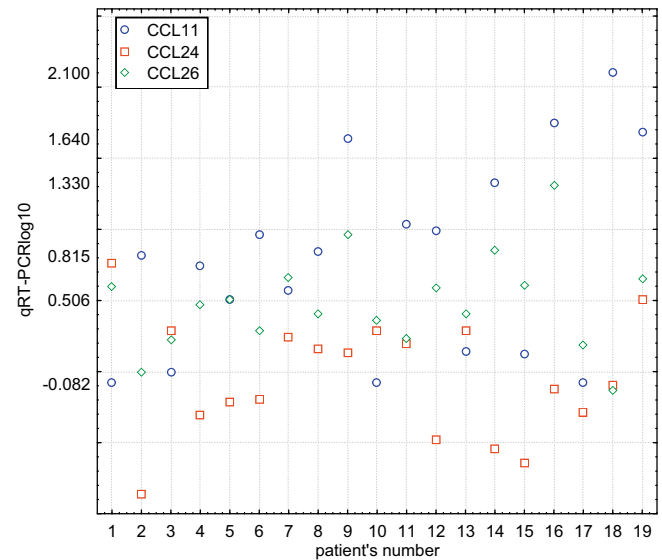


Fig. 3. The change of gene expression level of the investigated eotaxins of particular AD patients, calculated as $\log_{10}$ skin lesions of AD patients/healthy control.

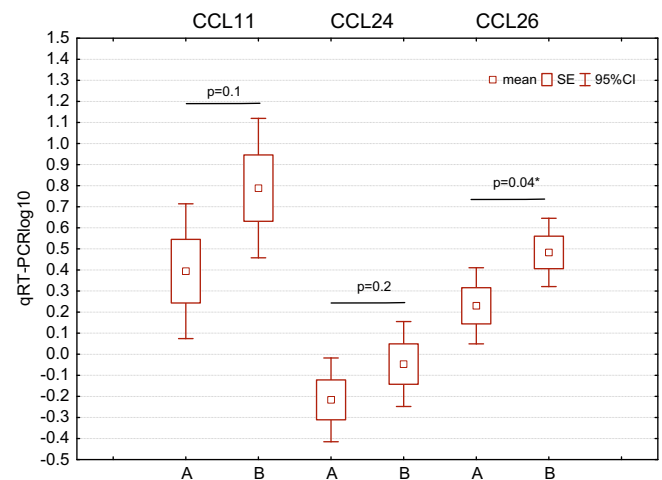


Fig. 4. A comparative analysis of the mean changes of gene expression level for investigated eotaxins when referred to the control, calculated as $\log_{10}$ non-lesional skin of patients with AD/healthy control (A) and lesional skin of patients with AD/healthy control (B). *P-value statistically significant.

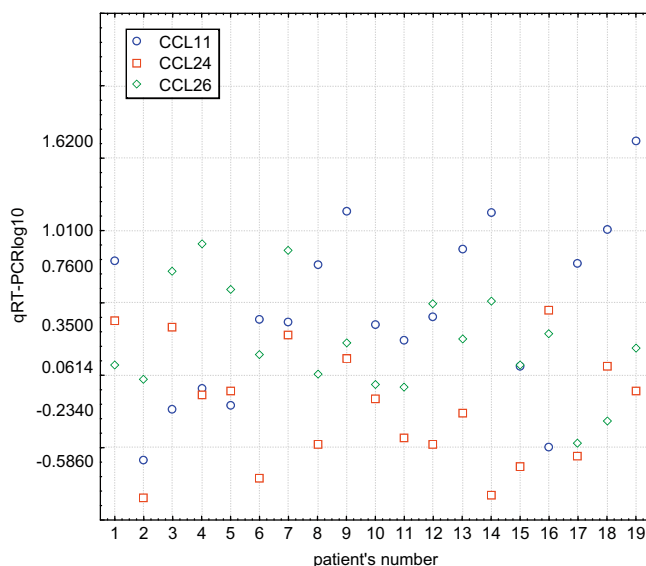


Fig. 2. The change of gene expression level of the investigated eotaxins of particular AD patients, calculated as $\log_{10}$ non-lesional skin of AD patients/healthy control.

(especially Th2 cell products IL-4, IL-5, IL-13), chemokines (e.g., RANTES and eotaxins) adhesion molecules (e.g., Beta₁, Beta₂, and Beta₇ integrins), and other molecules (e.g., acidic mammalian chitinase) [33–36]. However, only eotaxins and IL-5 have selective influence on eosinophils. CCR3 (chemokine cysteine–cysteine receptor 3), presented on eosinophils, is the specific receptor of eotaxins [20]. Following many reports, the ‘eotaxin–CCR3 receptor’ path is very important for the eosinophil accumulation and thus for enhancement of inflammatory reaction. Kinetic of eotaxins expression during an allergic reaction is different for each of them. It is suggested that increasing concentrations of eotaxin 2/CCL24 and eotaxin 3/CCL26 in patients with atopic asthma is important for the eosinophil influx in late asthmatic response (LAR) [31]. It was demonstrated that only the expression of eotaxin 3/CCL26 on the 24th hour after allergenic provocation differentiated patients with asthma from healthy subjects; a 100-fold increase of eotaxin 3 mRNA is observed in biopsies from lungs of asthmatic patients [32]. It was also demonstrated on an animal model that

eotaxin 2/CCL24 participated in the accumulation of eosinophils into the bronchoalveolar lavage fluid (BALF) [33]. It was also shown that eotaxin 2/CCL24 is locally secreted by lung epithelial cells due to the stimulation by proinflammatory cytokines produced by lymphocytes Th2 [37]. Despite many research programmes, trying to evaluate the role of eotaxins in atopic asthma, there has still been no unequivocal information, regarding the exact role of each of them in the pathogenesis of this disease.

Even less clear is the role of eotaxins in the pathogenesis of AD. It is assumed that eosinophils play an important role in the development of eczematous changes in patients with AD [9]. They migrate in the development phase from blood to the skin, releasing cytotoxic mediators, such as eosinophil cationic protein and protein-eosinophil X [8,38,39]. High levels of these proteins have been demonstrated in serum and urine of children with AD, while their levels positively correlated with the disease intensity [40]. The results of studies from the recent years indicate some correlation between serum levels of eotaxin 1/CCL11 with the activity of AD [28]. The presence of eotaxin 1/CCL11, as well as the expression of CCR3, are higher in case of skin changes, their further increase being observed in AD exacerbations [41]; such a relationship has also been demonstrated in the course of acute urticaria and angioedema disseminations [26,42].

The majority of performed studies have concentrated on the role of eotaxin 1/CCL11 in the development of AD, while less familiar are the relationships of eotaxin 2/CCL24 and eotaxin 3/CCL26. Following certain views, eotaxin 1/CCL11 is a constitutive eotaxin, while eotaxin 3/CCL26 is produced after allergenic stimulation – consequently, it is found in allergic and asthmatic patients. Eotaxin 1/CCL11 is present both in allergic and healthy subjects. The levels of protein eotaxin 1/CCL11 in asthmatic patients during remission of the disease are similar to those in healthy persons, not correlating with the maintained eosinophilia of the respiratory tract [43,44]. In *in vitro* experiments, eotaxin 2/CCL24 is secreted after 24 h from allergy initiation, and maintained for 96 h. This production depends on stimulation by Th2 cytokines. The concentration of eotaxin 2/CCL24 is considerably elevated in patients who suffer from asthma, however, it does not rise after allergen provocation [31]. Eotaxin 3/CCL26 seems to play the most important role in an allergic process. It has been confirmed that this cytokine is present only in allergic persons and in those suffering from asthma. Its expression begins to increase after 24 h from allergic reaction and is then continuously maintained at high level values [32].

There have been only few studies which measure cytokine concentrations and assess the dynamics of their occurrence in biopsates. The majority of studies are based on the concentrations of chemokines in peripheral blood, what does not provide a complete picture of local immunological reactions in the skin.

It has been demonstrated that serum levels of eotaxin 3/CCL26 are higher in patients with AD vs. those in healthy subjects, as well as in those affected by common psoriasis; moreover, the concentration of eotaxin 3/CCL26 in serum positively correlates with the intensity of the disease, while no such correlations have been observed for eotaxin 2/CCL 24 [11]. Some *in vitro* studies have shown eotaxin 3/CCL26 to be produced and more intensively expressed in the vascular endothelium together with interleukin 4 (IL-4) [45], while no such relationship has been found for eotaxin 2/CCL24 [11]. It seems that similar correlations between eotaxin 2/CCL24 and eotaxin 3/CCL26 were confirmed in our *in vivo* examinations.

In our study with the group of 19 patients with AD, the change of gene expression level for eotaxins was evaluated in two study systems. It was found in both study systems that, regarding the patients with AD, the mean gene expression level for all the evaluated eotaxins was higher in cases of skin changes than in those with uninvolved skin. It was demonstrated that higher expression levels were associated with eotaxin 1/CCL11 and eotaxin 3/CCL26 (Figs.

1–3). That observation was made both in the analysis of biopsates from skin changes, calibrated with a biopate of unchanged skin from the same patient or with the mean expression level of appropriate toxins in the skin of healthy volunteers, providing healthy control. In the case of eotaxin 3/CCL26, it was identified that the difference between the mean level of gene expression in skin changes and in uninvolved skin of patients with AD, calibrated with healthy control, was statistically significant (Fig. 4). Having analysed the change of gene expression level for the studied eotaxins in patients with AD, a lower expression level of medium level of eotaxin 2/CCL24 was demonstrated vs. the mean level in healthy volunteers, observed either in skin changes or in uninvolved skin (Figs. 2 and 3).

The above observations confirm the role of eotaxins, in particular eotaxin 3/CCL26 and eotaxin 1/CCL11 in the development of skin changes in patients with AD. The findings also indicate a considerably less important role of eotaxin 2/CCL24 in enhancing the course of AD. Following Watanabe et al., eotaxin 2/CCL24 is continuously produced by the blood circulating monocytes and its expression is stimulated by the proinflammatory factors such as IL-1beta or lipopolysaccharide (LPS). It was also presented, that monocytes lose this activity after their transformation into tissue macrophages. Furthermore, human dermal fibroblasts, even after IL-4, IL-13, TNFalpha or LPS stimulation, despite production of eotaxin 1/CCL11, do not produce eotaxin 2/CCL24 [46]. These observations might be the reason of the lower level of the expression of eotaxin 2/CCL24 genes in the skin lesions and the healthy skin of the atopic dermatitis patients in comparison to the healthy control of our study. An evaluation of cytokine expression level during inflammatory reaction may be extremely interesting and promising from the point of view of cytokine role interpretation in the development of skin changes in patients with AD; in fact, it will be included in the subsequent stage of the studies. The explanation of the mechanisms which control inflammatory reactions and the accumulation process of eosinophils may be of key importance for the understanding of AD pathogenesis. It may also turn out helpful in designing new protocols of AD therapy, taking into account a possibility of CCR3 receptor blocking, as prompted by the results of studies performed in patients with asthma.

5. Conclusion

It was demonstrated in our study, performed in various research systems, that the mean gene expression levels for eotaxin 1/CCL11, eotaxin 2/CCL24 and eotaxin 3/CCL26 were higher in skin changes of the patients with AD. The statistically significantly higher level of eotaxin 3/CCL26, demonstrated in skin changes, may indicate its important role in the development of eczematous changes, observed in patients with AD. Moreover, the studies showed that the mean gene expression level for eotaxin 2/CCL24 was lower, either in non-lesional skin or in skin lesions of the patients with AD, than in healthy subjects of the control group. That observation may suggest that destruction of the relations among particular eotaxins may also be significant in the pathogenesis of AD.

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