Oral Administration of the Immunomodulator JBT-3002 Induces Endogenous Interleukin 15 in Intestinal Macrophages for Protection against Irinotecan-mediated Destruction of Intestinal Epithelium

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ABSTRACT

We recently reported that p.o. administration of the new synthetic bacterial lipopeptide JBT-3002 can protect mice from irinotecan (CPT-11)-induced intestinal injury, but the mechanism was not known. Because interleukin-15 (IL-15) is associated with maintenance of intestinal epithelial cell integrity, we examined whether p.o. administration of JBT-3002 elevates expression of this monocytoid-derived cytokine. Four daily i.p. injections of 100 mg/kg CPT-11 were effective against liver metastases produced by CT-26 murine colon cancer cells, but severe damage to the intestinal epithelium and early death of the mice also resulted. Three consecutive daily p.o. doses of JBT-3002 prior to i.p. injection of irinotecan prevented the undesirable side effects of irinotecan without reducing its ability to eradicate liver metastases. Immunohistochemical analyses of the intestines of mice treated with JBT-3002 and CPT-11 demonstrated an increase in the number of dividing cells in the crypts and enhanced expression of IL-15 in lamina propria cells; the increase correlated with increased expression of the IL-15 gene as determined by semiquantitative reverse transcriptase-PCR. In vitro studies demonstrated that JBT-3002 induced expression of IL-15 in peritoneal macrophages but not in normal intestinal epithelial cells (IEC-6). Moreover, the presence of IL-15 decreased irinotecan-mediated cytotoxicity of IEC-6 epithelial cells. These data show that the p.o. administration of JBT-3002 induces expression of IL-15 by macrophages in the lamina propria, which can prevent irinotecan-induced injury to the intestinal mucosa.

INTRODUCTION

Irinotecan (CPT-11), a semisynthetic derivative of camptothecin, is a potent chemotherapeutic agent that is increasingly being used for the treatment of drug-refractory colorectal cancer (1–3). Unfortunately, the utility of irinotecan is limited by induction of severe diarrhea (2, 3). We and others have ascribed this toxicity to structural changes in intestinal architecture that resulted from disordered epithelial cell differentiation and apoptosis (4, 5).

Gastrointestinal mucosal integrity is regulated by several growth factors and cytokines (6, 7), such as transforming growth factor-β (8) and IL-2 (9, 10). IL-15 is a novel cytokine that shares the IL-2 receptor β- and γ-chains for signal transduction and, hence, many of the biological activities of IL-2 (11–14). In vitro studies have shown that IL-15 can be mitogenic for intestinal epithelial cells (7, 15), and recent reports have demonstrated that administration of rIL-15 can protect rats from intestinal toxicity induced by 5-fluorouracil (16) or irinotecan (17). IL-15 is expressed by intestinal epithelial cells and mononuclear cells in the lamina propria (7), and IL-15 transcription is known to be increased by macrophage-activating microbial agents such as LPS or Bacillus Calmette-Guérin (18). These findings suggest that IL-15 may have biological activities that promote recovery of epithelial integrity after wounding caused by chemotherapy drugs.

We have reported that the incubation of monocytes/macrophages with a new synthetic lipopeptide, JBT-3002 (N-acetylated derivative of 3-amino-C1-C3-alkanesulfonic acid), can induce production of monocyte-derived cytokines (19–21). Moreover, the p.o. administration of JBT-3002 to mice overcomes irinotecan-induced intestinal toxicity by an unknown mechanism (4). The purpose of this study was to determine whether up-regulation of endogenous IL-15 in the lamina propria prevents irinotecan-induced injury to the intestinal mucosa in mice given p.o. JBT-3002.

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3 The abbreviations used are: IL, interleukin; LPS, lipopolysaccharide; rIL-15, recombinant IL-15; rIFN-γ, recombinant IFN-γ; PEM, peritoneal exudate macrophage; BrdUrd, bromodeoxyuridine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
MATERIALS AND METHODS

**Animals.** Specific pathogen-free BALB/c mice were purchased from the Animal Production Area of the National Cancer Institute (Frederick, MD). The mice were maintained according to institutional guidelines in facilities approved by The American Association for Accreditation of Laboratory Animal Care, in accordance with United States Department of Agriculture, Department of Health and Human Services, and NIH regulations and standards.

**Reagents.** Eagle’s MEM, Ca\(^{2+}\)/Mg\(^{2+}\)-free HBSS, and fetal bovine serum were purchased from M. A. Bioproducts (Walkersville, MD). JBT-3002 was obtained from Jenner Biotherapies (San Ramon, CA). Irinotecan, produced by Yakult Honsha Co. (Tokyo, Japan), was used according to manufacturer’s instructions. Simian rIL-15 was obtained from R&D Systems (Minneapolis, MN). Murine rIFN-γ (specific activity, 10\(^7\) units/mg) was obtained from PharMingen (San Diego, CA). Salmonella LPS was purchased from Sigma Chemical Co. (St. Louis, MO). All reagents used in tissue culture (except for LPS) were free of endotoxin as determined by the Limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA).

**Cells.** CT-26 murine colon carcinoma cells, syngeneic to BALB/c mice (4), and IEC-6 normal rat intestinal epithelial cells obtained from the American Type Culture Collection (Manassas, VA) were grown as monolayer cultures in Eagle’s MEM supplemented with 5% fetal bovine serum, vitamins, sodium pyruvate, l-glutamine, and nonessential amino acids. The adherent monolayer cultures were incubated at 37°C in a humidified atmosphere containing 5% CO\(_2\) in air. PEMS were collected by peritoneal lavage of BALB/c mice given an i.p. injection of 1.5 ml of thioglycolate broth (Baltimore Biological Laboratories, Cockeysville, MD) 4 days before the harvest (4). One h after plating, the nonadherent cells were removed by washing with HBSS. At that time, >98% of adherent cell populations were macrophages according to morphology, phagocytic criteria, and positive staining with antibodies against scavenger receptor (22). All cultures were free of Mycoplasma, reovirus type 3, pneumonia virus of mice, K virus, encephalitis virus, lymphocyte choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD).

**Experimental Liver Metastasis.** To prepare tumor cells for inoculation, we harvested CT-26 cells in exponential-growth phase by a brief exposure to a 0.25% trypsin-0.1% EDTA solution. The cell suspension was pipetted to produce a single-cell suspension, washed, and resuspended in HBSS. Cell viability was determined by trypan blue exclusion, and only single-cell suspensions of >90% viability were used for injection. Tumor cells (1 \(\times\) 10\(^7\)) of HBSS) were injected into the spleens of BALB/c mice after laparotomy under methoxyflurane anesthesia. The incision was closed in one layer with wound clips (4). This protocol resulted in a 100% incidence of liver metastasis. The mice were euthanized when the control group became moribund. The volume of tumors (TV) in the spleens was calculated using the following formula: \(TV = L \times W / 2\), where \(L\) and \(W\) represent the length and the width of the tumor mass, respectively. The livers were harvested and placed in Bouin’s solution for 24 h before fixation in 10% buffered formalin. The number of experimental metastases on the surface of the liver was determined using a dissecting microscope. When the number of metastases exceeded 100, we assigned a value of >100.

**Preparation and Administration of JBT-3002.** Because our recent data have suggested that JBT-3002 may induce activation of macrophages by interacting with micellar JBT-3002 (21), for this study, we used soluble JBT-3002 instead of JBT-3002 encapsulated in multilamellar vesicles-lysosomes (4). JBT-3002 was suspended in HBSS at 1 mg/ml, sonicated for 5 min, and stored at 4°C. The solution was mixed on a vortex machine prior to each experiment. JBT-3002 suspended in 0.2 ml of HBSS was administered by mouth using a rigid, curved feeding tube (4). In a preliminary study, we determined the biological effective dose of oral JBT-3002. BALB/c mice were, therefore, given p.o. JBT-3002 at doses of 0.0001, 0.001, 0.01, 0.1, or 1.0 \(\mu\)g per dose (per mouse) once a day for 3 consecutive days. Seven days later, the mice were given i.p. injections of 100 mg/kg irinotecan once a day for 4 days. The mice were monitored for morbidity until day 14. The administration of irinotecan (CPT-11) alone produced severe diarrhea and corresponding loss of body weight. In contrast, all of the mice given 0.01, 0.1, or 1.0 \(\mu\)g/dose of JBT-3002 prior to the irinotecan survived with minimal to no diarrhea or reduction in body weight. In all of the following studies, we treated mice with 0.05 \(\mu\)g of JBT-3002 (2.5 \(\mu\)g/kg).

**Scanning Electron Microscopy.** The small intestines of control and treated mice were everted, rinsed in water, and immediately fixed overnight at 4°C in a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer. The samples were then treated with 1% buffered osmium tetroxide followed with 1% thiocarbohydrazide for 10 min and then with 1% aqueous osmium tetroxide for 10 min. The samples were then washed with distilled water, treated with 1% aqueous tannic acid for 30 min, and rinsed again with distilled water; they were then incubated for 12 h with 1% aqueous uranyl acetate. The samples were then washed with distilled water three times, dehydrated in increasing concentrations of ethanol, and placed in hexamethyldisilazane for 10 min according to Adams et al. (23). The samples were dried overnight under vacuum, and the specimens were mounted directly on double-stick carbon tabs and then mounted on aluminum specimen mounts. The samples were coated with platinum/palladium alloy and examined in a Hitachi S520 scanning electron microscope at an accelerating voltage of 5 kV.

**Immunohistochemistry.** Mice were injected i.v. with 0.2 ml of saline containing 250 \(\mu\)g BrdUrd 1 h before they were killed (24). Samples from the small intestines were fixed in 10% buffered formalin. Immunohistochemical staining was performed by the immunoperoxidase technique, as described previously (4). Paraffin sections (5 \(\mu\)m thick) were dewaxed in xylene and then rehydrated in graded ethanol. For S-phase labeling with BrdUrd, the sections were incubated with 2 \(\times\) 1HCl for 30 min at 37°C. For labeling with IL-15, the sections were incubated with Pepsin Reagent (Biomedia Corp., Foster City, CA) for 20 min at room temperature. The slides were rinsed twice with PBS, and endogenous peroxidase was inactivated by treatment with 3% hydrogen peroxide in methanol for 10 min. Nonspecific reactions were blocked by incubating the sections...
in a solution containing 5% normal horse serum in PBS. The sections were incubated overnight at 4°C with a 1:50 dilution of monoclonal mouse anti-BrdUrd antibody (Becton Dickinson, Mountain View, CA) or a 1:100 dilution of goat polyclonal anti-IL-15 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The sections were then rinsed three times with PBS and incubated at room temperature for 1 h with the appropriate dilution of peroxidase-conjugated secondary antibodies. Secondary antibodies were antirat IgG1 antibody (PharMingen) and antioctopus IgG1 antibody (Jackson ImmunoResearch, West Grove, PA) at a 1:200 dilution for IL-15. After three rinses with PBS, the sections were incubated overnight with the appropriate secondary antibodies were antimouse IgG1 antibody (PharMingen) and antioctopus IgG1 antibody (Jackson ImmunoResearch, West Grove, PA) at a 1:200 dilution for IL-15.

To ensure that equivalent amounts of cDNA were used in each reaction, we also performed PCR for glyceraldehyde-3-phosphate dehydrogenase. The primers used were: sense, 5'-TTTACGACCTATGGAGGCGT-3'; and antisense, 5'-CTGTTGAGCTAGCTAG-3'. The PCR product was electrophoresed through a 1.2% agarose gel and visualized with ethidium bromide.

**In Vitro Drug Sensitivity Assay.** Tumor cells (CT-26) and normal intestinal epithelial cells (IEC-6) suspended in 0.1 ml of EMEM were seeded into 38-mm² wells of 96-well microculture plates (Falcon Plastics, Oxnard, CA) at a density of 2000 cells per well and incubated for 96–120 h with different concentrations of HL-15 in the absence or presence of IC₅₀ irinotecan (8 µg/ml for CT-26 cells and 2 µg/ml for IEC-6 cells). MTT (1 mg/ml in PBS) was added to the cultures at 0.025 ml/well during the final 2 h of incubation. The medium was removed, and the cells were lysed in DMSO. The conversion of MTT to formazan by metabolically viable cells was monitored by a microtiter plate reader at 570 nm (27).

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spleen tumor</th>
<th>Liver metastasis</th>
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<tbody>
<tr>
<td></td>
<td>Incidenceᵃ</td>
<td>Mean tumor volume (mm³)</td>
</tr>
<tr>
<td>Control</td>
<td>10/10</td>
<td>594 ± 51</td>
</tr>
<tr>
<td>CPT-11</td>
<td>6/10ᵇ</td>
<td>79 ± 38ᵈ</td>
</tr>
<tr>
<td>JBT-3002</td>
<td>10/10</td>
<td>88 ± 34ᵈ</td>
</tr>
<tr>
<td>JBT-3002 + CPT-11</td>
<td>4/10</td>
<td>47 ± 26ᵈ</td>
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ᵃ No. of mice with tumor/no. of mice injected.
ᵇ Seven mice died during therapy. They were free of metastasis.
ᶜ Data of surviving mice.
ᵈ P < 0.001 compared to control.
ᵉ P < 0.0001 compared to control.
ᶠ P < 0.05 compared to control.
sis was calculated by the following formula: cytostasis (%) = \((B - A)/B \times 100\), where \(A\) is the absorbance of treated cells and \(B\) is the absorbance of the control cells.

**Statistical Analysis.** The significance of the differences in *in vitro* data was analyzed by the unpaired Student’s *t* test (two-tailed). The significance of the *in vivo* differences was analyzed by the Mann-Whitney *U* test and Student’s *t* test.

**RESULTS**

**Protection of Mice from Irinotecan-induced Toxicity by p.o. JBT-3002.** In the first set of experiments, we examined whether the p.o. administration of soluble JBT-3002 could protect mice against lethal diarrhea induced by high-dose irinotecan. Mice were given intrasplenic injections of CT-26 cells on day 0. Groups of mice were then given three daily p.o. administrations of 0.05 μg of JBT-3002 starting on day 3. Seven days after tumor cell injection, groups of mice (\(n = 10\)) were given four daily i.p. injections of 100 mg/kg irinotecan. The mice were killed 4 days after the last irinotecan treatment. The ileum was everted, fixed, and processed for scanning electron microscopy.

**Morphological and Histological Alterations in the Intestines of Treated Mice.** To examine the architectural changes in the intestinal mucosa, we harvested tissues from the ileum 4 days after the final treatment with irinotecan and processed them for examination by scanning electron microscopy. Fig. 1 shows that irinotecan-treated mice had severe damage of the mucosal surface of the ileum characterized by stubby villi and patches bare of villi. In mice given p.o. JBT-3002 without irinotecan, the villi were taller than normal. In mice treated with JBT-3002 prior to injections of irinotecan, the mucosa was thoroughly covered with villi and an appearance similar to that of control mice.

Tissues from these intestinal samples were processed for histopathological analysis (Fig. 2). H&E staining revealed severe morphological changes consisting of loss of villi, epithelial
vacuolation, and inflammatory cell infiltrate in the ileum of mice treated with irinotecan alone. In mice treated with JBT-3002 without irinotecan, the intestinal epithelium exhibited mild hyperplastic changes. Of note, in mice treated with JBT-3002 prior to injections of irinotecan, the morphology of the ileum was similar to that found in normal mice. Enlargement of the crypts and lamina propria was also observed in this group.

Proliferation of epithelial cells was evaluated by immuno-

Fig. 2 Histopathological changes in the ileum of BALB/c mice treated with irinotecan (CPT-11), JBT-3002, or JBT-3002 prior to injections of irinotecan (JBT-3002/CPT-11). Tissue sections from the ileum were analyzed for morphological integrity by H&E staining and immunohistochemistry to detect S-phase labeling (with anti-BrdUrd antibody) and expression of IL-15. Scale bar, 50 μm.
histochemical staining for S-phase labeling with anti-BrdUrd antibody (Fig. 2). The number of BrdUrd-positive cells in mice given irinotecan alone (mean, 24 ± 6 cells per 0.25 mm²) was significantly lower than the number in control mice (mean, 78 ± 13 cells per 0.25 mm²; P < 0.01). In mice given JBT-3002 without irinotecan, the number of BrdUrd-positive cells in the crypts was significantly increased (mean, 110 ± 11 cells per 0.25 mm²; P < 0.05). Notably, mice treated with JBT-3002 prior to injections with irinotecan had a nearly 6-fold increase in the number of dividing epithelial cells (mean, 153 ± 20 cells per 0.25 mm²; P < 0.01).

Expression and Localization of IL-15 in the Intestines of Mice Receiving p.o. JBT-3002 Prior to Injections with Irinotecan. In the next set of experiments, we examined whether the expression of IL-15 correlated with alterations in the mucosal epithelium. Immunohistochemistry, using antibodies against IL-15, depicted only a faint staining in intestinal mucosa of mice given irinotecan alone. In contrast, the ileum of mice treated with JBT-3002 before injections of irinotecan exhibited intense staining with the anti-IL-15 antibody (Fig. 2). The expression of the IL-15 protein was most prominent in cells associated with the lamina propria.

To examine expression of mRNA specific for IL-15, total RNA was isolated from the ileum 4 days after the final treatment with irinotecan and analyzed by reverse transcriptase-PCR. The results, shown in Fig. 3A, closely agreed with those obtained by immunohistochemistry. A nearly 90% reduction in IL-15 transcripts was observed in the ileum from mice treated with irinotecan alone. The ileum of mice treated with JBT-3002 prior to injections with irinotecan had a 1.8-fold increase in expression of IL-15 mRNA, which decreased by 20% after 7 days (data not shown).

Induction of IL-15 in Macrophages Activated by JBT-3002. Because both lamina propria mononuclear cells and intestinal epithelial cells can release IL-15 (7), we determined...
whether exposure of these cells of JBT-3002 would lead to enhanced expression of IL-15. PEMs and IEC-6 cells were cultured for 6 h with medium (control) or medium containing 10 ng/ml JBT-3002. Total RNA was isolated from the PEMs and IEC-6 cultures and the expression of IL-15 mRNA was analyzed by reverse transcription-PCR (Fig. 3B). Activation of PEMs with rIFN-γ (10 units/ml) and LPS (10 ng/ml) for 6 h produced a 2.4- and 7.1-fold increase in expression of IL-15 mRNA, respectively. Treatment of PEMs with JBT-3002 (10 ng/ml) induced a 4.2-fold increased expression of IL-15 mRNA. IEC-6 cells constitutively expressed IL-15 mRNA, but treatment with the stimuli did not enhance expression of the cytokine. The level of gene expression closely correlated with secretion of IL-15 protein as determined by an immunoperoxidase assay (Fig. 3C).

**DISCUSSION**

We have recently reported that the p.o administration of JBT-3002, a new synthetic macrophage-activating agent encapsulated in phospholipid liposomes (19–21), can prevent irinotecan-induced toxicity to the intestinal epithelium of mice, thus allowing irinotecan dose intensification to raise the therapeutic efficacy against liver metastasis produced by CT-26 murine colon carcinoma cells (4). Although efficient in situ activation of macrophages is achieved by the encapsulation of immunomodulators in phospholipid liposomes (28), our recent data show that JBT-3002 suspended in aqueous solution to form micelles produces superior activation of macrophages (21) and that the in vivo use of this micellar formulation of JBT-3002 requires only a 1/20 equivalent dose of liposome-encapsulated JBT-3002.

Topoisomerase I inhibitors such as irinotecan are highly S phase specific; therefore, diarrhea induced by high-dose irinotecan may result from direct toxicity against dividing epithelial cells in the intestinal crypts (29). Indeed, four consecutive daily administrations of 100 mg/kg irinotecan severely disrupted the intestinal architecture, producing a nearly 3-fold decrease in proliferating crypt cells as measured by BrdUrd labeling. In contrast, p.o. administration of JBT-3002 prior to injections with irinotecan increased cell division in the crypts and preserved epithelial organization in the villi. These findings suggest that JBT-3002 can prevent chemotherapy-induced injury of the intestinal mucosa by affecting epithelial stem cell integrity (4).

Recent studies have demonstrated that a variety of growth factors and cytokines regulate the proliferation of intestinal epithelial cells (6, 7). For example, IL-2 has been shown to modulate proliferation of both IEC-6 cells and rat primary intestinal epithelial cells under in vitro conditions (9). IL-15 is a novel cytokine that shares the IL-2 receptor β- and γ-chains for signal transduction and, hence, many of the biological activities of IL-2 (11–14). In addition, the α-chain specific to the IL-15 receptor transduces IL-15 signals that contribute to epithelial organization by enhancing tight junction formation (15). Unlike IL-2, IL-15 is produced by intestinal epithelial cells and lamina propria macrophages (7). These results show that tissues from the intestines of mice given p.o. JBT-3002 before injections of irinotecan overexpressed the IL-15 gene. Immunohistochemical analyses revealed that lamina propria leukocytes rather than epithelial cells were responsible for this overexpres-
sion. These findings were further confirmed by in vitro experiments in which PEMs but not IEC-6 cells exposed to JBT-3002 had evidence of increased expression of IL-15 mRNA and protein. Exposure of macrophages to LPS is known to enhance expression of IL-15 (18). Because JBT-3002 shares many intracellular signaling pathways for cytokine expression with LPS, it is not surprising that exposure of macrophages to JBT-3002 leads to enhanced expression of IL-15 (21). Studies have shown that the binding of nuclear protein to the nuclear factor kB binding site is required for transcriptional activation of the IL-15 gene in LPS-stimulated macrophages (30). Recent data show that administration of human rIL-15 can protect rats from intestinal toxicity produced by 5-fluorouracil (16) or irinotecan (17). Thus, the p.o. administration of JBT-3002, which activates lamina propria macrophages to produce IL-15, may well be involved in the prevention of irinotecan-induced injury to the intestinal mucosa.

Several lines of evidence suggest that intestinal epithelial cells, including IEC-6 cells, possess IL-15 receptor (9, 10, 15). Nevertheless, the mechanism by which IL-15 affects intestinal integrity is still unknown. Treatment of IEC-6 cells with IL-15 at concentrations of 1.5–100 ng/ml did not promote proliferation. However, IL-15 decreased irinotecan-mediated cytotoxicity, suggesting that IL-15 may promote the recovery of epithelial integrity after wounding. IL-15 may function by up-regulating other cytokines such as transforming growth factor-β, which is involved in epithelial cell migration (8). In that case, IL-15 would have an indirect effect on recovery from intestinal injury.

At higher concentrations (250 ng/ml), IL-15 stimulates the proliferation of intestinal epithelial tumor cells, such as Caco-2 (7). In this study, however, IL-15 (tested at 1.5–100 ng/ml) did not stimulate the division of CT-26 colon cancer cells or protect these tumor cells from irinotecan-mediated cytotoxicity. These differences have a tremendous therapeutic benefit, and indeed, treatment of mice with oral JBT-3002 followed by i.p. irinotecan significantly reduced the size of spleen tumors and eradicated liver metastases. IL-15 has also been shown to induce activation of CTLs and natural killer cells (11, 31). JBT-3002 activates macrophages to become tumoricidal and produce inflammatory and immunomodulatory cytokines (4, 19–21). JBT-3002 is also a potent activator of inducible nitric oxide synthase in macrophages, resulting in production of high concentrations of nitric oxide (19, 20) shown to induce apoptosis in tumor cells to which activated macrophages bind (32, 33). The release of IL-15 by JBT-3002-activated macrophages may, therefore play a role in antitumor activity.

In summary, we have demonstrated that p.o. administration of JBT-3002 prevents irinotecan-induced dose-limiting intestinal toxicity. One of the mechanisms for this protection may involve the production of endogenous IL-15 by activated macrophages within the lamina propria. The p.o. administration of JBT-3002 therefore allows the use of high-dose irinotecan that could produce superior therapeutic results against colon cancer liver metastases.

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REFERENCES


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