Plasticity of IL-2 and IL-2 receptor chains in rat lymphoid tissues in situ after stimulation with staphylococcal enterotoxin A

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Abstract

Although the effects of mitogens on the synthesis of interleukin-2 (IL-2) and IL-2 receptor (IL-2r) have been described, a detailed in situ analysis of the spatio-temporal changes of the expression of the IL-2 gene and the three IL-2r components in lymphoid tissues is still missing. Therefore, we analyzed the IL-2 and IL-2r expression after a staphylococcal enterotoxin A (SEA)-induced T cell activation on a cellular and anatomical basis in the Wistar rat. SEA caused a rapid induction of IL-2 mRNA in T cells of spleen, lymph node, and thymus, followed by the appearance of high systemic IL-2 serum levels (5 ng/ml), and a significant increase of CD25 on CD4+ and CD8+ lymphocytes. The histotopographic analysis of the IL-2r chains revealed a strong upregulation of IL-2r alpha (α) and IL-2r beta (β) mRNAs in similar T cell specific compartments of spleen, lymph node, and thymus as seen for IL-2 mRNA. The abundant constitutive expression of IL-2r gamma (γ) mRNA was unaffected by SEA. The parallel upregulation of IL-2, IL-2rα, and β chains in conjunction with the continuous presence of the IL-2rγ chain predominantly in T cell regions of immune organs suggests that the biological effects of IL-2 are essentially limited to T cells, at least after superantigen stimulation.

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Abbreviations: IL-2r, IL-2 receptor; PALS, periarteriolar lymphoid sheet; SEA, staphylococcal enterotoxin A

1. Introduction

The immune response to infectious agents or pathogens is accompanied by activation of different cells. This process is coordinated by numerous soluble factors (e.g. cytokines), which modulate the functional activities of individual cells and tissues (for an overview see Ref. [1]). Cytokines are important positive or negative regulators of an appropriate immune response. The cytokine interleukin-2 (IL-2) exhibits numerous key functions in the generation of a target-directed immune response. IL-2 is known to act as an autocrine and paracrine mediator, which is predominantly secreted by activated CD4+ T helper cells [2], and promotes proliferation, growth, and differentiation of respective effector functions in T- and B-lymphocytes [3–5]. Besides its autocrine function on T cells [6], IL-2 exhibits biological activities relating to B cells [7], lymphokine-activated killer cells [2], and it facilitates the differentiation of cytotoxic responses of monocytes [8,9] and NK cells [10]. In addition to the growth-promoting effects of IL-2, the IL-2/IL-2 receptor (IL-2r) system may have an essential role in the down-regulation of T cell activation. Mice deficient in either IL-2 [11] or the IL-2rβ chain [12] have hyperactive T cells
that appear to drive a generalized and severe autoimmune pathology affecting the gut [11]. A genetic defect in the IL-2rγ chain leads to an X-linked SCID [13]. Target deletion of the IL-2rγ chain severely affects B and T cell generation and function and NK cell development [14].

The effects of IL-2 are mediated by an IL-2 specific receptor. On the molecular level three different IL-2r chains, α (CD25), β (CD122), and γ (CD132) have been described, which can be expressed individually or together in different combinations forming dimeric or trimeric receptor complexes with varying affinities for IL-2. The IL-2rβ and IL-2rγ chains are constitutively expressed on most mature lymphocyte populations [15–17] forming an IL-2rβγ dimeric low-affinity receptor [18,19]. Binding of IL-2 to its functional IL-2rαβγ trimeric high-affinity receptor promotes antigen-induced expansion of T cells, whereas a block in IL-2 transcription or secretion or an insufficiency in IL-2 production during activation may be responsible for T cell unresponsiveness or anergy [20,21].

Numerous cell types have been reported to express IL-2 in vitro, e.g. stimulated eosinophils [22], activated murine B-lymphoma line A20-HLB [23], or virus transformed B cells [24]. Furthermore, dendritic cells have been suggested to be able to produce IL-2 in response to Gram-negative bacteria in vitro [25] and it is speculated that IL-2 might be an important co-stimulatory molecule by which dendritic cells influence T cell priming and activation of the adaptive immune system [25].

Despite this plethora of information about the cellular source of IL-2 and its effect on specific cell types, surprisingly little is known about the cellular expression and plasticity of the IL-2 system in lymphoid organs in situ. Often, the effect of IL-2 on a defined cell type by itself represents the basis to define this cell type as an IL-2r positive cell but little is known about actual IL-2r expression during immune responses in vivo in situ.

The aim of our study was to characterize the spatiotemporal patterns of the response of IL-2 and IL-2rα, β, and γ expressing cells in lymphoid organs to a strong T cell activation in vivo. We addressed the question whether the activation of T cells in vivo induces the expression of IL-2 and IL-2r chains exclusively in T cell compartments of lymphoid organs or whether the expression of the IL-2/IL-2r system is restricted to or regulated beyond T cell populations. We analyzed the in situ expression and regulation of IL-2 and IL-2r chains α, β, and γ in lymphoid organs of Wistar rats in response to staphylococcal enterotoxin A (SEA), known as a superantigen with potent T cell stimulatory properties [26–28]. Here we demonstrate that during an enterotoxin-induced immune response in vivo, IL-2 and IL-2rα and β are strongly induced in T cells, but that there is no evidence for an IL-2/IL-2r expression beyond the T cell population.

2. Results

To define an efficient T cell stimulus in rats, measurement of IL-2 serum concentrations was used. Intraperitoneal (i.p.) injection of 100 μg SEA into an adult Wistar rat induced IL-2 in rat serum (Fig. 1A) whereas in the control animals, no IL-2 serum levels were detectable. Maximal IL-2 serum concentrations (about 12 ng/ml) were measured 3 h after stimulation. Twenty-four hour post-injection concentrations of IL-2 were still measurable, though at lower levels. The appearance of IL-2 in the serum was paralleled by a strong induction of IL-2 mRNA expression in the spleen and lymph node, and a moderate induction of IL-2 mRNA in the thymus (Fig. 1B and C). IL-2 mRNA was not present in lymphoid tissue of sham-treated control rats, which received a saline injection. In contrast abundant amounts of IL-2 mRNA were seen in spleen and lymph nodes of stimulated rats 3 h after SEA-challenge (Fig. 1B). In the thymus of stimulated animals only weak signals for IL-2 were found. Minor amounts of IL-2 mRNA were present up to 8 h post-injection in the spleen and lymph node, whereas in the thymus no IL-2 mRNA was present at this time point. Semi-quantitative Northern blot analysis revealed a significant increase in the amount of IL-2 mRNA in the spleen and lymph node (Fig. 1C). Thus, spleen and lymph nodes are the main organic sources of SEA-induced systemic IL-2 in serum.

The IL-2r chains were upregulated in the spleen and lymph node in parallel to the IL-2 expression (Fig. 2). Northern blot analysis of IL-2rα and β chain mRNA revealed a significant induction of IL-2rα (Fig. 2B) and a significant enhancement of IL-2rβ chain mRNA (Fig. 2C) at 3 h after stimulation in the spleen, lymph node, and, to a lower extent, at 8 h in the thymus. No significant increase of IL-2rα mRNA levels in the thymus was measured (Fig. 2B). In all organs examined, the expression of IL-2rα and β chain mRNAs remained high within the observed time schedule of 24 h. While IL-2rα mRNA decreased after 3 h post-injection in the spleen, IL-2rα mRNA in the lymph node remained high up to 24 h. Thus, SEA induced the transcription of the IL-2rα and β chain mRNAs in all peripheral lymphoid organs of the rat.

To unravel the cellular source of the induced or enhanced IL-2/IL-2r, we determined the distribution pattern of IL-2 and IL-2r mRNAs in the lymphoid organs by performing in situ hybridization (Figs. 3–5). A basal expression of IL-2rα, IL-2rβ, and IL-2rγ was found in all lymphoid organs, though at different levels. In vivo stimulation with SEA resulted in an induction of IL-2 mRNA and a dramatic upregulation of IL-2rα and β mRNA in all organs (Fig. 3). The increased levels of IL-2, IL-2rα, and β mRNA remained high up to 24 h in all lymphoid organs (Fig. 3). The already high
expression levels of IL-2γ were unaffected. In spleen IL-2, IL-2α, IL-2β, and IL-2γ mRNAs were mainly localized in the periarteriolar lymphoid sheet (PALS) (Fig. 4A–H), a region containing T cells at a high density as shown by the presence of CD4 and CD8 mRNA expressing cells (Fig. 4I and K). In addition, IL-2γ mRNA was present in numerous cells of the red pulp and at the inner surface of the central artery (Fig. 4G insert). In the lymph node (Fig. 5), IL-2, IL-2α, and IL-2β mRNA were expressed in cells mainly located in
the paracortex, a region known to contain T cells at high density, but additionally some cells positive for IL-2rβ were found in the lymphoid follicle and in the medulla. The IL-2rγ mRNA was constitutively expressed in virtually all cells of the lymph node. In the thymus all three IL-2r mRNAs were expressed in the medulla, a region typically characterized by the presence of mature T cells (Fig. 5). The already high basal expression of the IL-2rγ mRNA was not altered by SEA injection as seen by in situ hybridization.
Fig. 4. High power microscopy of emulsion coated tissue section of a rat spleen 3 h after SEA-challenge. The distribution of IL-2 mRNA (A and E), IL-2Rα mRNA (B and F), IL-2Rβ mRNA (C and G), and IL-2Rγ mRNA (D and H) was analyzed in serial section by dark field (upper lane) and bright field (lower lane) microscopy. Detail in (H) shows the expression of IL-2Rγ mRNA in the central artery at higher magnification. CD4 mRNA (I) and CD8 mRNA (K) expressing cells in the spleen were localized in emulsion coated tissue section (upper part of (I) and (K)) and in digitized autoradiograms of the corresponding spleen tissue section from which the high power parts were taken (lower part of (I) and (K)). Abbreviations: PALS, periarteriolar lymphoid sheet; RP, red pulp. Asterisk marks the central artery.
In order to show that SEA induced the IL-2r expression in numerous T cells we performed FACS analysis of the IL-2r specific alpha chain (CD25) on CD4+ or CD8+ T cells. We revealed a significantly enhanced numbers of CD25+CD4+ and CD25+CD8+ T cells in the spleen at 3 and 48 h and in lymph node at 48 h post-injection (Fig. 6). The number of both CD4+ and CD8+ T cells expressing the IL-2r chain was reduced to control levels at 10 days in spleen and lymph node. In the thymus no significant changes of CD25+CD4+ and CD25+CD8+ T cells could be measured (Fig. 6C and F). This showed that SEA promotes the expression of IL-2r on CD4+ as well as CD8+ T cells and might lead to an IL-2 dependent polyclonal expansion of T cells.

3. Discussion

In spite of ample evidence for biological effects of IL-2 on various cells of the immune system, the aim of our study was to bring more insight into the course of the IL-2 and IL-2r expression after T cell activation on a cellular and anatomical basis in vivo. Most data that demonstrate IL-2 expression are derived from in vitro studies and are often based only on the molecular or biochemical examination of in vitro stimulation of various defined cell lines or cells that have been cultured for some time. We here demonstrate that the T cell mitogen SEA enhanced IL-2 serum concentration comparable to that described for SEB in mice and therefore can be used as a capable stimulator for analyzing the molecular and cellular regulation of the IL-2 system in rats. Serum concentrations were one-tenth lower than in SEB stimulated mice[29,30] but still within a physiological range to stimulate the trimeric high-affinity IL-2r[31].

Our histotopographic analysis of the expression patterns of IL-2 and IL-2α, β, and γ chains in the spleen, lymph node, and thymus in the course of an enterotoxemia revealed that SEA induces IL-2 mRNA...
ubiquitously in activated T cells of the spleen, lymph node, and thymus. This is in accordance with in vitro studies in that the production of IL-2 production mainly by lymphocytes is a consequence of activation by mitogens or allogens, whereas resting cells do not produce IL-2 [2]. There was no evidence that the bacterial superantigen induced the expression of IL-2 in cell types other than T cells. The absence of IL-2 in B cell follicles of the spleen and lymph node makes it unlikely that B cells or follicular dendritic cells express IL-2 in response to superantigens, which could not have been expected. We obtained no evidence for an induction of IL-2 by SEA in dendritic cells of lymphoid organs. This is in contrast to effects of Gram-negative bacteria [25], which have been claimed to cause IL-2 production by dendritic cells in vitro. Gram-negative bacteria might stimulate dendritic cells by LPS via a soluble CD14-dependent pathway [32] whereas the exogenous binding of superantigens to MHC class II seems ineffective to stimulate dendritic cells for IL-2 production.

Prior to this report little was known about the IL-2r expression in vivo and most of the information was derived from isolated lymphocytes by FACS analysis. Our FACS analysis revealed a transiently enhanced number of CD4+ and CD8+ T cells from spleen and lymph node expressing the IL-2r (CD25). The observed time course of the clonal expansion of T cells around day 2 is similar to that found for superantigen-stimulated splenic T cells in mice [33]. Because of the lack of Vβ-specific antibodies against the SEA sensitive Vβ T cell isotypes 11, 12, 14, 17, and 19 in rats [34], the increase in CD25+ T cells was only determined as an increase in the total T cell population. Therefore, the significant enhancement of the IL-2r on specific Vβ T cell populations may be more apparent when measured for single Vβ T cell populations. The increase in CD25+ T cells was at least in part due to SEA-induced T cell proliferation. Proliferation analysis in vitro showed that superantigenes, e.g. SEA, SEB, or TSST led to a strong proliferation response of rat spleen cells [35].

The effect of superantigens on T cell activation was recently monitored by expression analysis using the gene array technique [36]. T cell activation resulted in changes of a wide spectrum of genes including a transient expression of IL-2α and β chain mRNAs in T cells isolated from lymph node within 8–48 h [36]. The SEA-induced increase in IL-2rα and β mRNA measured by Northern blot and the pronounced enhancement of IL-2rα and β mRNA in T cell areas of the lymph node revealed by in situ hybridization are in concordance with these findings. The observed induction of IL-2rα is in line with observations that IL-2rα is not expressed by resting lymphocytes but is potentially induced after

**Fig. 6.** Expression of CD25 (IL-2rα) on CD4+ T-cells (A–C) and CD8+ T-cells (D–F) was measured by double-labeling FACS analysis. The ratios between CD25+CD4+ double positive and CD4+ single positive T-cells or between CD25+CD8+ double positive T-cells and CD4+ single positive T-cells were calculated for T-cells isolated from the spleen, lymph node, or thymus. Number of animals per group n = 3. Statistical significance: *p < 0.05; **p < 0.05.
antigen encounter or mitogen stimulation [37,38]. Functionally, the IL-2rα seems to be essential for regulation of both the size and content of the peripheral lymphoid compartment [39]. Mice deficient in the IL-2rα chain exhibit polyclonal T and B cell expansion [39] and develop severe immune dysregulation and consequent autoimmunity. The IL-2rβ is constitutively expressed in a variety of cells and is enhanced after stimulation in vitro [15]. We here also observed low basal expression of IL-2rβ in lymphoid organs and a dramatic upregulation of this receptor chain after mitogen injection. In vivo the IL-2rβ seems to function as an immune regulator because IL-2rβ-deficient mice develop spontaneously activated T cells, which results in high levels of autoantibodies and plasma cell accumulation [12]. The strong constitutive expression of the IL-2rγ chain mRNA in T cell areas observed in this study fulfils the requirements of the presence of all three receptor chains to express the functional IL-2r in mitogen-stimulated lymphoid organs. In this context it is interesting to note that the IL-2rγ chain is constitutively expressed as an immature form but only activation of T cells—at least CD4+—leads to the presence of the functional form of the IL-2rγ chain on the surface of T cells [40]. The enhanced expression of the IL-2rα and IL-2rβ chain mRNAs restricted to the T cell areas of spleen and lymph node and the already strong basal expression of the IL-2rγ chain mRNA in corresponding tissue regions supports the suggestion that activated T cells represented the only cell type able to express the trimeric high-affinity IL-2r in response to SEA.

The presence of the mRNAs for IL-2rα, β, and γ chains in the medulla of the thymus provides the possibility that a functional IL-2r is available, but an unchanged number of CD25+ cells makes it unlikely that a high-affinity IL-2r is activated on thymocytes after SEA injection. In this context, IL-2 has been suggested to be an important factor in proliferation events in the thymus during ontogeny [41]. However, recent studies using knockout and double knockout mice clearly demonstrated that IL-2 plays no or only a minor role in thymic T cell development [42], whereas the presence of a functional IL-2rγ chain is essential for a normal development of the thymus [43]. Here, other common γ chain-specific cytokines, e.g. IL-7 and IL-15 seem to be essential for physiological T cell development [42,44]. One possible function of the intrathymic derived IL-2 could be to react as a negative regulator of immune activation. Recent findings have shown that IL-2 triggers activation-induced cell death in antigen-activated T cells via the Fas–FasL apoptotic pathway [45]. The role of IL-2 as a negative regulator had been detected by the findings that IL-2 deficient mice suffer autoimmunity and severe immune dysregulation without having major impairments in immune development or activation [11,39].

4. Materials and methods

4.1. Treatment of rats

Male Wistar rats (weighing 200–250 g; Charles River, Sulzfeld, GER) received an injection of 400 μg/kg body weight SEA i.p. (S9399; Sigma, Munich, GER). Animals were killed at 3, 8, 24, 48 h, and 10 days after treatment and serum samples were taken by heart puncture. For in situ hybridization and Northern blot analysis mesenteric lymph nodes, spleen, and thymus were rapidly removed and deep-frozen. For FACS analysis lymphoid cells from the different lymph organs or the blood were immediately isolated and stained.

4.2. ELISA

Rat IL-2 ELISA from Endogen Inc., USA (LER-IL2; delivered from Biomar, Marburg, GER) was used. The procedure was performed according to the instruction manual given by the manufacturer. For reference the recombinant IL-2 included in the ELISA kit was used. Serum samples were diluted 1:2 and measured in duplicates. Optical density was measured at a wavelength of 450 nm with a reference wavelength of 605 nm using a Benchmark micro plate reader (BIO-RAD Laboratories, Munich, GER) and IL-2 concentration was calculated.

4.3. Generation of cDNA probes

Rat cDNA for IL-2, IL-2rα, β, γ chains CD4, and CD8 were cloned from cervical lymph nodes and spleen of SEA-stimulated Wistar rats using RT-PCR. The following cDNA fragments were generated: rIL-2, 505 bp, base 138–542 (Acc.: M22899); rIL-2rα chain, 851 bp, base 17–658 (Acc.: AI178808); rCD4 cDNA,630 bp, base 626–1255 (Acc.: M15768); rCD8, 458 bp, base 164–139 (Acc.: X04310). All cDNAs were cloned into the pGEM-T easy vector (Promega, Mannheim, GER). For Northern blotting, an 18S cDNA fragment coding for ribosomal RNA (S18) was used as an internal control.

4.4. In vitro transcription

All plasmids were linearized with the appropriate restriction enzymes and transcribed to antisense or sense cRNA using SP6 or T7 polymerases in the presence of 35S-UTP (Amersham, Dreieich, GER) when used for in situ hybridization, or in the presence of 32P-UTP when used for Northern blot analysis. Labeled cRNAs were treated with RNase-free DNase (Boehringer Ingelheim,
GER) for 15 min at 37 °C, hydrolyzed to 250 bp in sodium carbonate at 60 °C, and immediately purified over Micro Bio-Spin chromatography columns as described in the instruction manual (Bio-Rad, München, GER). The labeled cRNAs were diluted in hybridization buffer (100 mM Tris pH 7.5, 600 mM NaCl, 1 mM EDTA, 0.5 mg/ml tRNA, 0.1 mg/ml sonicated salmon sperm DNA, 1× Denhardts, 10% dextran sulfate, 50% formamide) to a final concentration of 5 × 10⁶ cpm/µl when used in situ hybridization, or to 10⁵ cpm/ml when used for Northern blotting. All in situ hybridization experiments were performed with both antisense and sense cRNA probes.

4.5. Northern blot

Total RNA was extracted using TRIzol reagent according to manufacturer’s specification (Gibco BRL, Karlsruhe, GER). The integrity of the RNA was evaluated by estimating the 28S/18S ratio after fractionation on a denaturing 2 M formaldehyde agarose gel. Ten microgram of each RNA samples and 2 µg of RNA ladder (Gibco BRL, Karlsruhe, GER) were submitted to electrophoresis on a 6% formaldehyde agarose gel. Gels were blotted onto Hybond-N membranes (Amersham, Dreieich, GER). Filters were stained in 0.02% methylene blue, 0.3 M sodium acetate (pH 5.5) to identify the position of 18S and 28S ribosomal RNA (rRNA) and RNA markers and hybridized with 32P-labeled cRNA probes for 16 h at 60 °C in 0.4 M NaH₂PO₄,1 mM EDTA, 50% (v/v) formamide, 0.5% SDS, and 1 mg/ml BSA. Filters were washed three times for 10 min at room temperature in 0.1× SSC, 0.2% SDS, and once for 2 h at 65 °C. X-ray film (Hyperfilm MP, Amersham, Dreieich, GER) exposure ranged from one to three days using an intensifier screen. Autoradiograms were digitized for densitometry using the MCID image analysis system (Imaging Research, Ontario, Canada). Quantification analysis was performed using the NIH image software 1.57 written by Wayne Rasband (NIH, Bethesda, USA).

4.6. In situ hybridization

Freshly prepared lymphoid organs were immediately embedded in Tissue Tek (Miles, Elkhart, IN, USA), frozen in −30 °C cold isopentane (Fluka, Deisenhofen, GER), and stored at −75 °C until use. In situ hybridization was performed on 14 µm thick serial cryostat sections as previously described [27]. In brief, pre-hybridization steps included: fixation with 4% paraformaldehyde in PBS at 4 °C for 1 h, washing in PBS, permeabilized by 0.4% Triton X-100 in PBS for 5 min, and acetylation for 10 min in 0.1 M triethanolamine pH 8.0 with 0.25% acetic anhydride. Then, tissues were washed in 2× SSC, dehydrated in ethanol, and hybridized with 35 µl 35S-labeled cRNA on each tissue section for 16–18 h at 60 °C in a moist chamber. For post-hybridization tissue sections were washed in 2× and 1× SSC for 10 min each. Single stranded RNA was digested for 1 h at 37 °C by RNase A1 (10 µg/ml) and RNase T1 (1 U/ml, both from Boehringer Mannheim, GER) diluted in Tris/EDTA, pH 8.0, 150 mM NaCl. After that, sections were desalted by passing them through 1×, 0.5×, and 0.2× SSC for 10 min each and washed in 0.2× SSC at 60 °C for 1 h followed by washing in H₂O for 10 min, dehydration by ethanol, and air drying. Autoradiograms were taken by exposing the sections to an autoradiography film (Hyperfilm MP, Amersham, Dreieich, GER) for one to three days. Sections were then coated with Kodak NBT-2 film emulsion, exposed for 12–21 days, and were then developed, fixed (D19 developer, Kodak, Stuttgart, GER), and counterstained with hematoxylin/eosin. Although not shown in the figures, sense probes were used in all experiment and never displayed any signal above background.

4.7. Cell purification

For the isolation of single cells, spleen, lymph node, and thymus were removed from non-stimulated Wistar rats. Organs were immediately suspended in 10 ml per organ of ice-cold Ca²⁺−Mg²⁺-free PBS and mashed through a sterile metal sieve. The suspension was left to settle on ice for 10 min before the supernatant, containing single cells, was removed. The supernatant was centrifuged (1000 × g, 5 min, 4 °C), washed in Ca²⁺−Mg²⁺-free PBS and then resuspended in Iscoves medium (Gibco BRL, GER) supplemented with 5% FCS (Gibco BRL, Karlsruhe, GER), 2 mM glutamine, and 50 µg/ml gentamycin.

4.8. FACS analysis

All antibodies were purchased from Labgene (Frankfurt, GER). Lymphoid cells were isolated from freshly prepared spleen, mesenteric lymph nodes, or thymus by passing the tissue through a sterile steel mash. Cells (5 × 10⁶) were incubated with anti-rat CD4-FITC (W3/25, dilution 1:100) or anti-rat CD8-FITC (MRC OX-8, dilution 1:25) for 40 min at 4 °C followed by a washing step. For double staining, cells were again incubated with anti-rat CD25-APC (MRC OX-39, dilution 1:1.25) for 40 min at 4 °C. Following a final washing step, the cells were fixed with 4% paraformaldehyde. Ten thousand events were measured on a FACSscan (FACScalibur, Becton and Dickenson, Franklin Lakes, NJ, USA). The statistical significance was calculated by one-way analysis of variance (ANOVA).
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