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Vasoactive Intestinal Peptide Balances Pro- and Anti-Inflammatory Cytokines in the Pseudomonas aeruginosa-Infected Cornea and Protects against Corneal Perforation

Elizabeth A. Szliter, Shahrzad Lighvani, Ronald P. Barrett, and Linda D. Hazlett

Corneal infection with Pseudomonas aeruginosa perforates the cornea in susceptible C57BL/6 (B6), but not resistant BALB/c, mice. To determine whether vasoactive intestinal peptide (VIP) played a role in development of the resistant response, protein expression levels were tested by immunocytochemistry and enzyme immunoassay in BALB/c and B6 corneas. Both mouse strains showed constitutive expression of corneal VIP protein and nerve fiber distribution. However, disparate expression patterns were detected in the cornea after infection. VIP protein was elevated significantly in BALB/c over B6 mice at 5 and 7 days postinfection. Therefore, B6 mice were injected with rVIP and subsequently demonstrated decreased corneal opacity and resistance to corneal perforation compared with PBS controls. rVIP- vs PBS-treated B6 mice also demonstrated down-regulation of corneal mRNA and/or protein levels for proinflammatory cytokines/chemokines: IFN-γ, IL-1β, MIP-2, and TNF-α, whereas anti-inflammatory mediators, IL-10 and TGF-β1, were up-regulated. Treatment with rVIP decreased NO levels and polymorphonuclear neutrophil (PMN) number. To further define the role of VIP, peritoneal macrophages (Mφ) and PMN from BALB/c and B6 mice were stimulated with LPS and treated with rVIP. Treatment of LPS-stimulated Mφ from both mouse strains resulted in decreased IL-1β and MIP-2 protein levels; PMN responded similarly. Both cell types also displayed a strain-dependent differential response to rVIP, whereby B6 Mφ/PMN responded only to a higher concentration of VIP compared with cells from BALB/c mice. These data provide evidence that neuroimmune regulation of the cytokine network and host inflammatory cells functions to promote resistance against P. aeruginosa corneal infection. The Journal of Immunology, 2007, 178: 1105–1114.

Pseudomonas aeruginosa has been implicated as one of the most prevalent organisms associated with the induction of microbial keratitis. Complications of bacterial keratitis caused by this common, opportunistic, Gram-negative pathogen primarily consist of structural alterations to the cornea, but secondary glaucoma and cataract may also develop. P. aeruginosa-induced bacterial infections progress rapidly and are characterized by inflammatory epithelial edema, stromal infiltrate, and corneal ulceration, which can lead to significant tissue destruction and vision loss (1). This sight-threatening disease is in large part a consequence of the inflammatory response invoked by the host.

In this regard, the eye is a site of immune privilege, an evolutionary modification whereby immune effectors provide local protection without the disruption of visual function. In general, control of an inflammatory state depends on the regulation of immune cells, such as macrophages (Mφ) and polymorphonuclear neutrophils (PMN), as well as the local balance between pro- and anti-inflammatory factors released by these and other host cells. Immunoregulation is even more consequential in light of ocular immune privilege and the subsequent conservation of the visual field and these inflammatory events can be characterized using murine models of ocular inflammatory disease. Infection induced experimentally by P. aeruginosa causes corneal perforation in C57BL/6 (B6) mice, a dominant Th1 responder, classified as susceptible, while BALB/c, a Th2 dominant strain, classified as resistant, are able to effectively resolve the infection (2). Previous studies using the susceptible/resistant models have provided essential information regarding the roles of inflammatory cells (e.g., PMN, T cells, Mφ, and Langerhans cells), as well as cytokines and chemokines in modulating inflammation, innate immunity, and Th1- vs Th2-like responses to P. aeruginosa in the eye (1). Yet, little information is available regarding the roles of neuropeptides in this disease (3), despite the fact that the cornea is among the most densely innervated tissues in the body (4).

Vasoactive intestinal peptide (VIP) is an endogenous neuropeptide that has recently received considerable attention for its anti-inflammatory role in both innate and acquired immune responses. VIP has been shown to regulate inflammatory mediators via transduction pathways and several transcription factors essential for gene activation (5), the effects of which are initiated by a family of receptors that interact with VIP as an endogenous ligand (6). Studies have shown that the immunological actions of VIP are evocative of cytokines, functioning in the development and maintenance of an immune response and regulating inflammatory mediators such as Mφ and T cells (7, 8). VIP is a strong inhibitor of Th1 T cell differentiation and it is thought that this influence on T cell responses may limit cellular-mediated inflammation. VIP has also been coined a “Mφ-deactivating factor” (5) by inhibiting...
endogenous proinflammatory cytokines, yet stimulating anti-inflammatory cytokines, produced by the Mϕ. These effects have been shown to contribute to the down-regulation of immune responses related to such pathologic conditions as Crohn’s disease and septic shock syndrome (5).

The studies described herein provide evidence that VIP promotes resistance against P. aeruginosa ocular infection through the regulation of pro- and anti-inflammatory cytokine/chemokine production and the differential effect on host inflammatory cells such as Mϕ and PMN. Results of these studies demonstrate not only the potential therapeutic effects of VIP, but also begin to elucidate its mechanistic actions in vivo using the aforementioned murine models of bacterial keratitis.

Materials and Methods

Experimental animal protocol

Eight-week-old female C57BL/6 (B6) and BALB/c mice, purchased from The Jackson Laboratory, were housed in accordance with the National Institutes of Health guidelines. Mice were anesthetized with ethyl ether and the cornea of the left eye was wounded as previously described (9). A 5-μl aliquot containing 1 × 10^6 CFU of P. aeruginosa American Type Culture Collection strain 19660 was topically delivered to the ocular surface. At 24 h postinfection (p.i.) and/or at times described below, eyes were examined to ensure that mice were similarly infected and to monitor disease.

Wounded corneas were included as an additional control for selected analyses, as specified below. BALB/c and B6 mice were anesthetized with ethyl ether and corneal epithelium of the left eye was debrided using a corneal epithelium spatuila (World Precision Instruments). Eyes were immediately examined by slit-lamp microscopy to ensure removal of epithelium (>75%). All animals were treated in accordance with, and all research was approved by, the Wayne State University Animal Institutional Review Board.

Corneal VIP detection

Whole eyes were removed before infection and at 1, 5, and 7 days p.i. from BALB/c and B6 mice (n = 3/group/time), embedded in OCT, frozen in liquid nitrogen, and stored at −20°C for immunohistochemical analysis. Wounded, uninfected corneas were included as an additional control and comparably analyzed. Ten-micrometer-thick sections were incubated with primary rabbit anti-VIP (1/500; Peninsula Laboratories) for 1 h, then with a biotinylated secondary goat anti-rabbit Ab (1/300; BD Pharmingen) for an additional hour, and stained was visualized using diaminobenzidine (DAB). Control sections were similarly treated, but with omission of the primary Ab. Representative sections were observed and photographed using a Zeiss Axioshot with Axioscam digital imagery (Carl Zeiss).

VIP protein levels in corneas of BALB/c and B6 mice before infection and at 1, 5, and 7 days p.i. were determined using a competitive enzyme immunoassay (EIA) kit (Peninsula Laboratories). Wounded, uninfected corneas of BALB/c and B6 mice at similar time points were also included as an additional control. Individual corneas were collected and homogenized in 250 μl of normal saline. Samples were centrifuged at 5000 × g (10 min) and an aliquot of each supernatant was assayed in triplicate for VIP protein according to the manufacturer’s instruction. Assay sensitivity was 2–3 pg/ml. Results are expressed as average picograms of VIP per milliliter ± SEM.

rVIP treatment

Regarding in vivo studies, B6 mice received daily i.p. injections of rVIP (5 nM in 100 μl) (Bachem) starting 1 day before infection (day −1) through a maximum of 7 days p.i., as indicated below. Control mice were similarly injected with PBS.

Ocular response to bacterial infection

Corneal disease was graded using an established scale (10): 0, clear or slight opacity, partially or fully covering the pupil; +1, slight opacity, fully covering the anterior segment; +2, dense opacity, partially or fully covering the pupil; +3, dense opacity, covering the entire anterior segment; and +4, corneal perforation or phthisis. Mice (n = 5/group/time) treated with rVIP or PBS were examined and a clinical score was calculated for each group to express disease severity. Slit-lamp photography was used to illustrate the disease response.

Bacterial load

Corneas from rVIP- and PBS-treated groups were collected (n = 5/group/time) at 3 and 5 days p.i. and the number of viable bacteria was quantitated. Individual corneas were homogenized in sterile 0.9% saline containing 0.25% BSA. Serial 10-fold dilutions of the samples were plated on Pseudomonas isolation agar (Difco) in triplicate and plates were incubated overnight at 37°C. Results are reported as log_{10} number of CFU per sample ± SEM.

Myeloperoxidase (MPO) assay

An MPO assay was used to quantitate PMN number in the cornea from both rVIP- and PBS-treated animals. Corneas (n = 5/group/time) were excised at 3 and 5 days p.i. and homogenized in 1.0 ml of 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl-ammonium. Samples were freeze-thawed and after centrifugation, 0.1 ml of the supernatant was added to 2.9 ml of 50 mM phosphate buffer containing o-dianisidine dihydrochloride (16.7 mg/100 ml) and hydrogen peroxide (0.0005%). The change in absorbancy (460 nm) was monitored for 5 min (0.0005%). The absorbancy (460 nm) was monitored for 5 min at 30-s intervals. The slope of the line was determined for each sample and used to calculate units of MPO per sample. One unit of MPO activity is equivalent to 2 × 10^6 PMN (11).

Griess reaction

NO levels were determined by measurement of its stable end product, nitrite, using a Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride 12.5% H3PO4) for rVIP- vs PBS-treated B6 mice (n = 5/group/time). Briefly, P. aeruginosa-infected corneas were homogenized in 500 μl of degassed PBS and microcentrifuged at 3500 rpm (5 min). Next, 100 μl of supernatant was added to an equal volume of Griess reagent in triplicate on a 96-well microtiter plate and incubated at room temperature (r.t.) (15 min). Absorbance (570 nm) was measured and nitrite concentrations estimated using a standard curve of sodium nitrite. Data are represented as the mean micromoles of nitrite per cornea ± SEM.

Table I. Primer sequences used for real-time RT-PCR, as described in Materials and Methods

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>IL-1β</td>
<td>5′-CGC AGG AGG ACA TCA ACA AGA GC-3′ sense</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5′-GGT CCT CTT GCT GAG AGG TCC-3′ antisense</td>
</tr>
<tr>
<td>MIP-2</td>
<td>5′-GAG AGG CAG ATT ATC TCT TCT CTC AGA C-3′ sense</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5′-ACC GGC CTC AGC AGA TCA TCT T-3′ sense</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>5′-GAG TAG ATG GAG GA-3′ sense</td>
</tr>
<tr>
<td>IL-10</td>
<td>5′-TCC TTA CCG ACT CTT TAC TGC CAG CAG C-3′ sense</td>
</tr>
<tr>
<td>β-actin</td>
<td>5′-GAC TCA TCG TAC TCC TGC TCG C-3′ antisense</td>
</tr>
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compared with B6 levels. seen only at 5 and 7 days p.i. showed an increase in VIP protein expression, but significant elevation was mice showed similar levels of VIP protein. At 1 day p.i., BALB/c mice remained unchanged in B6 mice. Controls, in which the primary Ab was omitted, were negative for immunostaining of VIP nerve fibers. Original magnification, ×54. B, VIP protein levels in the cornea of BALB/c and B6 mice as detected by EIA. Normal, uninfected cornea of BALB/c and B6 corneas of BALB/c and B6 mice. Distribution patterns for VIPergic fibers were similar in normal corneas of BALB/c and B6 mice (top panels). More fibers appeared detectable at 1 day p.i. in the corneal epithelium of BALB/c over B6 mice. At 7 days p.i., VIP-containing fibers became increasingly evident in both the epithelium and stroma of BALB/c corneas, yet remained unchanged in B6 mice. Controls, in which the primary Ab was omitted, were negative for immunostaining of VIP nerve fibers. Original magnification, ×54. B, VIP protein levels in the cornea of BALB/c and B6 mice as detected by EIA. Normal, uninfected cornea of BALB/c and B6 mice showed similar levels of VIP protein. At 1 day p.i., BALB/c mice showed an increase in VIP protein expression, but significant elevation was seen only at 5 and 7 days p.i. (p = 0.041 and 0.016, respectively), when compared with B6 levels.

Real-time RT-PCR

Total RNA was either isolated from individual corneas for in vivo analysis or collected from cultured cells for in vitro analysis (as indicated below) using RNA-Stat 60 (Tel-Test) according to the manufacturer’s recommendations and quantitated by spectrophotometric determination (260 nm). One microgram of total RNA was reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase. The 20-μl reaction mixture contained: 200 U of MMLV-reverse transcriptase, 10 U of RnaseH, 500 ng of oligo dT primers, 10 mM dNTPs, 100 mM DTT, and MMLV reaction buffer. Next, cDNA was amplified using SYBR Green Master Mix as suggested by the manufacturer. Briefly, 20-μl reactions contained: 10 μl of 2× SYBR Green PCR Master Mix, 0.5 μM primers, 2 μl of cDNA (diluted 1/10), and diethyl pyrocarbonate water. All primers for the PCR, except for VIPR1, were designed using PrimerQuest (Integrated DNA Technologies) and primer sequences are listed in Table I. VIPR1 was purchased from SuperArray Bioscience. Quantitative real-time RT-PCR was processed using the MyiQ Single Color Real-Time RT-PCR Detection System (Bio-Rad). Optimal conditions for PCR amplification of cDNA were established using routine methods (12).

Relative mRNA levels were calculated using the relative standard curve method that compares the amount of target normalized to an endogenous reference, β-actin. Briefly, the mean and SD values of replicate samples were calculated. Samples were then normalized to β-actin. Results are expressed as the relative amount of mRNA between experimental test samples and normal control samples (all normalized to β-actin). Before using this method, a validation experiment was performed comparing the standard curve of the reference and the target to demonstrate that efficiencies were approximately equal. The correct size of the amplified products was checked by electrophoresis using an agarose gel.

ELISA

Cytokine protein levels were selectively tested using ELISA kits (R&D Systems). For in vivo experiments, corneas from rVIP- and PBS-treated B6 mice were individually collected (n = 5/group/time) at 3 and 5 days p.i. Corneas were homogenized in 0.5 ml of PBS with 0.1% Tween 20. For in vitro experiments, supernatants were collected 18 h poststimulation. All samples were centrifuged at 5000 × g (10 min) and an aliquot of each supernatant was assayed in triplicate for IL-1β, MIP-2, and/or TGF-β1 protein per the manufacturer’s instruction. Assay sensitivity was 5.0, 1.5, and 1.7 pg/ml for IL-1β, MIP-2, and TGF-β1, respectively. Results are expressed as average picograms of each cytokine per milliliter ± SEM.

Cell isolation and culture

Peritoneal Mφ were isolated from BALB/c and B6 mice that had been injected i.p. with 1.0 ml of 3% Brewer’s thiglycolate medium (Difco) 5–7 days before cell harvest (13). Peritoneal exudate cells were collected by peritoneal lavage with DMEM, stained with trypan blue (1:1), and viable cells (> 95%) were enumerated using a hemocytometer. After a differential cell count, cells were seeded in 12-well plates at a density of 1 × 10⁶ cells/well. Nonadherent cells were removed 24 h later and isolated Mφ were used for in vitro stimulation assays as described below.
PMN from BALB/c and B6 mice were harvested as previously described (14). Briefly, mice received an i.p. injection of a 9% casein solution (1.0 ml), followed by a second injection 24 h later. PMN were lavaged from the peritoneal cavity 3 h following the second injection, washed, and isolated using a Percoll gradient. Viable cells (>95%) were counted and resuspended at a concentration of 5 × 10⁶ cells/well for in vitro stimulation assays as described below.

**In vitro stimulation assay**

Thioglycolate-elicited peritoneal Mφ or casein-induced PMN from B6 and BALB/c mice were stimulated with LPS (Mφ, 25 μg/ml; PMN, 0.5 μg/ml) in the presence and absence of rVIP (10⁻¹⁰ to 10⁻⁸ M) for 18 h (15, 16). Supernatants were collected and assayed by ELISA for selected cytokines (described above).

**Immunofluorescent staining**

Mφ expression of VIPR1 was evaluated by immunofluorescent dual-label staining using confocal laser scanning microscopy of corneal tissue sections. Whole eyes were enucleated at 5 days p.i. from PBS- and rVIP-treated B6 mice (n = 3/group), fixed, dehydrated, and embedded in paraffin as previously described (17), then stored at −20°C for immunofluorescent analysis. Ten-micrometer-thick sections were deparaffinized, then rehydrated through graded alcohols. Sections were incubated with Image-iT Signal Enhancer (Molecular Probes) for 30 min (r.t.). After blocking with 2.5% goat and 2.5% donkey serums (30 min, r.t.), slides were incubated with primary rat anti-F4/80 (1/100; Novus Biologicals) for 1 h, followed by Alexa Fluor 546-conjugated donkey anti-rat IgG (1/200; Molecular Probes) for an additional hour. Next, primary rabbit anti-VIPR1 (1/100; Novus Biologicals) was applied and incubated for 1 h, then with Alexa Fluor 594-conjugated donkey anti-rabbit IgG (1/200; Molecular Probes) for an additional hour. Slides were then incubated for 2 min with SYTOX Green nuclear acid stain (1/5000; Fisher Scientific). Negative controls were similarly treated, but with omission of the primary Abs. Sections were visualized and digital images were captured with a Leica TSC SP2 confocal laser scanning microscope (Leica Microsystems).

**Statistical analysis**

The difference in clinical score between two groups at each time point was tested by the Mann-Whitney U test. An unpaired Student’s t test was used to determine the significance of viable bacterial counts, MPO, and Griess assays, real-time RT-PCR, and protein assays. Data were considered significant at p < 0.05. All experiments were repeated at least twice and representative data from a typical experiment are shown unless otherwise indicated.

**Results**

**VIP expression in BALB/c vs B6 mice**

Immunostaining (Fig. 1A) of BALB/c and B6 mouse corneas showed a similar distribution pattern of VIP-containing nerve fibers in normal, uninfected eyes (top panels). However, at 1 day p.i. (middle panels), BALB/c mice showed slightly more intense staining beneath the epithelium, both centrally and in the corneal periphery (data not shown) compared with similar areas in the cornea of B6 mice. At 7 days p.i. (bottom panels), VIPergic fibers were present in the corneal epithelium and appeared more prominent in the stroma of BALB/c vs B6 mice, whose cornea was demoded of epithelium. Control sections (bottom panels) were similarly incubated, but in the absence of the primary Ab and were negative for nerve fiber immunostaining.

EIA analysis for VIP protein levels in the cornea of BALB/c vs B6 mice showed no difference between the two strains before infection (normal) and were elevated, but not significantly, at 1 day p.i. (Fig. 1B). VIP protein levels were significantly elevated in BALB/c vs B6 mouse corneas at both 5 and 7 days p.i. (p = 0.041 and 0.016, respectively).
Debrided corneas (epithelium removed) were included as an additional control to the infection model to determine whether elevated VIP levels and VIPergic nerve fiber regeneration was a result of infection or in response to corneal wounding alone. Immunostaining and EIA analysis of wounded corneas from BALB/c and B6 mice are provided in Fig. 2. At 1 and 7 days postwound (note epithelium is healed at both these times), distribution of VIP nerve fibers appeared similar to normal, uninfected controls for both BALB/c and B6 mice (Fig. 2A). In addition, corneal VIP protein levels in BALB/c and B6 mice at 1, 5, and 7 days postwound showed no difference when compared with normal, uninfected controls (Fig. 2B).

**Ocular response after bacterial infection**

Based upon the differential expression of VIP in resistant vs susceptible mice as indicated by immunostaining and protein levels, B6 mice were injected with rVIP and disease response compared with PBS-treated controls (Fig. 3). By 7 days p.i., rVIP-treated corneas consistently displayed a reduced level of disease (grade = +2/+3), whereas all control corneas perforated (+4), as indicated in Fig. 3A. Clinical scores showed that rVIP treatment significantly improved the disease response at 3, 5, and 7 days p.i. (3 days, \( p = 0.01 \); 5 and 7 days, \( p < 0.001 \)).

**Cytokine expression with rVIP treatment**

To begin investigating whether VIP modulated disease during bacterial keratitis, mRNA levels of select pro- and anti-inflammatory cytokines in the cornea were analyzed by real-time RT-PCR. Because B6 mice are Th1 responders (typically perforating by 5–7 days p.i.) and treatment with rVIP ameliorated the disease response even at 7 days p.i., classic type 1 and other proinflammatory cytokines and chemokines including: IFN-\( \gamma \), IL-1\( \beta \), TNF-\( \alpha \), and MIP-2, were evaluated at this time in normal and rVIP- vs PBS-treated animals (Fig. 4, A and D, respectively). At 7 days p.i.,
treatment with rVIP resulted in a significant decrease in mRNA transcripts for all proinflammatory cytokines/chemokines tested when compared with PBS-treated controls. Conversely, corneal mRNA expression of anti-inflammatory cytokines, TGF-β1 and IL-10, was significantly enhanced with rVIP treatment (Fig. 4, E and F, respectively).

Expression of IL-1β, MIP-2, and TGF-β1 was further examined at the protein level by ELISA and results are provided in Fig. 5, respectively. Protein expression in the corneas of rVIP- and PBS-treated animals was assessed at 3 and 5 days p.i. Results at the protein level corroborated effects observed at the mRNA level at 7 days p.i. Regarding proinflammatory mediators, IL-1β and MIP-2, protein levels were significantly decreased as a result of rVIP treatment at both time points (IL-1β; p < 0.001 and 0.003; MIP-2; p = 0.003 and 0.048, respectively). Furthermore, TGF-β1 protein levels were significantly elevated in contrast to controls (p = 0.003 and <0.001), thus providing direct evidence that VIP regulates an immune response by balancing these pro- and anti-inflammatory mediators in the cornea.

Quantitation of viable bacteria
To assess the effect of rVIP treatment on the bacterial component of disease pathogenesis, direct plate counts were used to detect viable bacteria in rVIP- vs PBS-treated corneas at 3 and 5 days p.i. and the results are shown in Fig. 6A. No difference was detected in bacterial load in the cornea between the two treatment groups at 3 days p.i. However, by 5 days p.i., bacterial counts were significantly decreased in rVIP-treated B6 mice (p < 0.001).

Effects of VIP treatment on Mφ and PMN in vivo
NO is a reactive free radical produced by Mφ during an inflammatory response. To examine the anti-inflammatory influence of rVIP on Mφ activation, nitrite (a stable oxidized product of NO) was measured at 1 and 5 days p.i. and results are shown in Fig. 6B. Corneas of rVIP-treated animals consistently showed decreased levels of nitrite at 1 and 5 days p.i. when compared with PBS-treated controls (p = 0.038 and 0.046, respectively).

To ascertain whether VIP treatment also has an influence on PMN number, MPO was quantitated in the cornea of rVIP- and PBS-treated mice at 3 and 5 days p.i. and results are presented in Fig. 6C. MPO activity was significantly decreased with rVIP treatment at both 3 and 5 days p.i. when compared with PBS controls (p < 0.001).

Effects of VIP treatment on Mφ and PMN in vitro
Because VIP treatment resulted in reduced nitrite levels and VIP is also known as a “Mφ-deactivating factor” (5), Mφ were collected from both BALB/c and B6 mice to directly determine the effects of rVIP treatment in vitro. Mφ from both strains of mice were stimulated with LPS, with or without rVIP (10^-10 to 10^-8 M), then tested for IL-1β and MIP-2 protein production (Fig. 7, A and B, respectively). IL-1β was significantly increased in LPS-stimulated Mφ from both mouse strains when compared with medium controls (p < 0.001) (Fig. 7A). Treatment with rVIP at 10^-10 and 10^-9 M down-regulated the effects of LPS stimulation on BALB/c Mφ resulting in significantly less IL-1β protein (p < 0.001). B6 Mφ-derived levels were also significantly decreased after rVIP treatment, but only at the highest concentration (10^-8 M) (p < 0.001).

In regard to MIP-2, LPS stimulation significantly increased protein levels in Mφ from both groups of animals when compared with medium controls (p < 0.001) (Fig. 7B). BALB/c Mφ appeared more sensitive to rVIP treatment, showing significantly decreased MIP-2 protein levels at all concentrations (10^-10 to 10^-8 M) (p < 0.001). However, B6-derived Mφ only responded to the highest concentration of rVIP (10^-8 M) (p < 0.001).
Expression of VIPR1 mRNA in BALB/c- and B6-derived Mφ and PMN. Peritoneal Mφ (A) and PMN (B) were stimulated with LPS (Mφ, 25 μg/ml; PMN, 0.5 μg/ml) for 18 h and VIPR1 transcript levels were determined by real-time RT-PCR. A, Constitutive expression of VIPR1 mRNA was detected in both BALB/c and B6 Mφ and was up-regulated in response to LPS stimulation. VIPR1 expression levels were significantly higher in BALB/c-derived Mφ under normal and LPS-stimulated conditions when compared with B6. B, PMN from BALB/c and B6 mice showed similar levels of VIPR1 expression under normal, unstimulated conditions. After LPS stimulation, VIPR1 transcripts were significantly elevated in cells from both strains of mice.

Because PMN are the predominant cellular infiltrate involved in bacterial keratitis and MPO results indicated that rVIP treatment reduced PMN number, these cells also were isolated from BALB/c and B6 mice for in vitro analysis (Fig. 7C). MIP-2 protein was not detectable from medium-treated (control) PMN of either mouse strain; however, LPS stimulation significantly increased MIP-2 protein levels in PMN from both groups of animals when compared with controls (p < 0.001). LPS-stimulated BALB/c PMN were more responsive to rVIP treatment compared with B6, showing significantly decreased MIP-2 protein levels at all concentrations (10⁻¹⁰ to 10⁻⁸ M) (p < 0.001). B6-derived PMN responded only to the highest concentration of VIP (10⁻⁸ M) (p < 0.001).

VIPR1 expression in BALB/c vs B6 mice

VIPR1 (VPAC1) is constitutively expressed on Mφ and is up-regulated upon antigenic stimulation (16, 18, 19). VIPR1 mRNA expression levels were determined by real-time RT-PCR in both BALB/c and B6 Mφ to determine whether disparate receptor expression could explain the differential response to rVIP treatment (Fig. 8A). Unstimulated Mφ isolated from both strains showed constitutive expression of VIPR1 transcripts, however, levels were significantly higher in BALB/c vs B6 mice (p < 0.001). VIPR1 transcripts were increased in both groups of LPS-stimulated cells, yet again BALB/c-derived Mφ displayed significantly higher VIPR1 mRNA levels when compared with cells from B6 mice (p = 0.029).

Regarding PMN, rVIP treatment resulted in decreased MIP-2 protein expression (in vitro) and less cellular infiltrate into the cornea (in vivo). Because VIPR1 is the predominant receptor mediating the effects of VIP on Mφ, PMN were examined for similar effects (Fig. 8B). Constitutive expression of VIPR1 mRNA was not detected in unstimulated BALB/c or B6 PMN. VIPR1 transcripts were elevated after LPS stimulation; however, there was no difference in receptor expression between the two strains.

Next, the influence of rVIP treatment on VIPR1 expression by Mφ in the cornea of B6 mice was examined at 5 days p.i. using fluorescent confocal laser scanning microscopy. Negative controls (primary Abs omitted) showed no detectable F4/80⁻/VIPR1⁺ immunostaining (Fig. 9A). Mφ from the corneas of PBS-treated animals immunostained (red) with the F4/80 Ab as shown in Fig. 9B, while few cells also showed detectable coexpression of VIPR1 (blue). C, Numerous Mφ in the cornea of rVIP-treated mice were positive for both F4/80 (red) and VIPR1 (blue) markers. Inset illustrates this at higher magnification.

Discussion

VIP is a pleiotropic neuropeptide that has been implicated as a regulatory molecule in a wide variety of immune events, which ultimately function to maintain or restore immune homeostasis. In regard to the eye, efficient transition from a state of ocular inflammation to immune homeostasis is essential for preservation of the visual axis. Therefore, the role of VIP in ocular infectious disease
provides attention not only to further elucidate the mechanisms of inflammation and ocular immune privilege, but also as a potential therapeutic target. The present study provides substantive evidence that VIP directly promotes and contributes to resistance against *P. aeruginosa*-induced keratitis. In addition, regulatory mechanisms/interactions have been explicated, which further indicate a major role for Mφ in the development of resistant vs susceptible phenotypes.

Previous studies have revealed the presence of VIPergic nerve fibers in both central and peripheral lymphoid organs, including: thymus, spleen, and lymph nodes (5). VIP is also delivered by neurons to the heart, lung, thyroid, gastrointestinal tract, kidney, urinary tract, and genital organs (20). More recently, it has been shown that VIP is expressed by human corneal nerves (4), is produced by human corneal endothelial cells, and is present in the aqueous humor of humans and rabbits (21). The current study, however, not only shows the presence of VIPergic nerve fibers and VIP protein in the normal murine cornea, but profiles VIP expression and nerve fiber distribution during the course of infection. Although both BALB/c and B6 corneas express comparable levels of VIP protein under normal conditions, levels were increased significantly at 5 and 7 days p.i. in BALB/c vs B6 mice. These data are supported by immunostaining for VIPergic fibers, which indicate that as the disease response progresses, BALB/c mouse cornea has a greater distribution of nerve fibers compared with B6. Furthermore, epithelial debridement of either BALB/c or B6 mouse cornea did not result in up-regulation of VIP protein production or regeneration of VIPergic fibers over normal controls, suggesting that induction of VIP requires the presence of foreign Ag and/or more intensive injury/damage. In view of the fact that resistant BALB/c mice intrinsically respond with higher levels of the anti-inflammatory molecule, these data suggest a pivotal role for VIP in the development of the resistant vs susceptible phenotype. Therefore, susceptible B6 mice were treated with rVIP to test whether this neuropeptide could promote resistance by ameliorating disease pathogenesis to *P. aeruginosa*-induced corneal infection. Indeed, rVIP treatment resulted in a dramatic decrease in subsequent disease development, as slit-lamp and clinical score visually indicated that corneal perforation in B6 mice was prevented. This was accompanied by a reduction in the production of proinflammatory cytokines/chemokines IFN-γ, IL-1β, TNF-α, and MIP-2 at the mRNA and/or protein levels, while production of potent anti-inflammatory cytokines, IL-10 and TGF-β1, were enhanced. These data are consistent with previous studies indicating that VIP inhibits production of several proinflammatory cytokines and chemokines, including TNF-α, IL-6, IL-1β, IL-12, MIP-1, and MIP-2, while stimulating the production of anti-inflammatory cytokines IL-10 and IL-1R antagonist (15, 16, 19, 22, 23). In addition, we have previously used Mφ depletion with clodronate and provided evidence that IL-10 is an important modulator of the resistant phenotype, functioning to balance IFN-γ levels in the BALB/c cornea (24).

Control of an inflammatory state depends not only on the local balance between pro- and anti-inflammatory factors, but also on proper activation/deactivation of host immunocompetent cells such as Mφ and PMN. Mφ participate in both innate and acquired immunity and are essential in the regulation of immune responses via a number of immunologic functions, including phagocytosis, Ag processing/presentation, cytokine secretion, and production of reactive oxygen and nitrogen intermediates. Despite their beneficial role in host defense, unregulated and sustained production of Mφ-derived proinflammatory cytokines and NO can result in serious pathological conditions such as septic shock and respiratory distress syndrome (25). Therefore, regulation of Mφ activity is essential in the overall modulation of the immune response. Our in vivo studies showed that rVIP treatment significantly reduced nitrite levels in the cornea compared with PBS-treated controls during infection. Nitrite, a stable oxidized product of NO, is a marker used to measure Mφ activation and inflammation. Because VIP is a well-known “Mφ-deactivator,” these results prompted us to more closely examine how VIP affects Mφ activity. For this, peritoneal Mφ were isolated from both BALB/c and B6 mice and stimulated with LPS in the presence and/or absence of rVIP (10^-10 to 10^-8 M), then analyzed for protein expression of two cardinal proinflammatory mediators known to contribute to disease pathogenesis in this model: IL-1β and MIP-2. Protein levels of IL-1β and MIP-2 were significantly reduced when LPS-stimulated Mφ from both BALB/c and B6 mice were treated with rVIP. However, results indicated a differential effect between the two mouse strains in that Mφ from BALB/c mice responded to 10^-10 to 10^-8 M rVIP, whereas B6 Mφ required a higher concentration to achieve an anti-inflammatory effect. In addition, to evaluate concentration-dependent effects of VIP, we also tested 10^-12 and 10^-6 M rVIP. We found that neither of these concentrations had any effect on BALB/c-derived Mφ and PMN or B6-derived Mφ (data not shown); however, B6-derived PMN responded to 10^-6 M (data not shown) similar to 10^-8 M rVIP (Fig. 7). It is of interest to note that not only were BALB/c-derived Mφ more sensitive to the lower concentrations of rVIP (10^-10/10^-9 vs 10^-8 M) compared with B6, but when stimulated with LPS alone, BALB/c Mφ also displayed a significantly more robust response than B6 via proinflammatory cytokine/chemokine production of both IL-1β and MIP-2. It has been reported by this laboratory that depletion of the Mφ in both BALB/c and B6 mouse strains before ocular infection with *P. aeruginosa* results in an exacerbated disease response, suggesting that Mφ play a key role in regulating/balancing pro- and anti-inflammatory activity in the resistant and susceptible models (24). Previous studies also have also suggested that Mφ from Th1 T cell responder mouse strains (B6) are different regarding functional behavior (phagocytic activity, NO generation) when compared with those from Th2 strains (BALB/c) (26, 27). Considering this study and our own results from rVIP-treated Mφ, it can be hypothesized that Mφ from Th1 and Th2 T cell responder mouse strains not only demonstrate differences in Mφ activation, but also deactivation, both of which have an intense influence over the progression and development of inflammation and the innate immune response.

As Mφ might be considered the “sentinels” of inflammation, PMN are the “infantry,” quickly recruited to sites of infection by Mφ-secreted cytokines, such as MIP-2. PMN are essential for effective clearance of microbial pathogens (28) and resolution of disease, as suggested by studies with leukopenic mice (29). Although most PMN clear from the site of infection (presumably by apoptosis) within 24–48 h, it is the persistence of these cells within the cornea that contributes to the extensive stromal destruction through continuous release of their intracellular proteolytic enzymes (30). Therefore, unless PMN are tightly regulated and cleared from the area of inflammation in a timely manner, disease pathogenesis is exacerbated, resulting in tissue necrosis and organ failure, as is manifested in the susceptible-B6 response. Data from our in vivo experiments revealed through MPO assay that PMN numbers were reduced in the cornea of rVIP-treated vs PBS-treated mice after 3 and 5 days p.i. Previous studies by this laboratory suggest that Mφ play a major role in regulating the influx and persistence of PMN in the cornea (24). Delgado and Ganea (31, 32) also have shown that VIP inhibits the production of MIP-2, a potent chemotaxtrant for PMN, by BALB/c-derived Mφ. Although evidence indicates VIP indirectly regulates PMN
activity via Μφ influence, we asked whether VIP also had any direct influence on PMN as well. In vitro stimulation assays using isolated PMN from BALB/c and B6 mice showed that rVIP reduces LPS-induced levels of MIP-2 protein in both BALB/c- and B6-derived PMN. The concentration-dependent response seen in the Μφ was also exhibited in the PMN, whereby B6 PMN were sensitive only to the higher concentration of rVIP treatment. This concentration-dependent response by both Μφ and PMN of Th1 vs Th2 mouse strains suggests the importance of availability of endogenous VIP at adequate levels within the corneal microenvironment to provide modulation of the inflammatory response.

VIP is known to exert its effects through three receptors: VPAC1 (or VIPR1), VPAC2, and PAC1. Studies have shown that peritonal Μφ constitutively express VIPR1 mRNA and that many of the VIP-induced effects on Μφ are predominantly conducted through this receptor (16, 18, 19, 33). In addition, it has been found that VIP inhibits CXC and CC chemokines such as MIP-1 and MIP-2 through VIPR1 (31). Because our in vitro data provide evidence for a differential response to VIP between the two mouse strains, indicating that BALB/c-derived Μφ are more sensitive than B6, we also examined VIPR1 expression. Although both BALB/c- and B6-derived Μφ showed constitutive expression of VIPR1 mRNA, BALB/c had significantly higher VIPR1 levels than B6. Furthermore, LPS-stimulated Μφ also displayed differential expression, whereby Μφ from B6 mice again exhibited a significantly elevated level of VIPR1 mRNA when compared with cells from B6 mice. We also looked at VIPR1 expression levels in PMN and found that constitutive levels of receptor mRNA were not detectable in normal PMN, yet were similarly up-regulated in both mouse strains upon LPS stimulation. These results strongly suggested that the effects of VIP are largely mediated via receptors on the Μφ, therefore we evaluated whether rVIP treatment enhanced VIPR1 expression in the in vivo model of infection. At 5 days p.i., corneas were examined for F4/80, a Μφ marker, and VIPR1 by dual-labeling fluorescent immunohistochemistry. Although corneas from PBS-treated B6 mice showed the presence of F4/80+ Μφ, very few of these cells demonstrated coexpression of VIPR1. In contrast, corneas from rVIP-treated mice exhibited Μφ that predominately coexpressed both F4/80 and VIPR1. These data suggest that the effects of rVIP are mediated primarily through VIPR1 and that rVIP enhances VIPR1 expression on the Μφ, thus further contributing to the anti-inflammatory effects exerted by this neuropeptide.

In addition to Μφ and PMN, T cell-mediated pathogenic mechanisms are also important in the development of the susceptible vs resistant phenotype during ocular infection (1, 34). Previous studies by our laboratory have shown that in B6 mice, CD4+ T cells (Th1 type) contribute directly to PMN persistence and stromal destruction, ultimately resulting in the susceptible response (35). Conversely, in BALB/c mice, T cells do not infiltrate the cornea. PMN do not persist and cytokines/chemokines are better balanced, resulting in decreased stromal destruction and the resistance response. In this regard, Delgado et al. (8) has shown that Ag-stimulated Th2, but not Th1, cells synthesize and secrete VIP (5), which is also known to inhibit Th1 T cell differentiation (36). It has been hypothesized that this influence on T cell responses may limit the threat of immunogenic inflammation to the eye (37). Thus, the effects of VIP on host immune cells such as T cells, Μφ, and PMN illustrate how this neuropeptide functions to restore immune homeostasis, largely in an anti-inflammatory manner.

In conclusion, the data from this study provide evidence that the following factors are influential in the development of resistant vs susceptible responses: 1) availability of endogenous VIP, BALB/c Μφ have greater nerve fiber distribution and protein expression in the cornea; 2) receptor expression on immune cells, BALB/c Μφ not only express higher VIPR1 mRNA constitutively, but more efficiently up-regulate VIPR1 mRNA expression upon LPS stimulation; and 3) overall sensitivity to VIP, B6 mice are less sensitive to VIP, requiring a higher (perhaps unavailable) concentration of endogenous VIP to modulate inflammation and restore immune homeostasis. We hypothesize that the therapeutic potential to manipulate disease outcome using rVIP provides an attractive addition to current modalities.

Disclosures
The authors have no financial conflict of interest.

References


