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Oral Use of Interferon-α Stimulates ISG-15 Transcription and Production by Human Buccal Epithelial Cells

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ABSTRACT

ISG-15 is a 15-kDa protein encoded by an interferon (IFN)-stimulated gene (ISG), which is transcriptionally regulated by IFN-α and IFN-β. Considered as part of the cytokine network, ISG-15 has the potential to amplify the immunomodulatory effects of these IFNs by enhancing IFN-γ production, natural killer cell proliferation, and lymphokine-activated killer cell cytotoxicity. To understand better the mechanism(s) of action of orally administered IFN-α, we have studied the effect of IFN-α on ISG-15 gene expression by human buccal epithelial cells (BEC). For *in vitro* studies, ISG-15 mRNA and protein levels were measured in BEC incubated for 0.5, 2, and 9 h with 100 or 1,000 IU/ml of human lymphoblastoid IFN-α. For *in vivo* studies, ISG-15 mRNA was measured in BEC samples collected at baseline, and 0.5, 2, and 9 h after 5–20 min of oral rinsing with 10 ml of IFN-α (1,000 IU/ml). ISG-15 mRNA was measured by reverse transcriptase polymerase chain reaction (RT-PCR), and ISG-15 protein production by Western Blot analysis. IFN-α augmented BEC ISG-15 gene expression in a concentration dependent manner both *in vivo* and *in vitro*. We conclude that orally administered IFN-α exerts its immunomodulatory effects in humans in part by upregulating the production of ISG-15 by BEC, thereby enhancing the immune reactivity of mucosa-associated lymphocytes.

INTRODUCTION

ORAL ADMINISTRATION of human interferon-α (HuIFN-α) has been reported to protect against infection with mucosally administered viruses in experimental animals and to enhance systemic resistance to viral invasion, including infection due to the feline leukemia virus and human immunodeficiency virus-1 (HIV-1).[1–7] In addition, orally administered natural (n) HuIFN-α in doses ranging from 50 to 200 IU/day has been reported to provide a safe and effective treatment for chronic active hepatitis B virus (HBV) infection.[8–10] Several of these studies suggest that low-dose orally administered IFN-α may be as effective as high-dose parenteral administration in eradicating HBV from chronically infected patients.[8,9]

Oral use of IFN-α also has shown promise in the treatment of Sjögren's syndrome,[11] an autoimmune disease characterized by inflammation of the lacrimal, salivary, and parotid glands with consequent reduction in tear and saliva production. Additionally, ingested IFN-α has been reported to be effective in suppressing the activity of chronic relapsing experimental autoimmune encephalomyelitis (CR-EAE) in mice, and to have favorable systemic immunomodulatory effects in humans with relapsing-remitting multiple sclerosis (RR-MS).[12,13] Promising results have also been obtained in the treatment of experimentally induced melanoma in mice.[14] Interestingly, in experimental animals, the immunomodulatory effects of ingested IFN-α on cells derived from the spleen and lymph nodes may differ from those observed following parenteral administration.[12]

Taken collectively, these reports suggest that orally administered IFN-α exerts both topical and systemic immunomodulatory effects, some of which may prove to be unique for this route of administration. The molecular mechanism(s) whereby orally administered IFN-α exerts these effects, however, is incompletely understood.

In previous *in vitro* studies on the topical effects of IFN-α on the mucosal barrier, we found that human lymphoblastoid IFN-α increased type II major histocompatibility complex (MHC) (HLA-DR) expression by human buccal epithelial cells (BEC), possibly by enhancing IFN-γ production by mucosa-as-

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sociated lymphocytes.[15] Other investigators reported that pretreatment of a cultured human oral mucosal epithelial cell line KB with nHuIFN-α augmented the expression of two adhesion molecules, intercellular adhesion molecule-1 and very late antigen-2, and induced adhesion of the epithelial cells to peripheral T cells by an unidentified ligand, all in a dose-dependent manner.[16] Hence, IFN-α may augment both antigen recognition and cell–cell interaction between mucosal epithelial cells and the mucosa-associated lymphoid tissue by upregulating the expression of MHC II and cell adhesion molecules on BEC.

We now report the results of studies on the effect of human lymphoblastoid IFN-α on ISG-15 transcription and production by human BEC *in vivo* and *in vitro*. ISG-15 is a 15-kDa protein that is transcriptionally regulated by type I IFNs.[17,18] The protein has been shown to induce the production of IFN-γ by T cells and augment natural killer cell proliferation and cytolytic activity of lymphokine-activated killer cells. Thus, it can be considered as part of the cytokine cascade, with the potential to amplify the immunomodulatory effects of IFN-α and IFN-β.[17]

MATERIALS AND METHODS

Cell collection

After oral rinsing with three 10-ml samples of sterile phosphate-buffered saline (PBS, pH 7.4, 0.1 *M*), BEC were collected by gently rubbing the buccal mucosa with sterile Dacron-tipped applicators and dispersing the samples in sterile siliconated tubes each containing 10 ml of PBS. Cells were centrifuged at 4°C for 20 minutes at 1,300 × *g*, washed three times with PBS containing penicillin (50 U/ml), streptomycin (50 μg/ml), and gentamicin (50 μg/ml) (PBS+), and resuspended at a concentration of 1.5 × 10^5 BEC/ml in RPMI 1640 containing 5% heat-inactivated AB+ human serum (vol/vol), glutamine (2 m*M*), penicillin (50 U/ml), gentamicin and streptomycin (50 μg/ml each) (RPMI+). All reagents were kept at 4°C.

Cell counts

Cell counts were done in a hemocytometer. Differential counts were done on cell preparations stained with Diff-Quik (Baxter Heathcare Corp, Miami, FL).

Density gradient separation

Only samples containing ≥98% BEC were used for analysis. When necessary, separation of BEC from contaminating mononuclear cells was accomplished using a gradient of 10, 30, 50, 70, and 90% Percoll (Pharmacia, Sweden) in PBS+. Cells were layered over the gradient, and centrifuged at room temperature for 30 min at 1,300 × *g*. BEC were collected from the 10–30% interface, washed three times with PBS, and cell counts and differentials were done. This fraction was ≥98% BEC.

IFN-α assays

For *in vitro* assays, preparations containing 1.0 × 10^5 BEC/ml were incubated under 5% CO_2 at 37°C in the presence of 0, 100, or 1,000 IU/ml of human lymphoblastoid IFN-α (Hayashibara Biochemical Laboratories, Okayama, Japan) in RPMI 1640+. Samples were taken for analysis at baseline, and 0.5, 2, or 9 h after incubation.

The *in vivo* effect of IFN-α on ISG 15 transcription was determined by having a volunteer rinse his mouth with 10 ml of IFN-α (1,000 IU/ml in PBS) for 5–20 min. The solution was then expectorated, and the mouth washed three times with PBS. In three separate experiments, samples were taken from the buccal surfaces of both cheeks at baseline, and then at 0.5, 2, or 9 h after treatment.

Reverse transcriptase-polymerase chain reaction

Total cellular RNA was extracted by the sequential addition of RNAzol™ B (Tel-Test, Inc., Friendswood, TX) (0.4 ml/1 × 10^6 BEC) and chloroform (0.2 ml/2.0 ml homogenate). The suspension was vortexed, placed on ice for 5 min, and centrifuged at 12,000 × *g* for 15 min at 4°C. The aqueous phase was washed twice with 0.4 ml of phenol:chloroform (1:1, vol/vol), and once with 0.4 ml of chloroform, each time centrifuging the suspension at 12,000 × *g* for 15 min at 4°C. An equal volume of isopropanol was added to the aqueous phase, and the preparation stored at −70°C overnight. After centrifugation at 12,000 × *g* for 15 min at 4°C, the RNA pellet was washed with 75% ethanol. The RNA pellet was then dried under vacuum, and resuspended in 20–30 μl of DEPC-treated water. Samples were further processed if the 260/280-nm optical density ratio was >1.6.[19] RNA was quantitated by optical density readings at 260 nm, and the integrity of the 28S and 18S bands determined by electrophoresis in 2% ethidium bromide-stained agarose gel.

First-strand cDNA was synthesized in the presence of: murine leukemia virus reverse transcriptase (2.5 U/μl); 1 n*M* each of dATP, dCTP, dGTP, and dTTP; RNase inhibitor (1 U/μl); 2 μl 10× PCR buffer (500 m*M* KCl, 100 m*M* Tris-HCl, pH 8.3); and $MgCl_2$ (5 m*M*), using oligo$(dT)_{16}$ (2.5 μM) as a primer. The preparation was incubated at 42°C for 20 min, 99°C for 10 min, and 5°C for 5 min in a DNA thermocycler (Perkin-Elmer Co., Norwalk, CT), and stored at −70°C.

PCR amplification was done on aliquots of the cDNA in the presence of $MgCl_2$ (1.8 m*M*), dNTPs (0.2 m*M* each), AmpliTaq polymerase (1 U/50 μl), and paired ISG-15-specific and GADPH-specific primers (0.2 μM of each primer) to a total of 50 μl. For ISG-15 primers, 5′-ggctgggagctgacg gtgaag-3′ (forward), and 5′-gctccgcccgccagg ctctgt-3′ (reverse) oligonucleotides were designed based on the human ISG-15 cDNA sequence. PCR was done for 40, 50, and 60 cycles under the following conditions: initial denaturation at 95°C for 2 min, denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, and extension at 72°C for 90 sec. Final extension was at 72°C for 10 min. Fourteen microliters of the amplified products were subjected to electrophoresis on a 2% agarose gel containing ethidium bromide. Care was taken to ensure equal RNA loading, and negative DNA controls were run with each experiment to exclude contamination or nonspecific amplification.

Identity of gene products was determined by the sizes of the amplified fragments (570 bp for ISG-15 and 593 bp for GADPH) and, in the case of ISG-15, by nucleotide sequencing. For sequencing, the 570 bp band was excised, and the DNA purified using a GlassMax DNA Isolation Matrix System (Gibco BRL, Gaithersburg, MD). Purified DNA was then sequenced at the Molecular Genetics Facility, Uni-

versity of Georgia (Athens, GA) using the above two oligonucleotide primers. The 570-bp sequence was compared with that of human ISG-15 cDNA using GenBank and the BLAST program.[20]

Semiquantitation was done using an AlphaImager™ 2000 Digital Imaging System (Alpha Innotech Corp., San Leandro, CA) to calculate ISG-15/GADPH ratios.

Western blotting

BEC were homogenized in 8 *M* urea containing protease inhibitor mix (1 m*M* each of antipain, aprotinin, bestatin, chromostatin, pepstatin A, leupeptin, and phenylmethyl sulfonyl fluoride), centrifuged at 13,000 × *g* for 10 min, and the supernatant collected. To obtain an enriched ISG-15 fraction, the supernatant was passed through a 30,000 molecular weight cut-off Centricon Concentrator (Amicon, Millipore Co., Bedford, MA). Protein samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using the buffer system of Laemmli[21] and gels of 12% acrylamide

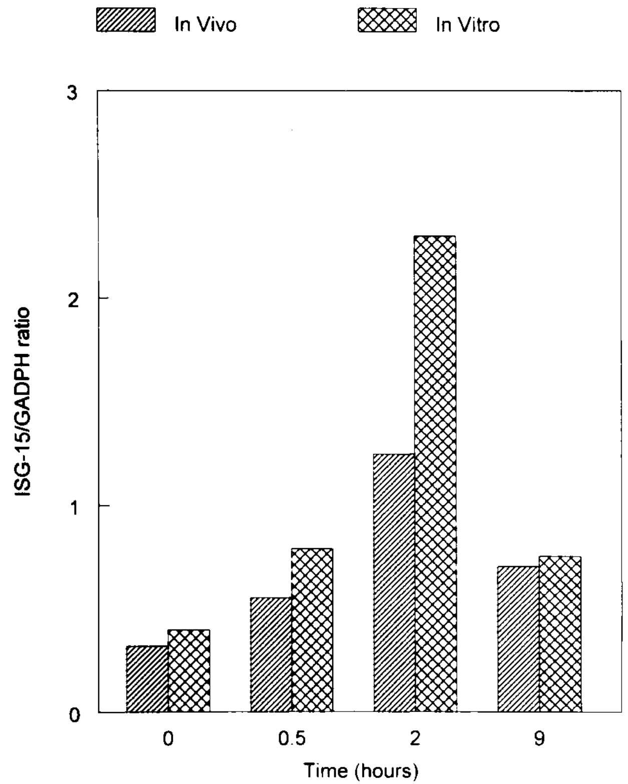

FIG. 2. *In vivo* and *in vitro* time-line effects of IFN-α on ISG-15 transcription by BEC. In three separate *in vivo* studies, BEC were exposed for 5–20 min to 10 ml of IFN-α (1,000 IU/ml), and samples were collected at baseline and 0.5, 2, or 9 h after treatment. In four *in vitro* studies, BEC were incubated with 1,000 IU/ml IFN-α, and samples removed for analysis at baseline and after 0.5, 2, or 9 h of incubation. Samples were analyzed for ISG-15 and GADPH transcripts by RT-PCR using ISG-15- and GADPH-specific primers, and 40–50 cycles of amplification. Semiquantitation was done by calculating ISG-15/GADPH ratios using a digital imaging system. Increases in ISG-15 transcription are evident as early as 0.5 h after IFN-α exposure and peak at 2 h both *in vivo* and *in vitro*.

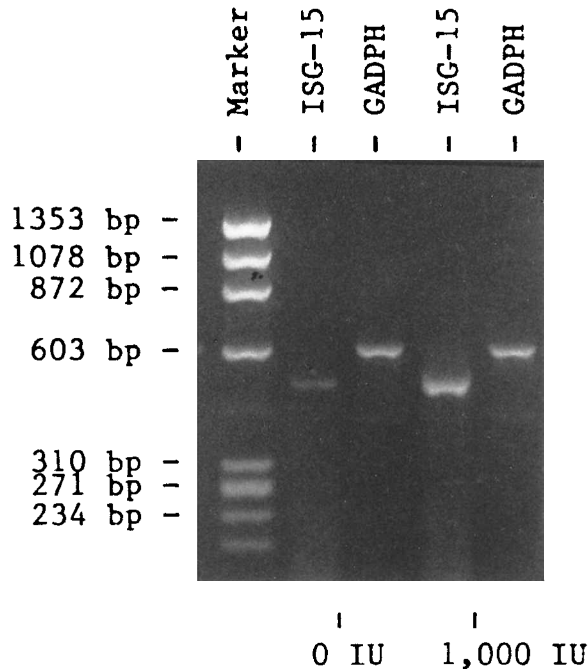

FIG. 1. *In vivo* effects of oral IFN-α on ISG-15 transcription by BEC. BEC were collected before and 2 h after 5 min of oral rinsing with 10 ml of IFN-α (1,000 IU/ml). Samples were analyzed for ISG-15 and GADPH transcripts by RT-PCR using ISG-15- and GADPH-specific primers, and 40 cycles of amplification. Semiquantitation was done by calculating ISG-15/GADPH ratios using a digital imaging system. Identity of gene products was confirmed by expected sizes of amplified fragments (570 bp for ISG-15 and 593 bp for GADPH) and sequencing of the 570-bp product, which showed a 96% identity with human ISG-15. Compared to the pretreatment sample, ISG-15 transcription increased by 384% following treatment with oral IFN-α. Results are representative of two other *in vivo* studies using BEC collected 0.5 and 9 h after treatment.

in a Bio-Rad Mini Gel System. Electroblotting of the SDS-PAGE separated polypeptides onto nitrocellulose membranes was carried out as described elsewhere.[22] Nitrocellulose containing immobilized samples were reacted with rabbit polyclonal antibody against recombinant ISG-15 that has been purified on an ISG-15 Affigel 10 column.[18] The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL). The bands were visualized using the SuperSignal Western Blotting Kit (Pierce, Rockford, IL) and Fuji RX X-ray film, and quantitated using the Alpha-Imager 2000 Digital Imaging System.

Statistical analysis

Probabilities were calculated using the Student's *t*-test (two-tailed). Some values are expressed as the mean ± 1 SEM.

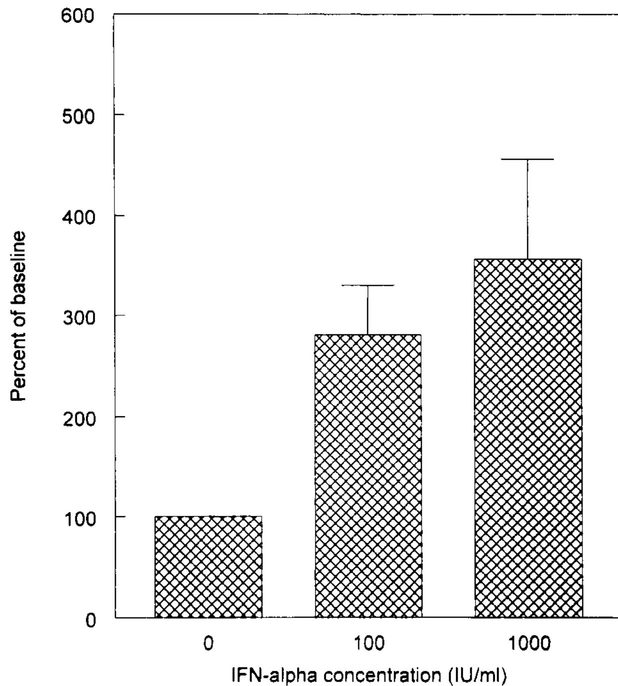

FIG. 3. Effect of IFN-α dose on ISG-15 transcription by BEC. The data are pooled from five *in vitro* studies in which BEC were incubated with 1,000 IU/ml of IFN-α for 9 h, and cells analyzed for ISG-15 and GADPH transcripts by RT-PCR using ISG-15 and GADPH-specific primers and 40–50 cycles of amplification. Semiquantitation was done by calculating ISG-15/GADPH ratios using a digital imaging system. Compared to pretreatment samples, ISG-15 transcription increased by 181 ± 49% in cultures containing 100 IU/ml IFN-α ($p = 0.0214$), and by 256 ± 100% in cultures containing 1,000 IU/ml of IFN-α ($p = 0.0630$).

RESULTS

Effect of IFN-α on ISG-15 transcription

ISG-15 transcript was constitutively expressed by all subject BEC samples, being detectable on reverse transcriptase (RT)-PCR after 40 amplifications in 1 subject, and 50 amplifications in 4 subjects. In three separate experiments involving a single BEC donor, oral rinsing for 5–20 min with 10 ml IFN-α (1,000 IU/ml) upregulated ISG-15 transcription in BEC samples taken 0.5, 2, and 9 h after treatment (Figs. 1 and 2). Similar results were obtained in four separate *in vitro* studies in which BEC taken from different donors were incubated with 1,000 IU/ml IFN-α for 0.5, 2, or 9 h (Fig. 2). Peak effects were evident 2 h after IFN-α exposure both *in vivo* and *in vitro*. In five separate *in vitro* studies employing BEC from 4 donors, augmentation of ISG-15 transcription was found to be dose dependent, with ISG-15 levels increasing over baseline by 181 ± 49% in cultures containing 100 IU/ml IFN-α, and by 256 ± 100% in cultures containing 1,000 IU/ml IFN-α following 9 h of incubation ($p \leq 0.0630$) (Fig. 3). Sequencing of the amplified 570-bp product revealed a 96% identity with human ISG-15.

Effect of IFN-α on ISG-15 production

Incubation *in vitro* for 9 h of four different BEC samples with 100 and 1,000 IU/ml IFN-α resulted in corresponding increases in ISG-15 production (Fig. 4). Compared to pretreatment measurements, ISG-15 production increased by 66 ± 10% in cultures containing 100 IU/ml of IFN-α, and by 117 ± 13% in cultures containing 1,000 IU/ml of IFN-α ($p \leq 0.0065$).

DISCUSSION

In this study, we have demonstrated that orally administered nHuIFN-α augments the transcription and production of ISG-15 by human buccal epithelial cells both *in vivo* and *in vitro*. Topically secreted ISG-15 has the potential to augment mucosal immunity by its effects on mucosa-associated lymphoid tissue, and, secondarily, by feedback effects of augmented lymphocyte activity on BEC function. In theory, this process may be enhanced by the reported ability of IFN-α to augment the expression of several BEC adhesion molecules that are capable of binding to ligands expressed on various leukocyte populations, including T lymphocytes.[16] The findings provide an explanation for our previous observation that IFN-α upregulates the expression of HLA-DR epitopes on BEC in preparations containing significant numbers of mucosa-associated mononuclear cells, but not in preparations containing purified BEC,[15] since this effect is most likely mediated via ISG-15-induced IFN-γ production by T cells.

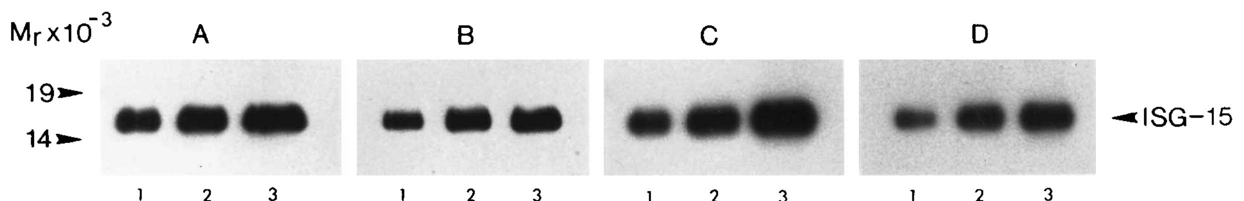

FIG. 4. *In vitro* effects of IFN-α on ISG-15 production by BEC taken from 4 donors (**A–D**). BEC were cultured for 9 h with 0 (#1), 100 (#2), or 1,000 (#3) IU/ml IFN-α, and analyzed for ISG-15 protein by Western blot. Blots containing immobilized BEC proteins were reacted with polyclonal rabbit anti-recombinant ISG-15 antibody. Bound antibodies were detected with horse radish peroxidase-conjugated goat anti-rabbit IgG using a SuperSignal Western Blotting kit and Fuji RX X-ray film. Quantitation was done using a digital imaging system. Compared to pretreatment samples, ISG-15 production increased by 66 ± 10% in cultures containing 100 IU/ml of IFN-α, and by 117 ± 13% in cultures containing 1,000 IU/ml of IFN-α ($p \leq 0.0065$).

The findings also provide a plausible explanation for the apparent ability of orally administered IFN-α to protect against mucosally administered viruses. The anti-viral effects of type I IFNs are felt to result from their ability to upregulate the expression of class I MHC glycoproteins, thereby facilitating recognition of viral antigens by the immune system,[23,24] and by their ability to inhibit viral replication by promoting the synthesis of antiviral proteins,[23] activities that presumably take place at the mucosal barrier. In addition, by augmenting the production of ISG-15 by mucosal epithelial cells and mucosa-associated lymphoid tissue, orally administered IFN-α also has the potential to enhance cell-mediated immune responses by stimulating IFN-γ production by T cells, by augmenting natural killer cell proliferation and cytolytic activity of lymphokine-activated killer cells, and by activating monocytes and macrophages and inducing HLA-DR expression by BEC via the induced IFN-γ.[17] IFN-α has also been shown to promote T helper type 1 (Th1) development from T helper progenitors.[25–27] All of these activities may assist in the eradication of viruses, a process that depends primarily on Th1-directed cell-mediated immune responses.[24]

The mechanism(s) by which orally administered IFN suppresses the activity of several autoimmune diseases is less clear. In the murine model of CR-EAE, ingested species-specific murine IFN-α resulted in systemic immunomodulatory effects manifested by an inhibition of concanavalin A (ConA)-induced proliferation and interleukin-2 (IL-2) and IFN-γ production by spleen cells.[12] Similar suppression of ConA-induced proliferation and cytokine production by blood lymphocytes has been reported in patients with RR-MS following administration of IFN-α orally, suggesting that ingested IFN-α may cause a functional inhibition of Th1-like T helper cells, a potential site of intervention at the level of effector T cells in EAE and MS.[13] However, these reported systemic immunomodulatory effects of IFN-α contrast with its ISG-15-mediated activities, which would be expected to augment rather than suppress T cell production of IFN-γ and IL-2. It seems clear, therefore, that further study will be necessary to clarify the mechanism(s) whereby ingested IFN-α alters the course of diseases such as CR-EAE.

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