Interleukin-8 Receptor Knockout Mice Have Subepithelial Neutrophil Entrapment and Renal Scarring following Acute Pyelonephritis

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Interleukin (IL)-8 receptor knockout (KO) mice were shown to have a dysfunctional neutrophil response to urinary tract infection and to develop renal scarring. Intravesical Escherichia coli infection stimulated epithelial chemokine secretion and IL-8 receptor expression in control mice. Neutrophils migrated through the tissues and crossed the epithelial barrier into the urinary tract lumen. In murine IL-8 receptor homologue (mIL-8R) KO mice, infection triggered a chemokine response, and neutrophils were recruited but failed to traverse the mucosal barrier and accumulated under the epithelium. After 7 days, control mice were healthy, and infection was cleared, but mIL-8R KO mice had swollen kidneys, with neutrophil abscesses and high numbers of bacteria. After 35 days, they developed kidney pathology and renal scarring. The results demonstrate that chemokine receptors drive transepithelial neutrophil migration. In their absence, the neutrophils are trapped, and the tissues are destroyed. This molecular deficiency may determine the progression from acute pyelonephritis to renal scarring.

Neutrophils migrate between tissue compartments and exert their effector functions at different sites. They circulate in the blood and interact with the endothelial lining that they cross to reach peripheral tissues. Much attention has focused on the molecular interactions of neutrophils with endothelial cells during the extravasation process [1–3], because this is the key to subsequent pathology and tissue destruction. There is increasing evidence that the fate of neutrophils outside the vascular compartment is governed by specialized molecular interactions distinct from those in the blood vessels [4], but those aspects have received less attention and are not well understood.

Mucosal pathogens trigger a rapid neutrophil response [5,6], and neutrophils are crucial effectors of the host defense at these sites [7,8]. The mucosal neutrophil response is initiated when bacteria stimulate the epithelial cells to secrete chemokines [6,9] and to increase their chemokine receptor expression. The neutrophils respond to the so-formed chemotactic gradient, leave the bloodstream, travel through the submucosa, and reach the basal side of the epithelial barrier, which they cross into the lumen [4,9].

Urinary tract infections (UTIs) are an excellent model to study neutrophil migration between tissue compartments and the fate of the activated neutrophils in peripheral tissues. In response to infection with uropathogenic Escherichia coli, human uroepithelial cells secrete interleukin (IL)-8–, and the expression of CXCR1 and CXCR2 receptors is increased [6,9,10]. Antibodies to IL-8 or CXCR1 both inhibit neutrophil migration across infected uroepithelial cell layers in vitro. Experimental UTI in the mouse causes the secretion of several CC and CXC chemokines into the urine. One of the murine IL-8 homologues, macrophage inflammatory protein (MIP)-2 [11], has been implicated in transepithelial neutrophil migration [4]. Anti–MIP-2 antibody treatment was shown to block this step and to cause neutrophil accumulation under the epithelium [4].

These observations suggest that the IL-8 homologues and their receptors are essential for neutrophil migration across the epithelial barrier. Proving this hypothesis would require stable deletion either of the chemokines or of their receptors. IL-8 does not exist in the mouse, but there are several murine IL-8 equivalents, all of which bind to the murine IL-8 receptor (mIL-8R). Cacalano et al. [12] recently developed the mIL-8R homologue (mIL-8Rh) knockout (KO) mouse and demonstrated that neutrophils from these mice have lost the ability to sense IL-8–like stimuli but retain the ability to migrate in response to other signals and to phagocytose microbes and microbial products [12].

This study examined the effect of chemokine receptor deletion on the neutrophil response to UTI and on tissue pathology, using mIL-8Rh KO mice.

Materials and Methods

Reagents. Polyclonal rabbit anti–murine MIP-2 antibodies were a gift from M. Burdick and R. Strieter (Institute of Internal...
Medicine, Division of Pulmonary and Critical Care Medicine, University of Michigan Medical Center, Ann Arbor). RB6-8CS, a rat IgG2b monoclonal antibody (MAb) specific for murine neutrophils, was a gift from A. Sjöstedt (Umeå University, Umeå, Sweden), W. Conlan (Trudeau Institute, Saranac Lake, NY), and R. Coffman (DNAX Research Institute, Palo Alto, CA). RB6-8CS hybridoma cells were cultured by the Diacult system, and MAb was purified by ammonium sulfate precipitation. The immunoglobulin concentration of the purified MAb was determined by ELISA. The murine MIP-2 ELISA kit was from R&D Systems (catalog no. MM200; Minneapolis), and polyclonal rabbit anti-mouse IL-8R antibodies were from Santa Cruz Biotechnology (catalog no. sc-683; Santa Cruz, CA).

**Bacteria.** *E. coli* 1177 (serotype O1:K1:H7) was isolated from a child with acute pyelonephritis [13]. The strain is virulent in the mouse UTI model and evokes a strong inflammatory host response [14]. It expresses P and type 1 fimbriae but is hemolysin negative. *E. coli* 1177 was maintained in deep agar slabs sealed with sterile paraffin, was passed on tryptic soy agar, was grown overnight in static Luria broth, and was harvested by centrifugation at 50,000 x g for 10 min. The pellet was resuspended in 0.01 M PBS, pH 7.2, to a concentration of 1–2 x 10^8 cfu/mL. The bacterial concentration was confirmed by viable counts.

**Experimental UTI.** Breeding pairs of IL-8R-deficient Balb/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). Because of insertional mutagenesis of the neomycin gene, mIL-8Rh KO mice lack a gene for the putative IL-8 receptor [12]. Mice were bred in the animal facilities at the Department of Microbiology, Immunology and Glycobiology, University of Lund, and female mice were used at 9–13 weeks of age.

Following anesthesia, mice were infected by intravesical inoculation with *E. coli* 1177 (0.1 mL) through a soft polyethylene catheter (outer diameter 0.61 mm; Clay Adams, Parsippany, NJ), as described elsewhere [15]. The catheter was immediately removed, and the mice were allowed food and water ad libitum for 2, 6, or 24 h or for 7 or 35 days. Animals were killed under anesthesia by cervical dislocation. Kidneys and bladders were removed and homogenized (Stomacher 80 homogenizer; Seward Medical, UAC House, London) in sterile disposable plastic bags. The homogenates were diluted in sterile PBS, and 0.1 mL of each dilution was plated on tryptic soy agar. The number of colonies was scored after overnight culture at 37°C.

Urination was induced by gentle pressure on the mouse abdomen, and urine was collected at the urethral orifice into sterile tubes. Urine samples collected before infection were cultured to ensure that the mice were uninfected and were examined for a preexisting neutrophil response. Neutrophils were quantified in unfixed urine, using a hemocytometer chamber. Tissue neutrophils were quantified by histology and immunohistochemistry. Urine samples were centrifuged and stored at –20°C for chemokine analysis. MIP-2 in the urine was quantified by a double ligand method [16].

**Histology and immunohistochemistry.** Kidneys and bladders were obtained from mice killed at 0, 2, 6, and 24 h and 7 days after inoculation. Tissues were cut (3 x 4 x 5 mm), were embedded in OCT compound (Tissue Tek; Miles, Elkhart, IN), were rapidly frozen in liquid nitrogen, and were kept at –80°C. Sections were cut (6 μm) with a steel knife and mounted on glass slides coated with poly-L-lysine. Samples were fixed in freshly prepared 4% paraformaldehyde in PBS (pH 7.4) for 15 min, were rinsed in PBS, and were air dried. For histology, the sections were stained with hematoxylin and eosin.

For MIP-2 immunostaining, the samples were treated with 0.1% saponin (Sigma, St. Louis) in PBS containing 5% normal mouse serum and were incubated with a 1:100 dilution of rabbit anti-mouse MIP-2 antiserum or preimmune serum overnight at 4°C. After incubation with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins (DAKO, Copenhagen), the sections were developed in Fast-Red substrate containing levamisole (DAKO) and finally were stained with Mayer hematoxylin (KEBO Laboratories, Stockholm) [4].

For staining of tissue neutrophils, samples were fixed in 50% and 100% acetone for 5 min each, were incubated with a 1:200 dilution of rat anti-mouse granulocyte MAb (RB6-8CS) or rat IgG (control), were developed with fluorescein isothiocyanate (FITC)-labeled goat anti-rat secondary antibody (STAR69; Serotec, Oxford, UK), and were examined in a fluorescence microscope (Nikon, Garden City, NY) [8].

Murine IL-8R expression was examined in acetone-fixed sections that were stained with polyclonal rabbit anti-mouse IL-8R antibody (3 h at 37°C), were counterstained with FITC-labeled anti-rabbit antibody for 1 h, and were examined in a fluorescence microscope. The normal rabbit IgG was used as a control.

**Definition of tissue damage and renal scarring.** Kidneys and bladders were collected at death and were inspected for the following macroscopic variables: size, color, contour, consistency, and abscesses. After longitudinal sectioning, the structure of the renal pelvis was inspected. Calyceal deformity, pelvis distension, and parenchymal thickness were recorded. Tissue sections were examined by light and fluorescence microscopy for tissue structure and integrity, neutrophil distribution, the presence of fibrosis, and focal scars. The tissue sections were also stained with trichrome, to detect fibrosis.

**Statistical analysis.** Groups were compared by use of the Mann-Whitney unpaired test. P < .05 was considered significant.

**Results**

**IL-8R expression increases after infection of Balb/c but not mIL-8Rh KO mice.** The IL-8R genotype of individual mice was confirmed by polymerase chain reaction, using primers specific for the wild-type murine IL-8R gene homologue or the inserted neomycin cassette (figure 1A). The positive and negative receptor phenotypes of the Balb/c and mIL-8Rh KO mice were confirmed by immunohistochemistry, using polyclonal anti-mIL-8Rh antibodies (figure 1B). Infection of control mice caused an increase in IL-8R expression, with intense staining of the epithelial cells and weaker staining of occasional inflammatory cells in the subepithelial compartment. Maximal epithelial staining was observed after 6 h. The mIL-8Rh KO mice were negative for the IL-8R staining at all times. There was no unspecific background staining of the tissues. The results demonstrate that normal mice up-regulate IL-8R ex pression after infection, with strong receptor staining in the epithelial layer of the renal pelvis.
The mucosal chemokine response to UTI is independent of the IL-8R genotype. The urine MIP-2 concentrations were quantified by ELISA (figure 2). After the infection of the control mice, an increase in urine MIP-2 concentrations was detected by 2 h, a peak was reached after 6 h, and the concentrations returned to low levels by 7 days. In the mIL-8Rh KO mice, urine MIP-2 levels had started to increase by 2 h and then continued to increase until day 7 with no evidence of a decline. Urine MIP-2 levels were higher in the mIL-8Rh KO than in the control mice from 6 h (P < .05).

The cellular origin of MIP-2 was analyzed by immunohistochemistry (figure 3). Infection of Balb/c controls caused an increase in epithelial MIP-2 staining that was strong after 6 h and still visible after 24 h but had disappeared by 7 days. A similar staining pattern was observed both in kidneys (figure 3) and in bladders. This increase in epithelial MIP-2 staining was also observed in the mIL-8Rh KO mice, but, in contrast to the controls, there was no evidence of a decrease over the 7 days. With time, MIP-2 staining spread from the epithelial lining into surrounding tissue compartments. After 7 days, staining was detected throughout kidney and bladder tissues. There was no unspecific staining with pre-immune serum in infected tissue sections. These results demonstrated that mIL-8Rh KO mice had an intact chemokine response to infection and suggested that the IL-8R influences the tissue distribution of the chemokine.

Difference in neutrophil recruitment into kidney and bladder tissues between mIL-8Rh KO and Balb/c mice. The neutrophil influx into the tissues was studied by histology and immunohistochemistry, using kidney and bladder sections from infected mice (figure 4A). Neutrophil numbers in the tissues in urine were quantified by microscopy.

In control mice, an increase in tissue neutrophil numbers
occurred at 2 h, with a peak at 6 h (95 × 10^4/section, mean of 5 sections). Large numbers of neutrophils were scattered throughout the renal tissue, adjacent to the pelvic epithelium, and neutrophils were seen crossing the epithelium into the lumen of the renal pelvis. In the bladders, the highest density of neutrophils was observed at 6 h (250 × 10^4/section, mean of 5 sections). By 7 days, the neutrophil response had declined in both kidneys and bladders.

The neutrophil influx was delayed in the mIL-8Rh KO mice (figure 4B), with few cells at 6 h (<10/section, mean of 5 sections). By 24 h, a few neutrophils were scattered throughout the renal tissue at a distance from the epithelium and were not seen crossing the barrier into the lumen. The neutrophils gradually accumulated in the submucosa and reached very high densities after 7 days (340 × 10^4/section, mean of 5 sections). A similar pattern was observed in bladder sections from mIL-8Rh KO mice. Few neutrophils were seen at 0 and 6 h after infection, but neutrophils accumulated in the tissues for the duration of the experiment.

The blocking of neutrophil migration across the epithelium in the mIL-8Rh KO mice was confirmed by the counting of neutrophils in urine samples obtained at different times after infection. In control mice, urine neutrophil numbers had increased by 2 h (55 × 10^4/mL) and reached a peak by 6 h (260 × 10^4/mL), which was followed by a decrease at 24 h (32 × 10^4/mL) and after 7 days. In mIL-8Rh KO mice, urine neutrophil numbers remained low at all time points (<10 × 10^4/mL). These results demonstrated that the IL-8R is required for transepithelial neutrophil migration.

**Difference in bacterial clearance between Balb/c and mIL-8Rh KO mice.** The bacterial counts in kidneys and bladders are shown in figure 5. In the control mice, infection was cleared after 7 days. The mIL-8Rh KO mice, in contrast, were unable to eliminate bacteria from the tissues. By day 7, the bacterial numbers increased to 10^6 cfu/mL and remained high in the mice that survived until day 35.

**Tissue damage in the mIL-8Rh KO mice.** Aging and dying neutrophils release their cytotoxic contents, so we were interested in studying tissue integrity in mIL-8Rh KO mice that survived infection. Most of the mice developed severe symptoms of acute pyelonephritis and were killed after 7 days, but the few surviving mice were killed on day 35. The macroscopic appearance of kidneys and bladders and the tissue integrity were examined after 7 and 35 days.

Kidneys from control mice (n = 10) were normal (on macroscopic and microscopic examination) after 7 days and had no further change after 35 days (figure 6). In contrast, kidneys and bladders obtained after 7 days from mIL-8Rh KO mice (n = 10) were red and swollen, and some had abscesses (macroscopic) in the pelvic or cortical areas. By microscopy, neutrophils were seen between the blood vessels and the pelvic epithelium. In some areas, the inflammation involved tubuli with edema and occasional obstruction, but the tissue architecture remained mostly intact (figure 6).

Macroscopic inspection of kidneys obtained after 35 days showed less edematous tissue, with pale color, many abscesses, and irregular contours of 1 or both kidneys. By microscopy, sections showed inflammation, with neutrophils throughout the tissues (figure 7). There was fibrosis under the epithelium, in interstitial tissues, and around arterioles and tubuli, and there were large areas of tissue destruction. Abscesses consisted of dead and disintegrated cells surrounded by viable inflammatory cells. The cortex was destroyed, with tubuli having replaced the glomeruli.

Bladders obtained after 35 days from mIL-8Rh KO mice were red and enlarged, with edema and accumulated neutrophils.
Figure 3. Tissue distribution of macrophage inflammatory protein (MIP)-2 in Balb/c control and murine interleukin-8 receptor homologue (mIL-8Rh) knockout (KO) mice after infection. Kidney sections were obtained at different times after inoculation and stained with rabbit anti-mouse MIP-2 antibody, followed by alkaline phosphatase-labeled secondary antibody. Strong epithelial MIP-2 staining was detected in the renal pelvis of control mice, with a peak after 6-24 h. In the mIL-8Rh KO mice, epithelial MIP-2 staining increased from 2 h to 7 days. At later times, MIP-2 staining was detected throughout the tissues. There was no unspecific staining of infected sections with the pre-immune serum. Magnification, ×100.
Figure 4. Neutrophil recruitment to the kidneys of infected mice. Kidney sections were obtained from Balb/c control and murine interleukin-8 receptor homologue (mIL-8Rh) knockout (KO) mice at different times after inoculation and were stained with hematoxylin and eosin (Htx-Eosin; left panels) and with the neutrophil antibody RB6-8C5 (right panels). Arrows indicate the lumen. Magnification, ×200. A, Neutrophil infiltration into the kidneys of Balb/c mice peaked 6 h after infection. B, Neutrophil infiltration into the kidneys was delayed in mIL-8Rh KO mice, but neutrophils accumulated under the epithelium.
Figure 5. Difference in resistance to infection between Balb/c control and murine interleukin-8 receptor homologue (mIL-8Rh) knockout (KO) mice. Bacterial counts in kidney and bladder homogenates from mice killed at 2, 6, or 24 h and 7 or 35 days after intravesical inoculation with Escherichia coli 1177. Bacteria were cleared from control mice after 7 days. Infection persisted in mIL-8Rh KO mice. Nos. are mean ± SE, 8–10 mice/time point, except for the 35-day end point when surviving mice were examined in each group.

Discussion

The neutrophil response to UTI results in so-called “pyuria.” For decades, neutrophils in urine have been recognized as a sign of UTI, and pyuria has been used as a diagnostic tool [17]. The neutrophils are clearly an essential element of the acute cellular response to UTI and were recently shown to be crucial for the clearance of bacteria from the urinary tract [8]. However, their role in chronic inflammation and renal scarring is not well understood. In mice with normal neutrophil function, infection is cleared, and there are no evident tissue sequelae. In the absence of neutrophils, renal tissue remains intact, despite the presence of infection. These observations suggest that tissue damage results not from the presence or absence of the neutrophils themselves but from anomalies of the neutrophil response.

Urinary tract epithelial cells were recently shown to express the CXCR1 and CXCR2 receptors. In vitro infection of human uroepithelial cells was shown to up-regulate chemokine receptor expression and the binding of IL-8 to the infected cells. Neutrophil migration across infected cell layers was blocked by anti-CXCR1 or anti–IL-8 antibodies, which suggests that CXCR1 and IL-8 are crucial for this process [18]. The present study demonstrates that inactivation of the murine IL-8Rh chemokine receptor causes a dysfunctional acute neutrophil response to UTI, which eventually leads to tissue destruction and renal scarring. In normal mice, UTI causes a mucosal chemokine response and an increase in epithelial IL-8R expression. Neutrophils were recruited into the tissues and were seen crossing the mucosal barrier into the lumen. During this time, infection was cleared. In addition, the mIL-8Rh KO mice had a neutrophil response to infection, but their neutrophils, which failed to cross the epithelial barrier, accumulated in the tissues and eventually caused tissue damage that resembles renal scarring in humans. Bacteria remained fully viable, and some animals died of systemic infection. These results show that neutrophil migration across the epithelial layer is IL-8R dependent and that abrogation of this response causes neutrophil accumulation and tissue destruction.

The mIL-8Rh KO mice had intact chemokine production, but with time, a difference in tissue distribution of MIP-2 was noticed in these mice, compared with control mice. The epithelial MIP-2 response and the secretion of MIP-2 into the urine was similar in mIL-8Rh KO and control mice during the first hours after infection, showing that the receptor deficiency did not influence the chemokine response per se. At later times, the MIP-2 response decreased in the controls, but the mIL-8Rh KO mice continued to produce MIP-2, which suggests impaired down-regulation and/or continued stimulation of this response. Parallel studies showed that bacterial clearance is impaired in the mIL-8Rh KO mice [10], which suggests that the bacteria provide a continuing stimulus for the chemokine response. We also noted a difference in MIP-2 tissue distribution between mIL-8Rh KO and control mice at later time points. In control mice, MIP-2 staining was restricted to the epithelium and the inflammatory cells. In the mIL-8Rh KO mice, the chemokine appeared to have diffused out from the epithelial cells into the surrounding tissues. The results suggest that the chemokine receptors are required to sequester the MIP-2 at the site of infection and to set up the chemotactic gradient.

Trapping of neutrophils in the tissues will invariably cause tissue damage as the short-lived neutrophils disintegrate and...
Neutrophils release their toxic components. Neutrophils contain a large arsenal of powerful molecules intended for antimicrobial host defense, but release of these molecules can cause tissue damage and organ failure [19]. Neutrophil granules contain >20 enzymes [20], but serine proteinase, elastase, and 2 metalloproteinases (collagenase and gelatinase) are probably the most powerful direct mediators of local tissue injury. Neutrophil-derived elastase may contribute to the development of acute and chronic tissue injury through cytotoxicity and extracellular matrix degradation [21]. In addition, neutrophil-derived phospholipase products—such as leukotrienes, prostanoids, and platelet-activating factor—and cytokines directly and indirectly cause vascular changes and amplify tissue damage.

This risk for tissue damage makes it crucial to ensure that neutrophils migrate through the tissues and can escape before damage is done. Our studies certainly illustrate this concept for the urinary tract mucosa and the kidney. Abrogation of neutrophil migration across the epithelial barrier was disastrous for tissue integrity, because the trapped neutrophils disintegrated and caused tissue damage. Similar mechanisms may well regulate neutrophil migration across other mucosal barriers, and microbial activation of chemotactic mediators and chemokine receptors may have evolved for this purpose as much for antimicrobial defense per se. Earlier studies have discussed if mucosal surfaces are the “graveyards” where effete neutrophils go to die [22]. Our results support this concept and provide a molecular basis for how this may occur.

Experimental UTIs in the mIL-8Rh KO mice cause both acute and chronic tissue changes that resemble acute pyelonephritis in children and adults. The acute changes included...
Figure 7. Neutrophil accumulation in the tissues of murine interleukin-8 receptor homologue (mIL-8Rh) knockout (KO) mice after 35 days. Sections were obtained from mIL-8Rh KO mice 35 days after inoculation and were stained with RB6-8C5 antibody, to detect neutrophils. A, Section from the renal medulla. B, Section from the renal cortex. Magnification, ×200.
edema, with an increase in overall size, hyperemia, neutrophil influx, and the formation of abscesses that were visible to the naked eye. Tissue sections of these areas showed accumulation of neutrophils but little destruction of surrounding tissue. The acute changes were followed by chronic tissue damage in the mIL-8Rh KO mice. After 35 days, a reduction in overall renal size occurred, and the kidneys were pale, reflecting a reduced blood flow. Areas of scar tissue caused an irregular kidney outline. There was parenchymal thinning, with loss of cortical tissue in large areas, as well as fibrosis and diffuse inflammatory infiltrates. These acute and chronic changes make the mIL-8Rh KO mice a unique model for studying the natural history and mechanisms of renal scarring.

Several experimental approaches have been taken to study the mechanisms of renal scarring. Earlier models have required surgical manipulations or other invasive procedures to render the tissues vulnerable to infection [23, 24]. Anti-inflammatory drugs or oxygen radical scavengers have been used to modify the inflammatory response to UTI and to link the inflammatory response to the scarring process [17], but tools to directly address the role of neutrophils have not been used. Different lymphocyte populations accumulate in the kidney, and immune mechanisms have been proposed to contribute to tissue destruction [23, 25]. Our findings show that the chemokine receptor dysfunction renders the mIL-8Rh KO mice susceptible to UTI, and that dysfunctional neutrophil recruitment into the kidney is a powerful mechanism of tissue destruction.

About 30% of children with acute pyelonephritis have >1 recurrence, and, of them, ~60% continue to recur, and some go on to develop renal scarring [26]. The defects that render these patients susceptible to infection and the molecular mechanisms of renal scarring are poorly understood. The present study suggests that defects in chemokine receptor expression cause dysfunctional neutrophil migration and tissue damage. In preliminary studies, we examined chemokine receptor expression on neutrophils from children with recurrent UTI and renal scarring. These studies demonstrated that cell surface expression of the CXCR1 receptor is low in children with recurrent episodes of acute pyelonephritis, compared with age-matched controls [10].

The following scenario may now be proposed. Infection of the urinary tract triggers the secretion of epithelial chemokines and expression of chemokine receptors. Neutrophils are recruited into the kidneys and bladders and kill the bacteria on their way through the tissues. There is no tissue damage as the neutrophils rapidly cross the mucosal barrier and are excreted in the urine. In the absence of functional chemokine receptors, neutrophils are trapped, tissues are damaged, and renal scarring may develop. In addition, the clearance of bacteria from the tissues is impaired. We have shown that one specific molecular deficiency may underlie both disease susceptibility and the tendency to develop tissue pathology. In addition, the results underline the important role of innate defense mechanisms in both the prevention and promotion of disease. This work therefore provides a new approach for studying the pathogenesis of scarring and to understand its basis in this important group of patients.

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