Prevention of Experimental Autoimmune Uveoretinitis by Vasoactive Intestinal Peptide

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Background: Vasoactive intestinal peptide (VIP), a neuropeptide that is known to be present in lymphoid tissue microenvironments, shows prominent anti-inflammatory actions.

Objective: To examine the potential effect of VIP on the development of experimental autoimmune uveoretinitis (EAU).

Design: We immunized C57BL/6 mice with human interphotoreceptor retinoid-binding protein peptide 1-20 (h-IRBP peptide). Vasoactive intestinal peptide was administered intraperitoneally on alternate days until day 21 after immunization (entire group). In some cases, VIP was injected at different time points after the induction of immunity with h-IRBP peptide (efferent group). In each experiment, a control group of mice was injected with phosphate-buffered saline instead of VIP. Development of EAU was evaluated by means of histological examination on day 21 after immunization. Furthermore, we determined whether intravenous injection of peritoneal exudate cells cultured with VIP overnight in vitro abrogated EAU. We analyzed delayed hypersensitivity for h-IRBP peptide and the occurrence and severity of EAU using evaluation of histopathological sections for inflammatory ocular disease.

Results: Treatment with VIP suppressed the expression of delayed hypersensitivity responses to h-IRBP peptide significantly (positive control vs entire group, \( P = .02 \); positive control vs efferent group, \( P < .001 \)). Mice treated with VIP (\( n = 10 \)) showed a lower occurrence (40%) and decreased severity of EAU (entire group mean score, 0.3; median score, 0) compared with untreated mice (occurrence, 80%; mean score, 0.85; median score, 0.75), as assessed by histopathological analyses (\( P = .049 \)). Suppressive effects of VIP on EAU were also observed, even when VIP was administered on days 8 through 20 after immunization (efferent group [\( n = 9 \)] occurrence, 11%; mean score, 0.1; median score, 0) (\( P = .003 \)). Moreover, expression of EAU was significantly suppressed when the animals were pretreated with peritoneal exudate cells pulsed with h-IRBP in the presence of VIP (control mean score, 1.2; median score, 1.0; occurrence, 80% [\( n = 10 \)]) compared with the VIP-treatment group (mean score, 0.3; median score, 0; occurrence, 30% [\( n = 10 \)]) (\( P = .004 \)). In addition, VIP-treated peritoneal exudate cells generated regulator T cells in the spleens of recipient mice that were able to interfere with the development of EAU (control group mean score, 0.5; median score, 0.5; occurrence, 63% [\( n = 8 \)]) compared with the VIP-treatment group (mean score, 0.08; median score, 0; occurrence, 17% [\( n = 6 \)]) (\( P = .08 \)).

Conclusion: Treatment with VIP is a highly effective therapy to suppress EAU.

Clinical Relevance: As a result of its efficacy in preventing EAU, VIP might be considered as a novel therapeutic modality for human uveitis.


The eye is an immune-privileged site that inhibits the induction of conventional immunological responses within its microenvironment. Studies of the immunosuppressive activity of aqueous humor, the fluid that fills the anterior chamber, have revealed the presence of soluble factors that actively suppress immune-mediated inflammation. Aqueous humor contains numerous potential immunomodulating factors such as transforming growth factor \( \beta_1 \), the neuropeptide \( \alpha \)-melanocyte–stimulating hormone, and calcitonin gene–related peptide. Another neuropeptide constitutively detected in aqueous humor is vasoactive intestinal peptide (VIP).

Vasoactive intestinal peptide, a neuropeptide that can be detected in lymphoid microenvironments, displays a broad spectrum of biological functions, including modulation of innate and adaptive immunity and suppression of inflammatory reactions. Recent reports have indicated that VIP promotes helper T cell...
type 2 (Tₐ₂) differentiation and inhibits helper T cell type 1 (Tₐ₁) responses by regulating macrophage costimulatory signals and probably interleukin 12/interferon-γ production. All of these activities are likely to have beneficial effects in autoimmune diseases such as Behçet syndrome. From these reasons, we elected to evaluate the therapeutic effects and immunomodulatory properties of VIP in experimental autoimmune uveoretinitis (EAU), an animal model for human uveitis.

Experimental autoimmune uveoretinitis is a T-cell–mediated autoimmune disease induced by immunizing susceptible strains of mice with interphotoreceptor retinoid-binding protein (IRBP) or its peptide. The inflammatory CD4⁺ T cells that mediate EAU are known to secrete Tₐ₁-type cytokines, including interleukin 2, interferon-γ, and tumor necrosis factor α, as well as cytokines responsible for expression of delayed-type hypersensitivity (DH) responses. Because EAU shares a number of clinical, histological, and immunological features with human uveoretinitis syndromes, including Behçet syndrome, Vogt-Koyanagi-Harada syndrome, sympathetic ophthalmia, and sarcoidosis, it is a suitable model to study the potential effects of VIP on the pathogenesis of uveitis. Herein we report that VIP prevents the development of and leads to an improvement in the pathological score and occurrence of EAU induced by human IRBP peptide 1-20 (h-IRBP peptide). In addition, peritoneal exudate cells (PECs) cultured in vitro with h-IRBP peptide in the presence of VIP-induced h-IRBP peptide–specific regulatory T cells were injected into syngeneic mice and suppressed the h-IRBP peptide–specific DH response and development of EAU. These results suggest that VIP might be an effective therapy for human uveoretinitis.

### METHODS

#### ANIMALS

Specific pathogen-free female C57BL/6 mice were obtained from Shizuoka Laboratory Service, Shizuoka, Japan, and maintained in our animal facilities. Mice aged 8 to 12 weeks were used in this study. Mice were maintained in accordance with the statement of the Association for Research in Vision and Ophthalmology regarding the use of animals in research. A mixture of ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (12.5 mg/kg) was used for anesthesia and administered by means of intraperitoneal injection.

#### REAGENTS

Human IRBP peptide 1-20 (GPTHLFQPSLVLDMAKVLLD) was synthesized by conventional solid-phase techniques on a peptide synthesizer (TakaraBio Inc, Shiga, Japan). Complete Freund adjuvant and Mycobacterium tuberculosis H37Ra were purchased from Difco Labs, Detroit, Mich. Purified Bordetella pertussis toxin was purchased from Sigma-Aldrich Corp, St Louis, Mo.

#### INDUCTION AND SCORING OF EAU

Mice were immunized subcutaneously in the thigh and neck with a peptide (200 µg) derived from h-IRBP peptide in a 0.2-mL emulsion in complete Freund adjuvant that had been supplemented with M tuberculosis strain H37Ra to 5 mg/mL and were given 100 µg of purified Bordetella pertussis toxin intraperitoneally as an additional adjuvant. Eyes were collected and assessed on day 21 after immunization. In another experiment, spleen cells were collected from immunized mice on day 21 after PEC transfer, and T cells were enriched using a magnetic, activated cell-sorting high-gradient separation column purchased from Miltenyi Biotec (Auburn, Calif) that yields 99% CD3⁺ cells, as confirmed by flow cytometry. Purified T cells were injected intraperitoneally (40×10⁶ to 50×10⁶ cells/mouse) into naive C57BL/6 mice. The presence of EAU was assessed by histopathological examination 14 days after the adoptive transfer. Eyes were fixed in Bouin solution. Sections of samples were embedded in paraffin and stained with hematoxylin-eosin for histopathological study. The EAU score was evaluated in a masked fashion. Occurrence and severity of EAU were examined for each eye and scored on a scale of 0 to 4 in half-point increments, according to a semiquantitative system described previously. The 0.0 grade of EAU means that inflammatory cell infiltration into the entire retina is observed, and anatomical retinal layers are partially destroyed. The 0.5 grade of EAU means that retinal layer structures are completely preserved, but slight cell infiltration into the retina and ciliary body is observed.

#### TREATMENT PROTOCOLS

Vasoactive intestinal peptide (3 nmol/mouse) was administered intraperitoneally on alternate days until day 21 after h-IRBP peptide immunization. In another experiment, VIP was administered at different times during the eff erent phase of EAU. In each experiment, a control group of mice was injected with phosphate-buffered saline (PBS) alone.

#### ASSAY OF DH

Mice received an intradermal injection of 20 µg/10 µg of h-IRBP peptide suspended in PBS into the right ear pinnae. After 24 and 48 hours, the ear swelling was measured by a micrometer (Mitutoyo, Tokyo, Japan).

#### PREPARATION OF PECs

Peritoneal exudate cells were obtained from naive B6 mice that had received 2 mL of intraperitoneal 3% thioglycolate solution 3 days earlier. The cells were washed and suspended, then placed in 24-well culture plates (8×10⁶/well). Based on the report by Taylor et al that the concentration of VIP in rabbit aqueous humor is 12 nM, we treated PECs with 12 nM VIP or PBS in serum-free medium at 37°C in an atmosphere of 5% carbon dioxide.

The h-IRBP peptide was added at concentrations of 10 µg/mL. After overnight culture, plates were washed 3 times with culture medium to remove VIP and nonadherent cells. Adherent cells were cooled for 30 minutes at 4°C, dislodged from the culture plate by vigorous pipetting, washed twice, and resuspended in PBS. When analyzed by means of flow cytometry for expression of F4/80, greater than 99% of these cells were positive (data not shown), and we used the cells as macrophages. A total of 100 µL of PBS containing 2×10⁶ PECs was injected into the tail vein of naive syngeneic mice. Thirty minutes later, the recipients received a subcutaneous injection of a uveitogenic regimen of h-IRBP peptide in complete Freund adjuvant.

#### STATISTICAL ANALYSIS

Analysis of ear swelling was performed by using analysis of variance and the Scheffé test. We used analysis of variance and the Scheffé test to determine any significant difference that existed among all 4 experimental groups. We chose these tests to correct for multiple comparisons. We compared the EAU scores using the Mann-Whitney test. Means were considered to be significantly different when P<.05.
Results

Preventive Effect of VIP on DH Response to h-IRBP Peptide

To determine whether VIP treatment suppresses h-IRBP peptide–specific immune responses in vivo, as revealed by suppression of antigen-specific DH, C57BL/6 mice were immunized with h-IRBP peptide as described. The mice then received intraperitoneal VIP on alternate days for 3 weeks after immunization. Twenty days after immunization, the ears of the animals underwent challenge with h-IRBP peptide. As the results in Figure 1A show, VIP impaired the capacity of mice immunized subsequently by means of h-IRBP peptide to display DH response (VIP entire group) 

\( P = .02 \).

Furthermore, to determine the potential effect of VIP on the DH response after the immune system had already been exposed to an uveitogenic regimen, panels of mice that received VIP on days 8 through 20 after immunization also underwent testing for DH. The results, depicted in Figure 1A, show that mice treated with VIP in this manner displayed feeble DH (VIP efferent group) 

\( P < .001 \).

Thus, pretreatment of B6 mice with VIP impairs the capacity of these mice to acquire DH responses. Furthermore, delayed VIP treatment suppresses the expression of DH responses.

Preventive Effect of VIP on Development of EAU

To estimate the potential effect of VIP on the development of EAU, we administered VIP intraperitoneally on alternate days for 3 weeks after immunization with h-IRBP peptide. The development of EAU was assessed by means of histopathological examination of the eye tissue sections. As shown in Figure 1B, EAU developed in 8 of 10 control mice (mean score, 0.85; median score, 0.75; range, 0-2.0). In contrast, EAU developed in only 4 of 10 VIP-recipient mice (mean score, 0.3; median score, 0; range, 0-1.0) (Figure 1B) 

\( P = .049 \).

As shown in Figure 2A, inflammatory cell infiltration was present in the vitreous and the retina, and damaged photoreceptor layer and retinal vasculitis were observed in control mice. In contrast, pathological findings in VIP-treated mice were distinctly milder (Figure 2B). These results indicate a potent preventive effect of VIP on the development of EAU. Furthermore, the result shown in Figure 1A encouraged us to attempt to suppress DH after the induction of immunity with h-IRBP peptide (on the efferent limb of immune response to h-IRBP peptide). Therefore, to determine the potential effect of VIP on EAU after the immune system had been exposed to an uveitogenic regimen, VIP was administered on days 8 through 20 after immunization. As shown in Figure 1C, VIP blocked disease development (mean score, 0.1; median score, 0; range, 0-0.5) compared with control group results (mean score, 0.85; median score, 0.75; range, 0-2.0) 

\( P = .003 \) and led to an improvement in the pathological score and occurrence of EAU (control group, 8/10 mice; VIP-treatment group, 1/9 mice). These results suggest that VIP has a suppressive effect on established EAU.

Capacity of In Vitro–Treated PECs to Prevent EAU

We next wished to determine whether intravenous injection of in vitro–treated PECs with VIP had the capacity to abrogate EAU. The PECs were harvested from C57BL/6 mice, pulsed with h-IRBP peptide, and incubated overnight with VIP. The cells were then harvested, washed free of h-IRBP peptide and VIP, and injected intravenously into naive B6 mice. Thirty minutes later, the recipients received a uveitogenic regimen, as described previously. The results are shown in Figure 3. Uveitis was detected in the eyes of only 3 of 10 mice (mean score, 0.3; median score, 0; range, 0-0.5) that received intravenous injection of h-IRBP peptide–pulsed PECs cultured overnight in VIP. In contrast, uveitis was detected...
in the eyes of 8 of 10 mice (mean score, 1.2; median score, 1.0; range, 0-2.0) that received intravenous injection of h-IRBP peptide–pulsed PECs cultured overnight in PBS only \( (P = .004) \). Thus, mice that received PECs treated with VIP and that carried an h-IRBP peptide–specific signal displayed significantly reduced evidence of EAU.

INDUCTION BY VIP-TREATED PECs OF CD3+ REGULATORY T CELLS THAT AMELIORATE EAU

To test the postulate that VIP-treated PECs generate regulatory T cells in the spleen that interfere with development of EAU, we examined whether the splenic T cells primed by VIP-treated PECs could transfer tolerance to naive recipient mice. Forty million to 50 million T cells obtained from spleens of mice that first received VIP-treated PECs were transferred to syngeneic recipients. Within 30 minutes, these recipients were immunized with h-IRBP peptide and complete Freund adjuvant. Mice that received T cells derived from mice injected with VIP-treated PECs displayed lower scores of uveoretinitis and a lower occurrence of disease \( (P = .08) \). These data demonstrate that VIP-treated PECs induce the generation of CD3+ regulatory T cells that are capable of suppressing the h-IRBP peptide–specific pathogenic T-cell responses of uveitis.

COMMENT

The data presented herein show that the neuropeptide VIP is effective in suppressing EAU, a murine experimental model for human uveitis. In this study, we demonstrated that (1) VIP had a significant prophylactic effect on both the occurrence and severity of EAU; (2) VIP was effective in ameliorating established EAU; and (3) PECs pulsed with h-IRBP peptide in the presence of VIP possessed the ability to ameliorate EAU via inducing h-IRBP peptide–specific regulatory T cells.

It is important to gain insight into the mechanisms by which VIP down-regulates DH to h-IRBP peptide. It is possible that VIP directly down-regulates immune effector T cells. Taylor et al\(^6\) reported that VIP suppresses antigen-specific lymph node cell proliferation and interferon \( \gamma \) production in vitro. Furthermore, it has been shown recently that mice deficient in VIP receptors \( (V_{PAC_2R}) \), which are highly up-regulated on TH1 cells, display enhanced DH.\(^{12}\) Our results showed that injection of VIP during the induction of immunity with h-IRBP peptide suppressed DH expression and EAU occurrence and severity. In addition, VIP even inhibited DH responses to h-IRBP peptide after the immune system had been exposed to a uveitogenic regimen, indicating that VIP impairs the capacity of mice immunized with...
h-IRBP peptide to mount DH responses during the afferent and efferent limbs of the immune response. These findings are in agreement with the view that VIP has the capacity to suppress induction and expression of \( T_H1 \) immune responses.

Hara et al\(^{13} \) showed that PECs incubated with IRBP in the presence of aqueous humor or supernatants from cultured iris/ciliary body induced anterior chamber–associated immune deviation in vivo (impaired DH and IgG2a antibody production) when injected into naive, syngeneic mice, and in vitro–treated PECs had the capacity to abrogate EAU. Similarly, the present study showed that PECs pulsed with h-IRBP peptide in the presence of VIP conferred on recipient mice impaired DH responses to h-IRBP peptide (data not shown) and protected those mice against EAU. Moreover, VIP–treated PECs induced regulatory T cells that were able to suppress the \( T_H1 \) response after immunization, which may explain the cure of uveitis after onset. However, how and where regulatory T cells are generated and alter the immune response in vivo is still unclear and needs further investigation.

This is the first report to suggest a role of VIP in the development of experimental intraocular inflammation. Although our data indicate that treatment with VIP is effective at inhibiting EAU, Zhang and colleagues\(^{14} \) reported that treatment with VIP exacerbated the inflammatory process of endotoxin-induced uveitis and that the treatment decreased the production of tumor necrosis factor \( \alpha \) and elevated the serum level of interleukin 10. This difference in the effectiveness of VIP in endotoxin-induced uveitis and EAU may be due to the different features of ocular inflammation in these models. Moreover, Katayama\(^{15} \) previously showed that high-dose intravitreal injection of VIP resulted in miosis and the breakdown of the blood–aqueous barrier, whereas low-dose injection of VIP induced mydriasis. These findings suggest that VIP plays a role as a proinflammatory factor at high concentrations and has the capacity for different immune responses at low concentrations.

However, in the present study, we demonstrated that the transfer of macrophage cultured with VIP at the intraocular concentration (12nM) significantly suppressed the induction of EAU. Also, systemic injection of VIP (5 nmol/mouse) was effective for amelioration of uveitis. These findings are supported by a recent report showing that VIP treatment (5-nmol dose) reduced the incidence and severity of collagen-induced arthritis.\(^{10} \) The in vitro study also showed that the VIP inhibition of messenger RNA expression of proinflammatory cytokines (such as tumor necrosis factor \( \alpha \) and interleukin 1\( \beta \)) induced by collagen-induced arthritis was found at concentrations of \( 10^{-8} \text{M} \) to \( 10^{-6} \text{M} \).\(^{10} \) Therefore, although VIP might exert dual effects on induction of ocular inflammation, our results indicate that VIP provides a highly suppressive effect on EAU at the intraocular concentration of VIP.

**CONCLUSIONS**

We have demonstrated that VIP has the ability to ameliorate EAU and to down-regulate established pathogenic \( T_H1 \) responses to h-IRBP peptide. Moreover, h-IRBP peptide–pulsed PECs, cultured in the presence of VIP, acquired the capacity to suppress DH responses to antigen and prevent the development of EAU by inducing regulatory T cells. Vasoactive intestinal peptide might offer a new approach for the management of clinical uveitis.

**REFERENCES**


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**Figure 4.** Vasoactive intestinal peptide (VIP)–treated human interphotoreceptor retinoid-binding protein peptide 1-20 (h-IRBP peptide)–pulsed peritoneal exudate cells (PECs) induce regulatory T cells that ameliorate experimental autoimmune uveoretinitis (EAU). The h-IRBP peptide–pulsed PECs treated with phosphate-buffered saline (positive control group) or 12nM VIP (VIP-treatment group) were injected intravenously into naive C57BL/6 mice and immunized with h-IRBP peptide. Twenty-one days later, spleen cells were collected, then T-cell enrichment was performed. Purified T cells were injected intraperitoneally (40 \( \times \) 10\(^6 \) to 50 \( \times \) 10\(^6 \) cells/mouse) into naive C57BL/6 mice. Thirty minutes later, these recipients received a uveitogenic regimen. Eyes were collected on day 14 and were graded by histopathological findings on a scale of 0 to 4. The number of mice with occurrence of EAU/total number of mice is shown over the columns. Bars represent mean; limit lines, standard error. The experiment was repeated twice with similar results.

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**Table 1:** EAU Score, Mean ± SE

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<tr>
<th>Group</th>
<th>EAU Score, Mean ± SE</th>
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<tbody>
<tr>
<td>Positive Control</td>
<td>1/6</td>
</tr>
<tr>
<td>VIP Treatment</td>
<td>5/8</td>
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Congratulations to the winner of our April quiz, Rupak Kanti Biswas, MD, Sankara Nethralaya, Tamil Nadu, India. The correct answer to our April challenge was central serous chorioretinopathy due to corticosteroid use. For a complete discussion of this case, see the Clinicopathologic Reports, Case Reports, and Small Case Series section in the May ARCHIVES (Karadimas P, Kapetanos A, Bouzas EA. Central serous chorioretinopathy occurring after local application of glucocorticoids for skin disorders. Arch Ophthalmol. 2004;122:784-786).

Be sure to visit the Archives of Ophthalmology Web site (http://www.archophthalmol.com) and try your hand at our Clinical Challenge Interactive Quiz. We invite visitors to make a diagnosis based on selected information from a case report or other feature scheduled to be published in the following month’s print edition of the ARCHIVES. The first visitor to e-mail our Web editors with the correct answer will be recognized in the print journal and on our Web site and will also be able to choose one of the following books published by AMA Press: Clinical Eye Atlas, Clinical Retina, or Users’ Guides to the Medical Literature.