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*J Immunol* 2004; 172:3736-3744; doi: 10.4049/jimmunol.172.6.3736

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The *Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Mice Transgenic for IL-1 Receptor Antagonist Protein Are Resistant to Herpetic Stromal Keratitis: Possible Role for IL-1 in Herpetic Stromal Keratitis Pathogenesis

Partha S. Biswas, Kaustuv Banerjee, Bumseok Kim, and Barry T. Rouse

Ocular infection with HSV may result in the blinding immunoinflammatory lesion stromal keratitis (SK). This represents a CD4+ T cell-mediated immunopathologic lesion in both humans and a mouse model. Early events in the pathogenesis that set the stage for SK are poorly understood. The present study evaluates the role of IL-1 using a transgenic mouse that overexpresses the IL-1 receptor antagonist (IL-1ra) protein. Such transgenic mice were markedly resistant to SK compared with IL-1ra−/− and C57BL/6 control animals. The resistance was shown to be the consequence of reduced expression of molecules such as IL-6, macrophage-inflammatory protein-2, and vascular endothelial growth factor, normally up-regulated directly or indirectly by IL-1. A critical event impaired in IL-1ra transgenic mice was vascular endothelial growth factor production with a consequent marked reduction in angiogenesis, an essential step in SK pathogenesis. Targeting IL-1 could prove to be a worthwhile therapeutic approach to control SK, an important cause of human blindness. The Journal of Immunology, 2004, 172: 3736 –3744.

Herpes simplex virus infection is a major cause of vision loss (1). This results mainly from a chronic immunoinflammatory reaction in the normally transparent and avascular cornea. A complex of humoral and cellular events is involved in the pathogenesis of stromal keratitis (SK), but the critical event responsible for clinically evident lesions is CD4+ T cell-mediated immunopathology (2, 3). Before the T cell-mediated immunoinflammatory phase, multiple events occur after virus infection that set the stage for the subsequent pathology. These events include the production of proinflammatory cytokines and chemokines and a prominent invasion of the cornea by polymorphonuclear leukocyte (PMN) (4, 5). The later response appears protective and helps clear virus (4, 5). However, PMN invasion also contributes to pathology because the cells are a major source of angiogenesis factors (6) and perhaps also damaging factors such as NO (7). Neovascularization represents a major step in SK pathogenesis, and multiple molecules are involved in this process (8). It is not clear how HSV infection, which in the mouse model is usually confined to the corneal epithelium, results in neovascularization into the underlying stroma. Thus, once a cell is productively infected by HSV, most cellular proteins cease production (9). Exceptions include IL-6 (10, 11) and probably IL-1 (12), which are briefly induced by HSV infection by mechanisms that remain undefined. These cytokines could represent key molecules that set off a cascade of events that culminate in the clinically evident immunoinflammatory lesions. After HSV infection, both IL-1 and IL-6 have been shown to be eminently present after peak levels at day 10, and then to diminish over the next few days (13, 14). In consequence, counteracting IL-1 and IL-6 could be a valuable control measure at least if performed early after infection. The aim of this study was to determine whether inhibition of IL-1, as could be achieved by using a transgenic mouse that overexpressed the IL-1 receptor antagonist (IL-1ra), had an effect on SK pathogenesis. Such transgenic mice have been used in other inflammatory models and were shown to block IL-1 and diminish disease expression (15–18). Our results with ocular infection with HSV demonstrate that IL-1ra transgenic (IL-1ra Tg) mice developed significantly milder disease and reduced corneal angiogenesis compared with IL-1ra−/− and C57BL/6 mice. This difference in disease phenotype was an indirect event and was shown to be the consequence of changed expression of molecules such as IL-6, macrophage-inflammatory protein-2 (MIP-2), and the angiogenesis factor vascular endothelial growth factor (VEGF) normally up-regulated by IL-1 during an inflammatory process (19–21). Our results demonstrate that IL-1 production is a critical event in SK pathogenesis and that inhibiting this cytokine represents a valuable approach for disease control.

Materials and Methods

Mice

IL-1ra Tg (T14 hemizygous line) and knockout mice were kindly provided by D. Hirsh (Department of Biochemistry and Molecular Biophysics, College of Physician and Surgeons, Columbia University, New York, NY). Wild-type female 4- to 5-wk-old C57BL/6 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). Animals were sex and age matched for all experiments. All manipulations involving the immunocompromised mice were performed in a laminar flow hood. To prevent bacterial superinfections, all mice received prophylactic treatment with sulfamethoxazole-trimethoprim (Barre-National, Baltimore, MD). All experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

Virus

HSV-1 RE (obtained from R. Hendricks Laboratory, University of Pittsburgh School of Medicine, Pittsburgh, PA) was used in the present study.
The virus was propagated and titered on monolayers of Vero cells (American Type Culture Collection, Manassas, VA; catalogue CCL81) using standard protocols (22). Infected Vero cells were harvested, titered, and stored in aliquots at −80°C until used.

**Corneal HSV-1 infection**

Corneal infections of all mice groups were conducted under deep anesthesia induced by i.p. injection of avertin (Sigma-Aldrich, St. Louis, MO). Mice were scarified on their corneas with a 27-gauge needle, and a 4-μl drop containing the required dose of virus was applied to the eye and gently massaged with the eyelids.

**Clinical observations and angiogenesis scoring**

The eyes were examined on different days postinfection by a slit-lamp biomicroscope (Kowa, Nagoya, Japan), and the clinical severity of keratitis of individually scored mice was recorded, as described before (8). Angiogenesis severity was measured, as described previously (23). Briefly, a grade of 4 for a given quadrant of the circle represents a centripetal growth of 1.5 mm toward the corneal center. The score of the four quadrants of the eye was then summed to derive the neovascularization index (range 0–16) for each eye at a given point.

**In vitro stimulation of corneal epithelial cell culture**

Isolation of corneal epithelial cells was performed, as described before (24). Briefly, corneal buttons were incubated at 37°C in PBS containing 10 mM sodium dithionitroacetate dehydrate (Sigma-Aldrich) for 45 min. After incubation, the epithelial cell layer was separated from the adjacent corneal tissue by gentle teasing with a fine forceps. The intact epithelial cells were washed several times in PBS, followed by treatment with collagenase D (Roche, Basel, Switzerland) at 37°C for 30 min. The disrupted epithelial cells were then passed through a 40-μm filter to make a single cell suspension. Cells were counted and suspended in F–12K medium (American Type Culture Collection) with 10% FCS and plated in 48-well tissue culture plates at 37°C in 5% CO₂. Purity of cultured corneal epithelial cells was determined by flow cytometry using anti-K12 Ab (kindly provided by W. Kao, University of Cincinnati, Cincinnati, OH). The corneal epithelial cell culture yielded 75–80% purity.

Adherent monolayer of epithelial cells was stimulated with different doses of murine rIL-1α and rIL-1β (R&D Systems, Minneapolis, MN) in serum-free F–12K medium for 24 and 48 h. After incubation, the supernatant was collected and stored at −80°C until further use. For blocking studies, different doses of anti-murine IL-1α Ab (BD Pharmingen, San Diego, CA) were mixed with 800 pg (dose selected on the basis of dose–response curve) of IL–1α. Rat IgG was used as isotype control.

**Conical intrastromal injection assay**

Conical intrastromal injection was performed, as described before (25). Under direct stereomicroscopic observation, a nick in the epithelium and anterior stroma of mouse cornea was made with a one-half-inch 30-gauge needle with a 30° bevel in the mid periphery. Eight eyes were injected per group. The needle was introduced into the corneal stroma and advanced 1.5 mm to the corneal center. Two microliters of solution containing the required concentration of murine rIL–1α (100, 200, and 400 ng) (BD Pharmingen) and anti-murine VEGF Ab (5 μg) (26) (R&D Systems) was forcibly injected into the stroma to separate the corneal lamellae and disperse the solution. PBS was used as control.

**Subconjunctival inoculations**

Subconjunctival inoculation of murine rIL–6 (50 ng) (27) (BD Pharmingen) and anti-murine IL–6 Ab (5 μg/μl) (27) was performed, as described previously (27). Briefly, subconjunctival inoculations were done using a 2-cc 23-gauge needle and syringe (Hamilton, Reno, NV) to penetrate the perivascular region of conjunctiva and deliver 4 μl into the subconjunctival space. Control mice received PBS. Mice received murine rIL–6 1 day before corneal infection with HSV–1 RE.

**Viral titration**

Eye swabs were taken from the infected corneas (three mice/group) using sterile cotton swabs soaked in DMEM containing 10 IU/ml penicillin and 100 μg/ml streptomycin. All manipulations are done under sterile conditions. Swabs were stored in tubes containing serum-free DMEM at −80°C. For detection of virus, samples were thawed and vortexed. Duplicate samples (200 μl) were plated on Vero cells grown to confluence in 24-well plates at 37°C in 5% CO₂ for 90 min. Medium was aspirated, and 500 μl of DMEM containing 1% low melting point agarose was added to each well. Titer was calculated as log₁₀ PFU/ml as per standard protocol (22).

**Histopathology**

For histopathological analysis, eyes were extirpated and fixed in 10% buffered neutral formalin. Staining was performed with H&E (Richard Allen Scientific, Kalamazoo, MI).

**RT-PCR**

Total RNA from four corneas per time point was extracted by using Tri-reagent (Molecular Biology, Cincinnati, OH). Total RNA (1 μg) was reverse transcribed using murine leukemia virus reverse transcriptase (Life Technologies, Bethesda, MD) with oligo(dT) as primer (Invitrogen, San Diego, CA). All cDNA samples were aliquoted and stored at −20°C until further use. PCR was performed in PTC-100 Programmable Thermal Controller (MJ Research, Cambridge, MA) using Hot Start PCR Master Mix (Promega, Madison, WI). The primers used were murine GAPDH forward, CACCTGCAACCACTGGTCAAG, and reverse, GCGTCGTTTACACCTTCTTGATG, and murine IL–1α forward, TAGACATGGTGCTATTTGAC, and reverse, GAGGTCACAGGAGCTTGAC.

**Flow cytometry**

Single cell suspensions were prepared from four corneas at 24 and 48 h postinfection, as described elsewhere (28), with some modifications. Briefly, corneal buttons were incubated with collagenase D (Roche) for 60 min at 37°C in 2% horse serum, 5% CO₂. All incubated eyes were disrupted by grinding with a syringe plunger and passing through a cell strainer. Cells were washed and suspended in RPMI 1640 with 10% FBS. The Fc receptors on the cells were blocked with unconjugated anti–CD16/32 (BD Pharmingen) for 30 min. Samples were incubated with FITC-labeled anti–Gr1 Ab (clone RB6-8C5; BD Pharmingen) and isotype controls for 30 min. All samples were collected on a FACSan (BD Biosciences, San Diego, CA), and data were analyzed using CellQuest 3.1 software (BD Biosciences).

**Cytokine ELISA of corneal lysate**

For preparation of corneal lysates, six corneas per time point were pooled and minced. All procedures were done on an ice bath. Minced pieces were collected in 1 ml of DMEM without FCS and homogenized using a tissue homogenizer (PRO Scientific, Monroe, CT) four times, 15 s each, with a gap of 1 min between homogenization to allow the sample to cool. The lysate was then clarified by centrifugation at 14,000 rpm for 5 min at 4°C. The supernatant was collected and used immediately or stored at −80°C until further use. Lysates were analyzed using a standard sandwich ELISA protocol. Anti–IL–6 capture and biotinylated detection Abs were from BD Pharmingen (clone MPS–20F3), and standard murine rIL–6 was from R&D Systems. Anti–MIP–2 and anti–VEGF164 capture and biotinylated detection Abs and recombinant standards for murine MIP–2 and murine VEGF164 were from R&D Systems. The color reaction was developed using ABTS (Sigma–Aldrich) and measured on an ELISA reader (SpectraMax 340; Molecular Devices, Sunnyvale, CA) at 405 nm. The detection limit was 2 pg/ml. Quantification was performed with Spectramax ELISA reader software version 1.2.

**Quantitative real-time PCR**

Total RNA from four corneas per time point was extracted by using RNeasy RNA extraction kit (Qiagen, Valencia, CA). Briefly, tissues were lysed in RLT buffer, and RNA was purified following manufacturer’s instructions (Qiagen). DNase treatment (Qiagen) was done to remove any contaminating genomic DNA. To generate cDNA, 1 μg of total RNA was reverse transcribed using murine leukemia virus reverse transcriptase (Life Technologies) with oligo(dT) as primer (Invitrogen), according to manufacturer’s instructions. All cDNA samples were aliquoted and stored at −20°C until further use. Real-time PCR was performed using a DNA Engine Opticon (MJ Research). PCR was performed using SYBR Green I reagent (Qiagen), according to manufacturer’s protocol. PCR amplification of housekeeping gene, murine GAPDH, was done for each sample as a control for sample loading and to allow normalization between samples. A standard curve was constructed with PCR-H topo cloning vector (Invitrogen) containing the inserted fragment amplified by the SYBR I system. PCR for each sample was analyzed in three dilutions in duplicate for both GAPDH and target gene. The target gene was then normalized to 10⁶ copies of GAPDH control, and data were represented as copy numbers/10⁶ GAPDH. The primers used were murine GAPDH forward, CACCTGCAACCACTGGTCAAG, and reverse, GCGTCGTTTACACCTTCTTGATG, and murine VEGFR–2 forward, TGTCACAGGGCTGGTAAAG and reverse, CAAACAGCTTTAAATCTCGAGACTTG.
**Results**

**IL-1ra Tg mice show reduced SK and corneal angiogenesis**

Three groups of genetically different mice, IL-1ra Tg, IL-1ra\(^{-/-}\), and wild-type C57BL/6, were evaluated clinically for the development of SK following HSV-1 (5 x 10\(^6\) PFU) corneal infection over a 20-day test period. Naive and infected corneas of IL-1ra Tg mice demonstrated higher level of IL-1ra mRNA in comparison with wild-type control (Fig. 1). As shown in Fig. 2A, whereas the pattern and severity of SK in both IL-1ra\(^{-/-}\) and wild-type C57BL/6 mice were similar (mean scores 3.5 and 2.7, respectively), SK in IL-1ra Tg mice was strikingly reduced (mean score of 1.4) (Fig. 2A). In addition, while 75% of eyes of IL-1ra\(^{-/-}\) mice developed clinically evident lesions (score 3 or greater), only 25% of IL-1ra Tg mice developed such lesions (data not shown). Attempts to infect IL-1ra Tg mice with a higher virus dose (10\(^7\) PFU) resulted in lethality, but still few developed SK of ≥3 (data not shown). Histopathological analysis of representative eyes of IL-1ra Tg mice revealed mild inflammatory changes and cellular infiltrations in the corneal stroma and epithelium at day 20 postinfection (p.i.) (Fig. 2B). However, both IL-1ra\(^{-/-}\) and C57BL/6 mice developed more severe inflammatory changes (Fig. 2B).

One characteristic of corneal HSV-1 infection is neovascularization into the normally avascular corneal stroma, an event deemed necessary for the full expression of SK (29). In comparison with the extent of angiogenesis following HSV infection in IL-1ra\(^{-/-}\) and C57BL/6, the eyes of IL-1ra Tg mice revealed marked reduction of this process (Fig. 3, A and C). By day 20 p.i., the angiogenesis score was greater than 10 in 13 of 16 IL-1ra\(^{-/-}\) eyes and 8 of 16 C57BL/6 mice (Fig. 3B), but only 4 of 16 eyes of IL-1ra Tg mice had such a score (Fig. 3B). Taken together, these results indicate that overexpression of IL-1ra protein led to diminished angiogenesis as well as SK.

**PMN inflammatory response in IL-1ra Tg mice**

A major event following ocular infection with HSV is a prompt PMN influx into the corneal stroma (4, 5). This reaction at 24 and 48 h p.i. was significantly less in IL-1ra Tg mice compared with either IL-1ra\(^{-/-}\) or wild-type mice (Fig. 4, Table I). PMN are considered as involved in antiviral defense (4, 5). In line with this notion, viral clearance was impaired in IL-1ra Tg mice compared with IL-1ra\(^{-/-}\) and wild-type mice (Table II). Accordingly, virus was present in ocular swabs for ~2 extra days in IL-1ra Tg mice (Table II).

One reason considered for the scant PMN response in IL-1ra Tg mice was that IL-1 must, in normal situations, serve to induce molecules that are directly or indirectly chemotactic for PMN. Based on the results of others (27, 30), prime candidates considered were IL-6 and MIP-2. Levels of these proteins were compared in the three mouse strains at various time points after HSV infection. Both IL-6 and MIP-2 protein levels in corneal extracts were significantly less in IL-1ra Tg mice compared with IL-1ra\(^{-/-}\) and wild-type animals at all time points analyzed (Fig. 5). In addition, if murine rIL-6 protein was injected subconjunctivally into IL-1ra Tg animals 1 day before corneal infection, there was a ~4-fold increase in PMN influx in HSV-infected IL-1ra Tg mice compared with controls given PBS (Fig. 6A, Table III). Such eyes demonstrated a higher IL-6 level in comparison with PBS control at day 2 postinjection (data not shown). Furthermore, IL-6 reconstitution also resulted in significantly higher levels (p < 0.05) of the chemokine MIP-2 in corneal extracts (Fig. 6B).

To evaluate which corneal cells might act as a source of IL-6 upon IL-1 stimulation, corneal epithelial cell cultures were stimulated in vitro with different doses of rIL-1\(\alpha\) and rIL-1\(\beta\). IL-1\(\alpha\), but not IL-1\(\beta\), could induce IL-6 production from corneal epithelial cells in a dose-dependent manner at 24 and 48 h (Fig. 7A). The presence of neutralizing Ab against murine IL-1\(\alpha\), but not control IgG, blocked the IL-6 production from these cells in a dose-dependent manner (Fig. 7B).

**Statistical analysis**

A standard Student’s t test was used for statistical analysis.
Environment favorable for corneal angiogenesis is severely compromised in IL-1ra Tg mice

As noted above, IL-1ra Tg mice developed markedly diminished angiogenic responses compared with either IL-1ra knockout (K/O) or C57BL/6 mice. Protein levels of VEGF were significantly lower (p < 0.05) in IL-1ra Tg mice compared with IL-1ra K/O and C57BL/6 animals at all time points analyzed (Fig. 8A). In addition, real-time PCR demonstrated a significantly higher level of VEGFR-2 mRNA transcript in IL-1ra K/O and C57BL/6 mice than in IL-1ra Tg animals (Fig. 8B).

Previous studies demonstrated that IL-1α could up-regulate the angiogenesis factor VEGF in human PBMC in vitro (20). To demonstrate the angiogenic activity of IL-1α in murine cornea, different doses of murine rIL-1α were injected intrastromally, and the extent of angiogenesis was measured at 2 and 4 postinjection. Corneal ELISA revealed a significant increase in IL-1α level at day 2 postinjection in comparison with PBS control (data not shown).

Table I. IL-1ra Tg mice cornea has less number of Gr-1<sup>+</sup> cells than IL-1ra knockout and C57BL/6

<table>
<thead>
<tr>
<th>Mice</th>
<th>24 h p.i.&lt;sup&gt;a&lt;/sup&gt;</th>
<th>48 h p.i.&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1ra Tg</td>
<td>750 ± 103&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1250 ± 169&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-1ra knockout</td>
<td>3233 ± 345</td>
<td>5523 ± 478</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>3002 ± 298</td>
<td>4172 ± 534</td>
</tr>
</tbody>
</table>

<sup>a</sup> Single cell suspensions of corneal cells were prepared from four eyes infected with 5 × 10<sup>6</sup> PFU HSV-1 RE at 24 and 48 h p.i. The cells were counted and stained with Gr-1 FITC Ab, and numbers of Gr-1-positive cells were enumerated by FACS. Results were shown as mean ± SD of three individual experiments.

<sup>b</sup> Statistically significant (p < 0.05) difference in comparison with IL-1ra<sup>−/−</sup> and IL-1ra Tg mice.
shown). Interestingly, IL-1α induced a dose-dependent angiogenic response (Fig. 9). In such eyes, both IL-6 and VEGF protein levels were elevated (Fig. 10). Administration of neutralizing Ab against

**Table II.** Delayed viral clearance in IL-1ra Tg mice in comparison with IL-1ra−/− and C57BL/6

<table>
<thead>
<tr>
<th>Days p.i.</th>
<th>IL-1ra Tg</th>
<th>IL-1ra−/−</th>
<th>C57BL/6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.3 ± 0.6</td>
<td>6.7 ± 0.3</td>
<td>5.8 ± 0.8</td>
</tr>
<tr>
<td>3</td>
<td>4.9 ± 0.5</td>
<td>3.8 ± 0.6</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>3.2 ± 0.3</td>
<td>2.1 ± 0.2</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>7</td>
<td>2.4 ± 0.5</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>9</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
</tr>
</tbody>
</table>

* Mice were infected with $5 \times 10^6$ PFU HSV-1 RE. Virus titer was estimated by standard plaque assay from swabs taken from infected corneas (n = 4) at indicated time points.

* p < 0.05.

* UD, Undetectable, i.e., below the sensitivity of the assay (<10 PFU/ml).

**Table III.** Recombinant murine rIL-6-reconstituted IL-1ra Tg cornea has more Gr-1+ cells than PBS control following HSV-1 RE infection

<table>
<thead>
<tr>
<th>Mice</th>
<th>24 h p.i.</th>
<th>48 h p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1ra Tg (IL-6 50 ng)</td>
<td>2388 ± 532</td>
<td>3996 ± 429</td>
</tr>
<tr>
<td>IL-1ra Tg (PBS)</td>
<td>913 ± 269</td>
<td>1447 ± 381</td>
</tr>
</tbody>
</table>

* IL-1ra Tg mice were subconjunctivally injected with murine rIL-6 (50 ng) 1 day before corneal HSV-1 RE ($5 \times 10^6$ PFU) infection. PBS was used as negative control. Suspensions of corneal cells were prepared from four eyes infected at 24 and 48 h p.i. The cells were counted and stained with FITC-labeled anti-Gr-1 Ab, and numbers of Gr-1-positive cells were enumerated by FACS. Results were shown as mean ± SD of three separate experiments (six corneas/time point).

* Significant difference (p < 0.05) compared with PBS treatment.
murine VEGF could abrogate IL-1α-induced angiogenesis, demonstrating the key involvement of VEGF in this process (Fig. 11A). Such eyes demonstrated diminished VEGF level in comparison with IgG control (data not shown). To rule out the possible involvement of IL-6 in the IL-1α-mediated corneal angiogenesis, anti-IL-6 Ab was administered with IL-1α protein. Interestingly,

FIGURE 7. IL-1α-induced IL-6 production from corneal epithelial cells. A, Corneal epithelial cells were stimulated with different doses of murine rIL-1α for 24 and 48 h. Supernatants were collected, and levels of IL-6 production were measured by an Ab capture ELISA, as outlined in Materials and Methods. Results are expressed as mean ± SD of three separate experiments. *, Significant difference (p < 0.05) compared with medium control. B, Corneal epithelial cells were stimulated with 800 pg of murine rIL-1α along with different doses of anti-IL-1α Ab for 24 h. Supernatants were collected, and levels of IL-6 production were estimated by an Ab capture ELISA, as outlined in Materials and Methods. Results are expressed as mean ± SD of three separate experiments (six corneas/time point). **, Significant difference (p < 0.05) compared with rat IgG control. ***, Significant difference (p < 0.05) compared with medium control.

FIGURE 8. Reduced VEGF protein levels and diminished expression of VEGFR-2 mRNA expression in HSV-1 RE-infected corneas of IL-1Ra Tg mice. A, At indicated time points, six corneas/group were processed to measure the VEGF protein levels. Levels of VEGF were estimated from supernatants of corneal lysates of mice infected with 5 × 10⁶ PFU HSV-1 RE by an Ab capture ELISA, as outlined in Materials and Methods. Results are expressed as mean ± SD of three separate experiments (six corneas/time point). *, Statistically significant differences in VEGF level (p < 0.05) were observed between IL-1α Tg mice to either IL-1α knockout (K/O) or C57BL/6 mice. B, At 2, 5, and 7 days postinfection, four corneas/group were processed for the extraction of cellular mRNA. Real-time PCR analysis was conducted to detect the VEGFR-2 mRNA expression in corneas of mice infected with 5 × 10⁶ PFU of HSV-1, as described in Materials and Methods. Results are expressed as mean ± SD of three separate experiments (six corneas/time point). **, Statistically significant differences in VEGFR-2 expression (p < 0.05) were observed between IL-1α Ta Tg mice to either IL-1α knockout (K/O) or C57BL/6 mice. B. Copy number of the target gene was normalized to 10⁶ copies of GAPDH.
the presence of neutralizing Ab against IL-6 reduced, but did not abrogate, IL-1α-induced corneal angiogenesis (Fig. 11B).

Discussion
This study deals with early events in the pathogenesis of the blinding immunoinflammatory lesion SK caused by ocular infection with HSV. Our results demonstrate that transgenic mice that overexpress the IL-1ra protein are markedly resistant to SK compared with control C57BL/6 mice. The resistance appeared to be the consequence of a reduced expression of molecules usually induced by IL-1, which in turn participate in inflammatory cell recruitment and angiogenesis. In the present study, we evaluate the critical role of IL-1 using a transgenic mouse that over expresses IL-1ra protein. Thus, IL-1ra Tg mice had reduced levels of IL-6, the chemokine MIP-2, as well as the angiogenesis factor VEGF and its receptor VEGFR-2. In consequence, the IL-1ra Tg mice showed less PMN influx and neovascularization than did control C57BL/6 animals. Our results imply that blocking IL-1 activity could represent a valuable therapeutic approach to control SK.

One striking difference noted between C57BL/6 control and IL-1ra Tg mice was a marked difference in PMN invasion 2 days post-HSV infection. This early PMN response was shown previously to be in part responsible for viral clearance (4, 5). This viewpoint was also supported by the present studies. Thus, IL-1ra Tg mice, which expressed a diminished PMN response, also showed a delay in viral clearance. How IL-1 in normal infected mice causes PMN influx remains unclear. However, because IL-1 itself is not chemotactic for PMN (31), the effect must be indirect, with IL-1 inducing one or more chemokines. A likely candidate is MIP-2, because MIP-2-neutralized mice develop minimal PMN responses to ocular HSV infection (30). The results of the present study indicate that IL-1 did induce MIP-2, but this was an indirect
This process may be brief because, as HSV infection results in the autocrine production of IL-1 as well as other chemokines, such as MIP-1, were shown by others to be induced by IL-6. This process can be largely inhibited by anti-VEGF Ab. Furthermore, intrastromal injection of IL-1α was shown to induce VEGF in the cornea. These observations as well as others in tumor systems (20, 36) indicate that IL-1 can cause cells to produce VEGF.

Even though IL-1 produced from HSV-infected cells may act as an inducer for VEGF production, an additional VEGF stimulant appeared to be mediated by the cytokine IL-6. Thus, we demonstrated that corneal cells exposed to IL-1 in vitro produce IL-6, and IL-6 was shown previously to be a strong agonist for VEGF production (25, 37, 38). Further support that IL-1-induced angiogenesis depended in part on IL-6 production was the observation that it was partially blocked by anti-IL-6 Ab. The relative importance of the direct and indirect effect of IL-1 on VEGF production and angiogenesis remains to be evaluated.

Finally, our results demonstrated that corneal RNA samples from transgenic mice showed diminished VEGFR-2 levels measured by real-time PCR. In tumor systems, IL-1 was shown to up-regulate VEGFR-2 expression on endothelial cells (39), the latter known to be critical for pathological angiogenesis (40). Evidently, the absence of IL-1 activity, as occurred in IL-1α Tg mice, results in reduced expression of VEGFR-2. The consequence is lack of stimulation for the endothelial cells to proliferate, resulting in reduced angiogenesis. However, it is not clear why transgenic mice demonstrated reduced VEGF expression. We believe there may be two possibilities: first, IL-1 may directly up-regulate VEGFR-2 expression on corneal endothelial cells, and blocking its activity by specific antagonist protein results in reduced receptor expression; second, reduced proliferation of endothelial cells as a consequence of diminished VEGF and inflammatory response in these mice. We are currently attempting to ascertain which of these two mechanisms accounts for the major effect of IL-1 on angiogenesis.

Taken together, our results support the hypothesis that the inflammatory milieu and angiogenic stimuli created early after infection play an important role in HSV-induced ocular lesions. An important participant of this environment is IL-1. Antagonizing the effect of IL-1 by a specific receptor antagonist protein abrogates consequences of IL-6 induction. In support, IL-1α was shown to induce IL-6 in vitro in corneal epithelial cells. In addition, MIP-2, as well as other chemokines, such as MIP-1α and monocyte chemotactrant protein-1, were shown by others to be induced by IL-6 (27, 32). Our studies also demonstrated that IL-1 was up-regulated in virus-infected corneal epithelial cells (data not shown). This observation as well as others (10–12) indicate that HSV infection results in the autocrine production of IL-1 as well as IL-6. This process may be brief because, as HSV infection proceeds, host mRNA synthesis and protein translation are inhibited in productively infected cells (9). However, IL-1 also drives IL-6 production by uninfected cells, which, together with that produced by infected cells themselves, sets off a cascade of events that result in PMN influx. Supporting the scheme, we could show that the provision of exogenous IL-6 intrastromally to the transgenic mice reconstituted the PMN invasion.

Although PMN play a protective function in HSV ocular infection, they also participate in lesion expression and act as a source of angiogenesis factors (6) as well as molecules such as NO (7) that damage corneal tissues. Thus, in line with the minimal early PMN response noted in IL-1α Tg mice, such animals showed a marked reduction in angiogenesis compared with controls. One factor derived from PMN, as well as from other cell types involved in neovascularization, is VEGF (33, 34). We demonstrated that VEGF was significantly down-regulated in IL-1α Tg mice in comparison with control animals. Similar observations were reported previously in a nonspecific model of ocular inflammation (35). The diminished VEGF response of transgenic mice may be explained by the fact that the presence of the IL-1α suppressed IL-1-induced VEGF production from uninfected corneal cells such as PMN. Previously, HSV infection was shown to cause VEGF production, but not from the cells actually infected with virus (8). Conceivably, cytokines such as IL-1 released from infected cells explain the paracrine induction of VEGF. Supporting this scheme, the presence of IL-1α suppressed the VEGF response. In addition, the angiogenic response induced in mice by intrastromal injection of IL-1 could be largely inhibited by anti-VEGF Ab. Furthermore, intrastromal injection of IL-1α was shown to induce VEGF in the cornea. These observations as well as others in tumor systems (20, 36) indicate that IL-1 can cause cells to produce VEGF.

Even though IL-1 produced from HSV-infected cells may act as an inducer for VEGF production, an additional VEGF stimulant appeared to be mediated by the cytokine IL-6. Thus, we demonstrated that corneal cells exposed to IL-1 in vitro produce IL-6, and IL-6 was shown previously to be a strong agonist for VEGF production (25, 37, 38). Further support that IL-1-induced angiogenesis depended in part on IL-6 production was the observation that it was partially blocked by anti-IL-6 Ab. The relative importance of the direct and indirect effect of IL-1 on VEGF production and angiogenesis remains to be evaluated.

Finally, our results demonstrated that corneal RNA samples from transgenic mice showed diminished VEGFR-2 levels measured by real-time PCR. In tumor systems, IL-1 was shown to up-regulate VEGFR-2 expression on endothelial cells (39), the latter known to be critical for pathological angiogenesis (40). Evidently, the absence of IL-1 activity, as occurred in IL-1α Tg mice, results in reduced expression of VEGFR-2. The consequence is lack of stimulation for the endothelial cells to proliferate, resulting in reduced angiogenesis. However, it is not clear why transgenic mice demonstrated reduced VEGF expression. We believe there may be two possibilities: first, IL-1 may directly up-regulate VEGFR-2 expression on corneal endothelial cells, and blocking its activity by specific antagonist protein results in reduced receptor expression; second, reduced proliferation of endothelial cells as a consequence of diminished VEGF and inflammatory response in these mice. We are currently attempting to ascertain which of these two mechanisms accounts for the major effect of IL-1 on angiogenesis.

Taken together, our results support the hypothesis that the inflammatory milieu and angiogenic stimuli created early after infection play an important role in HSV-induced ocular lesions. An important participant of this environment is IL-1. Antagonizing the effect of IL-1 by a specific receptor antagonist protein abrogates
the cascade of events that culminate in herpetic SK (HSK). This regulation is indirectly mediated by down-regulating various signaling molecules previously known to be important in HSK pathogenesis and corneal angiogenesis. It would seem that targeting IL-1 could prove to be a worthwhile therapeutic approach to control SK, an important cause of human blindness.

Acknowledgments

We express our thanks to Dr. Udayasankar Kumaraguru and Christopher Pack for helpful suggestions and critical reading of the manuscript. The help of Amy Cupples is gratefully acknowledged.

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