Spontaneously Increased Production of Nitric Oxide and Aberrant Expression of the Inducible Nitric Oxide Synthase In Vivo in the Transforming Growth Factor β1 Null Mouse

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Summary

Transforming growth factor β1 null mice (TGF-β1$^{-/-}$) suffer from multifocal inflammation and die by 3–4 wk of age. In these mice, levels of nitric oxide (NO) reaction products in serum are elevated approximately fourfold over levels in controls, peaking at 15–17 d of life. Short-term treatment of TGF-β1$^{-/-}$ mice with N^G-monomethyl-L-arginine suppressed this elevated production of NO. Expression of inducible NO synthase (iNOS) mRNA and protein is increased in the kidney and heart of TGF-β1$^{-/-}$ mice. These findings demonstrate that TGF-β1 negatively regulates iNOS expression in vivo, as had been inferred from mechanistic studies on the control of iNOS expression by TGF-β1 in vitro.

The three mammalian isoforms of TGF-β mediate a wide array of physiological processes (1). In addition to their functions in development, cell cycle control, and wound healing, the TGF-βs regulate the function of cells involved in the immune response to antigens, mitogens, and pathogens (2). Among the immunomodulatory effects attributed to the TGF-βs is the suppression of the production of nitrogen free radicals by macrophages in vitro (3). Nitric oxide (NO) and its derived nitrogen free radicals have been the subject of much investigation, owing to their pleiotropic effects (4). An enzyme inducible by cytokines and microbial products present in many cell types (iNOS; NOS type II) produces large quantities of NO (4). The cytotoxic or cytostatic effects of high levels of NO are beneficial during infection but can damage host tissue when unchecked (4). Accordingly, suppression of NO production by exogenously administered TGF-β1 can have both deleterious and beneficial effects in different contexts (5).

To discern whether endogenous TGF-β1 can control the expression of iNOS in vivo, we made use of the TGF-β1 null (TGF-β1$^{-/-}$) mouse (6). In contrast with normal (TGF-β1$^{+/+}$) or heterozygous (TGF-β1$^{+/-}$) littermates, these mice develop multifocal inflammation and die after a wasting syndrome (6). TGF-β1$^{-/-}$ mice exhibit high levels of circulating autoantibodies (7) as well as increased expression of MHC class I and II in the absence of increased expression of IFN-γ (8), suggesting that TGF-β1 may be an active repressor of inflammation even in the absence of proinflammatory stimuli. For these reasons, and owing to the multiple regulatory effects of TGF-β1 on NO produc-

tion in vitro (5), we examined whether production of NO is dysregulated in TGF-β1$^{-/-}$ mice.

Materials and Methods

TGF-β1$^{-/-}$ Mice. TGF-β1$^{-/-}$ mice were derived as described previously (6) and maintained in a specific pathogen-free animal room. By routine screening of the animal colony, the mice were determined not to be infected with any identifiable pathogens[1] (data not shown). TGF-β1$^{-/-}$ mice were identified by expressing the null allele and not the normal allele. Genotyping was performed by PCR from tail preparations as described previously (8, 9).

Treatment Protocols. Mice were injected intraperitoneally with N^G-monomethyl-L-arginine (L-NMA; 50–400 mg/kg; Sigma Chemical Co., St. Louis, MO), N^G-nitro-L-arginine methyl ester (L-NAME; 50 mg/kg; Sigma), or aminoguanidine (50 mg/kg; Sigma). All injected solutions were diluted in sterile PBS and sterile filtered before injection.

Preparation of Serum. Mice were killed by asphyxiation in CO_2, and blood was collected in microcentrifuge tubes after cardiac puncture. Serum was obtained after centrifugation of coagulated blood and stored at $-70°C$.

Quantitation of NO_2^- and NO_3^- in Serum. Serum NO_2^- and NO_3^- were assayed by reduction of NO_3^- to NO_2^- on Cd filings as described elsewhere (10). The samples were then assayed for NO_2^-

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[1] Mice were screened routinely for the following pathogens: murine hepatitis virus, pneumonia virus of mice, polioencephalomyelitis virus, lymphocytic choriomeningitis virus, mouse cytomegalovirus, reovirus type 3, ectromelia virus, and mouse polyoma virus, as well as several types of parovirus, ectoparasites, and endoparasites.

content by the Griess assay as described elsewhere (3, 11). The NO_2^- + NO_3^- content of selected serum samples was verified by a modification of the nitrate reductase method (12).

Preparation of Homogenates. Organs were removed from mice by dissection and frozen at $-70°C$, resuspended in 500 μl of sonication buffer (40 mM Tris, pH 8.0, containing 5 μg/ml pepstatin A, 5 μg/ml aprotinin, and 500 μM AEBSF [ICN Biomedicals Inc., Costa Mesa, CA]), and homogenized with a Kinematica electrical homogenizer (Brinkmann Instruments, Westbury, NY). The homogenates were centrifuged at room temperature, and the supernatant was stored at $-70°C$.

Northern Analysis. Tissues were homogenized in GTC buffer (4.0 M guanidine thiocyanate, 50 mM Tris, pH 7.0, 10 mM EDTA, 2% [wt/vol] sarkosyl, 1% [vol/vol] antifoam A [Sigma]) purified by the CsCl method as described previously (8, 9). 15 μg of total RNA was electrophoresed and blotted by standard methods (13). Northern blotting was carried out according to the procedure of Church and Gilbert (14) with the HincII–EcoRI fragment of the iNOS cDNA (15) and the 675-bp region within the TGF-β1 precursor cDNA starting at the ATG initiation codon (16). After hybridization, the filters were exposed to a Phosphor-Imager (Molecular Dynamics, Inc., Sunnyvale, CA).

Anti-iNOS Antibodies. For immunocytochemical detection of iNOS, we used a rabbit polyclonal antiserum raised against the carboxy terminal EEPKATRL peptide of mouse iNOS coupled to thyroglobulin as a carrier by use of sulfo-MBS chemistry (Pierce Chem. Co., Rockford, IL) (17). For immunoblotting, we used a mouse mAb raised against a fragment of mouse iNOS from amino acids 961–1144 (Transduction Laboratories, Lexington, KY).

SDS-PAGE and Immunoblotting. 150–250 μg of each homogenate was separated on precast 4–20% polyacrylamide gels (NOVEX, Encinitas, CA) along with prestained mol wt standards (Amersham Corp., Arlington Heights, IL). As a positive control for mouse iNOS, RAW 264.7 cells (American Type Culture Collection, Rockville, MD) were stimulated with IFN-γ (100 U/ml; GIBCO BRL, Gaithersburg, MD) and LPS (200 ng/ml; prepared by phenol extraction from *Escherichia coli* O11:5; List Biological Laboratories, Inc., Campbell, CA) as described elsewhere (18). The separated proteins were immunoblotted by standard methods. In brief, membranes were blocked with 5% nonfat dry milk

and incubated with a 1:5,000 dilution in 5% nonfat dry milk of anti-iNOS. Secondary antibody was a goat anti–mouse IgG coupled to HRP (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The membranes were visualized by enhanced chemiluminescence (Amersham).

Histopathology and Immunohistochemistry. Tissues were fixed in 10% buffered formalin and embedded in paraffin. 5-μm sections were stained with hematoxylin–eosin and analyzed for histopathology. Tissue sections were deparaffinized at 65°C, followed by incubation in xylene and ethanol. After endogenous peroxidase activity was blocked in H_2O_2–methanol, and nonspecific binding in 5% BSA, 1% normal goat serum was carried out, sections were incubated overnight at 4°C with anti-iNOS at a dilution of 1:1,000. Control for specificity of iNOS was normal rabbit serum. The adherent antibodies were reacted with biotinylated goat anti–rabbit or horse anti–mouse secondary antibodies followed by HRP conjugated to streptavidin (Vector Laboratories, Burlingame, CA). These complexes were visualized with 3,3'-diaminobenzidine (Sigma) as a chromagen and $CuSO_4$ as an enhancer. Sections were then counterstained with Meyers hematoxylin and coverslipped. The stained sections were imaged digitally.

Data Analysis. Data are presented as mean $\pm$ SEM unless stated otherwise and were analyzed for significance by the unpaired one-tailed Student's t-test.

Results and Discussion

The experiments described herein were carried out on TGF-β1$^{-/-}$ mice obtained sequentially, owing to the low frequency of TGF-β1$^{-/-}$ mice in most litters (6, 19). We first looked for evidence of increased systemic production of NO, by assaying for NO_2^- and NO_3^-, the predominant reaction products of NO found in serum (4). We also divided TGF-β1$^{+/+}$ and TGF-β1$^{-/-}$ mice into cohorts of equal age between 10 and 28 d to relate the age of the mice to serum NO_2^- and NO_3^- content as an indicator of systemic NO production. Serum levels of NO_2^- and NO_3^- of TGF-β1$^{-/-}$ mice were generally higher than those of
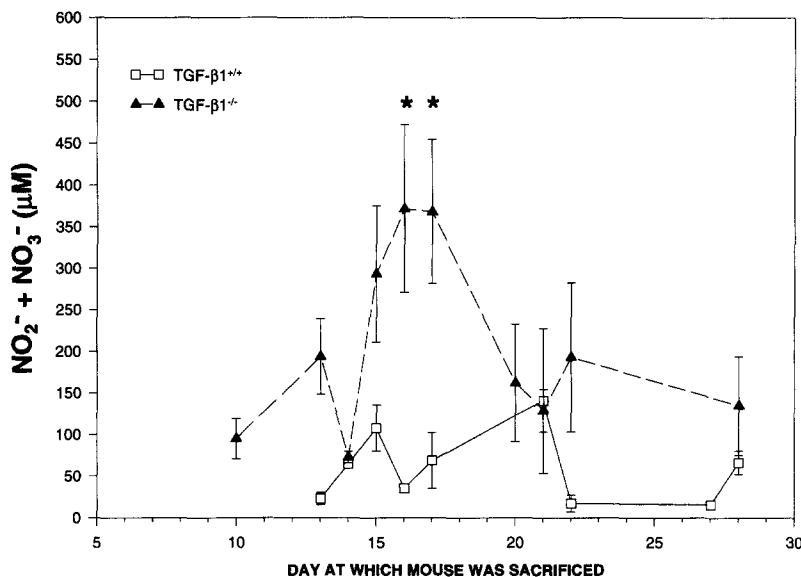

Figure 1. TGF-β1$^{-/-}$ mice exhibit increased levels of NO_2^- and NO_3^- in their sera as compared with TGF-β1$^{+/+}$ mice, peaking between days 15 and 17. TGF-β1$^{+/+}$ mice (*open squares*) or TGF-β1$^{-/-}$ mice (*filled triangles*) were killed upon reaching the indicated ages, and their serum levels of NO_2^- and NO_3^- were determined. Values represent mean $\pm$ SEM of serum samples from 3–5 mice at each time point. *Asterisk*, P <0.05 when compared with TGF-β1$^{+/+}$ mice.

TGF-β1$^{+/+}$ mice between 10 and 28 d of age, peaking between days 14 and 17 (Fig. 1; P <0.05 vs. TGF-β1$^{+/+}$ mice), ~1 wk before death and concomitant with the onset of severe wasting (6, 19).

Thus, the lack of a functional TGF-β1 gene product, with unaffected expression of TGF-β2 and TGF-β3 (A.B. Roberts, unpublished observations), is associated with a fourfold elevation of NO$_2^-$ and NO$_3^-$ in serum (Fig. 1). This spontaneously elevated systemic NO production is quite substantial: although all mice were free from infection (data not shown), the serum levels of NO$_2^-$ and NO$_3^-$ observed in TGF-β1$^{-/-}$ mice are comparable to those of mice injected with *C. parvum* (20) or staphylococcal enterotoxin B (21). The increase in systemic NO production in TGF-β1$^{-/-}$ mice is greatest approximately at the time of weaning (Fig. 1), suggesting an association with decreased maternally transferred TGF-β1 (9).

Systemic NO production in TGF-β1$^{+/+}$ mice was relatively constant over 10–28 d of age, though some fluctuation was observed (Fig. 1, *open squares*). There was a significant drop in the NO$_2^-$ plus NO$_3^-$ content in the serum of TGF-β1$^{-/-}$ mice at 20–28 d of age (Fig. 1). The drop in systemic NO production and iNOS expression after day 17 in the life of TGF-β1$^{-/-}$ mice suggests that mechanisms other than TGF-β1 decrease the expression of iNOS. Although TGF-β1 may be the most potent negative regulator of NO production (5) on the basis of its multiple mechanisms of action in vitro (11, 22, 23), it is only one of a multitude of agents that suppress the expression of iNOS in vitro: the cytokines TGF-β2, TGF-β3, platelet-derived growth factor, fibroblast growth factor, epidermal growth factor, insulin-like growth factor, and interleukins 4, 8, and 10 (5); endogenous corticosteroids (24); *N,N*-dimethyl-arginine (25); thrombin (26); angiotensin II (27); and osteopontin (28). It is unknown whether the absence of TGF-β1 leads to increased expression of any or all of these agents.

The observation that systemic NO production is dramatically increased in TGF-β1$^{-/-}$ mice suggested that this increase was in some way related to the wasting syndrome and death characteristic of these mice (6, 19). We therefore determined whether suppression of NOS enzyme activity would contribute to or correlate with an increase in the life span of these mice. We compared the serum levels of NO$_2^-$ and NO$_3^-$ of untreated TGF-β1$^{-/-}$ mice 14–17 d of age with those of mice that had been either untreated or treated for ~1 wk with L-NMA (Fig. 2), L-NAME (data not shown), or aminoguanidine (data not shown). As can be seen in Fig. 2, 14–17-d-old TGF-β1$^{+/+}$ mice had 69 ± 13 μM NO$_2^-$ plus NO$_3^-$ (*open bars*; n = 12), and TGF-β1$^{+/-}$ mice of similar age had 39 ± 13 μm NO$_2^-$ plus NO$_3^-$ (*gray bars*; n = 6; not significantly different from TGF-β1$^{+/+}$ mice). In contrast, untreated 14–17-d-old TGF-β1$^{-/-}$ mice (*black bars*; n = 15) had 290 ± 52 μM NO$_2^-$ plus NO$_3^-$ (P <0.005 vs. TGF-β1$^{+/+}$ mice). Serum NO$_2^-$ plus NO$_3^-$ levels in TGF-β1$^{-/-}$ mice were reduced to 43 ± 19 μM NO$_2^-$ plus NO$_3^-$ (n = 3) after treatment for 8 ± 1 d with L-NMA; Fig. 2, *diagonally hatched bar*; 14–18 d of age;
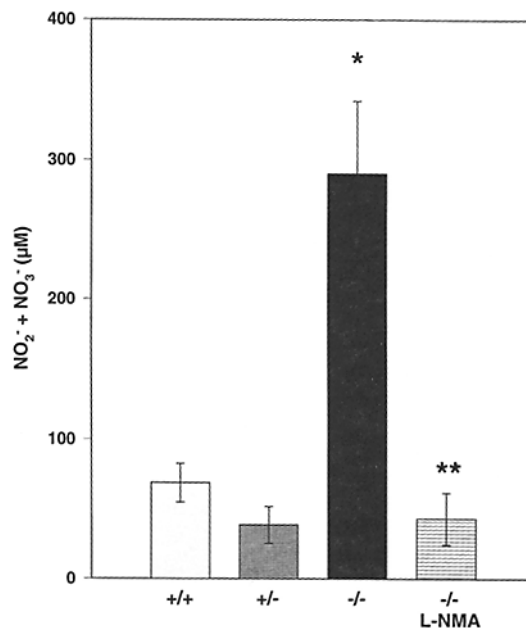

Figure 2. Treatment with L-NMA inhibits the elevated serum levels of NO$_2^-$ and NO$_3^-$ in TGF-β1$^{-/-}$ mice. TGF-β1$^{+/+}$ mice (+/+; *open bar*, n = 12), TGF-β1$^{+/-}$ mice (+/−; *gray bar*, n = 6), TGF-β1$^{-/-}$ mice (−/−; *black bar*, n = 15), or TGF-β1$^{-/-}$ mice treated with L-NMA (−/− + *L-NMA*; *hatched bar*, n = 3) were killed, and their serum levels of NO$_2^-$ and NO$_3^-$ were determined. *Single asterisk*, P <0.005 when compared with TGF-β1$^{+/+}$ mice. *Double asterisk*, P <0.001 when compared with TGF-β1$^{-/-}$ mice.
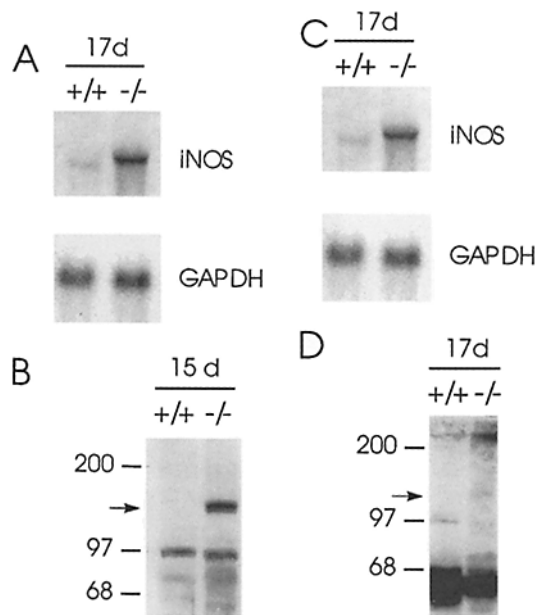

Figure 3. The expression of iNOS mRNA and protein is higher in the heart and kidneys of TGF-β1$^{-/-}$ mice relative to TGF-β1$^{+/+}$ littermates. Mice were sacrificed at the indicated ages, and the hearts (*A* and *B*) and kidneys (*C* and *D*) removed. The organs were processed for Northern blot analysis with a probe specific to iNOS or GAPDH as a control for loading (*A* and *C*) or immunoblot with anti-iNOS antibody (*B* and *D*). Migration of iNOS produced by the mouse macrophage-like cell line RAW264.7 (data not shown) is indicated by the arrow. Experiment is representative of three.

$P < 0.001$ or L-NAME or aminoguanidine (data not shown), all competitive inhibitors of NOS activity (4), demonstrating that the increased levels of NO_2^- and NO_3^- were due to aberrantly high NOS activity and not to another metabolic aberration. Treatment with these agents did not result in increased life span of TGF-$\beta 1^{-/-}$ mice, but long-term treatment with a glucocorticoid or rapamycin resulted in suppression of systemic NO production and was associated with increased life span of TGF-$\beta 1^{-/-}$ mice (Letterio, J.J., Y. Vodovotz, A.G. Geiser, L. Chesler, A. Campbell, and A.B. Roberts, manuscript in preparation).

Our analysis of the age dependence of systemic NO production suggested that either iNOS expression or its activity may be reduced by some mechanism in the days before the death of TGF-$\beta 1^{-/-}$ mice. Homogenates from mice 14–21 d of age showed that iNOS mRNA and protein were expressed at their highest levels in the heart (Fig. 3, A and B, respectively) and kidney (Fig. 3, C and D, respectively) between days 15 and 17 in the life of TGF-$\beta 1^{-/-}$ mice, in parallel with serum NO_2^- and NO_3^- levels (Fig. 1). At later time points, however, iNOS protein expression in the heart and kidney of TGF-$\beta 1^{-/-}$ mice decreased to levels almost indistinguishable from those of TGF-$\beta 1^{+/+}$ littermates (data not shown). We next characterized the expression of iNOS protein at the cellular level by immunocytochemistry. iNOS protein was expressed at low levels in the medulla of TGF-$\beta 1^{+/+}$ mice (data not shown). We did not find significant expression of iNOS in glomeruli of TGF-$\beta 1^{+/+}$ mice (Fig. 4 A). We found that the expression of iNOS protein was increased in the collecting ducts and tubular epithelial cells, but not the glomeruli, of TGF-$\beta 1^{-/-}$ mice relative to its expression in controls (Fig. 4; compare A and B).

The increased expression of iNOS mRNA (Fig. 3 C) and protein (Fig. 3 D and Fig. 4) in the kidney, an organ that is typically not inflamed in TGF-$\beta 1^{-/-}$ mice (6, 19), suggests that the increase in iNOS expression may not solely be due to the inflammatory infiltrate observed in TGF-$\beta 1^{-/-}$ mice. Two mouse models of autoimmunity, that of experimental autoimmune encephalomyelitis (29) and that

of the MRL-lpr/lpr mouse (30), are also associated with increased systemic NO production. Lowrance et al. (31), however, have reported that MRL-lpr/lpr mice have high circulating levels of TGF-$\beta 1$, and thus the relation between an autoimmune phenotype and increased systemic NO production is not clear.

Paradoxically, the increased systemic production of NO by TGF-$\beta 1^{-/-}$ mice may compensate partially for the lack of TGF-$\beta 1$ in maintaining normal lymphocyte proliferation. Like TGF-$\beta 1$ (2), NO can suppress lymphocyte proliferation (32), perhaps explaining the reduced proliferative responses of lymphocytes derived from 14-d-old TGF-$\beta 1^{-/-}$ mice as compared with controls (33). Florquin et al. (21) recently reported elevated systemic levels of NO in a model of shock induced by staphylococcal enterotoxin B. The role of NO in this system was to suppress the proliferation of T lymphocytes: inhibition of NO production in mice undergoing shock increased morbidity owing to increased T lymphocyte proliferation and subsequent production of TNF-α (21).

The increase in iNOS expression in TGF-$\beta 1^{-/-}$ mice is most probably mediated indirectly via increases in the expression of proinflammatory cytokines. Cytokines such as IFN-γ, TNF-α, IL-1, or IL-2 may induce iNOS (4) and are known to be regulated by TGF-$\beta 1$ (2). Shull et al. (19) have reported that TGF-$\beta 1^{-/-}$ mice exhibit increased expression of TNF-α and IFN-γ mRNA, but not IL-1α mRNA, as determined by PCR analysis of liver and lung tissues. Christ et al. (33) have reported elevated IL-2 levels in the thymus of TGF-$\beta 1^{-/-}$ mice. However, a previous study from our laboratory suggested that the expression of IFN-γ mRNA was not altered in TGF-$\beta 1^{-/-}$ mice relative to its expression in controls (8). Future studies must determine the cellular and organ source of the increased circulating levels of metabolites of NO, identify the mechanisms which shut off NO production late in the life of TGF-$\beta 1^{-/-}$ mice, and the direct physiological consequences the increased production of NO has on TGF-$\beta 1^{-/-}$ mice.
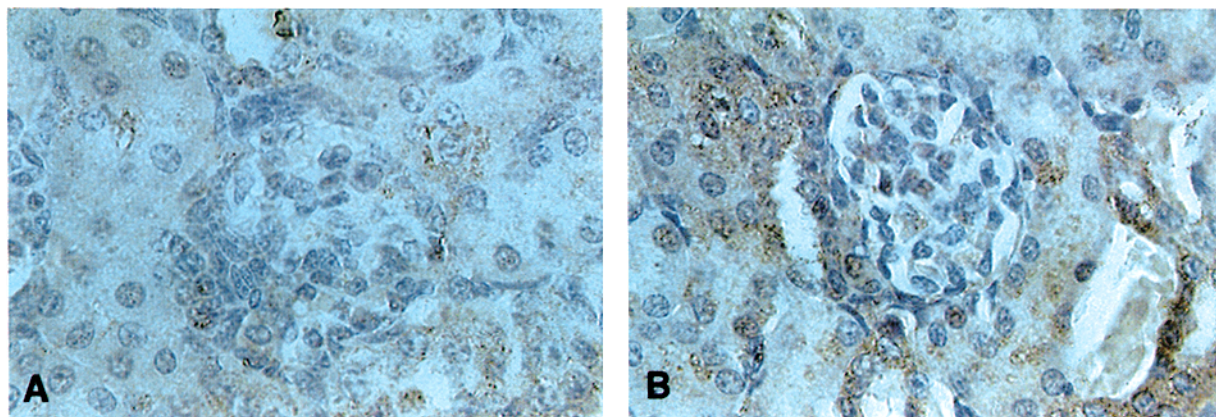

Figure 4. Expression of iNOS protein is increased in kidneys from TGF-$\beta 1^{-/-}$ mice. Mice were sacrificed at the indicated age, and the kidneys were removed and processed for immunocytochemistry with anti-iNOS antibody. (A) Glomerulus from the kidney of a 15-d-old TGF-$\beta 1^{+/+}$ mouse, $\times 1,000$. (B) Glomerulus from the kidney of a 15-d-old TGF-$\beta 1^{-/-}$ mouse, $\times 1,000$. Experiment is representative of four.

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