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Uveitopathogenic Site of the γ-Subunit of Cyclic Guanosine Monophosphate Phosphodiesterase in Lewis Rats

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Purpose: The γ-subunit of cyclic guanosine monophosphate phosphodiesterase (PDEγ) plays an important role in the phototransduction process of rod photoreceptors. A previous report indicated that experimental autoimmune uveoretinitis (EAU) could be induced in Lewis rats by immunization with PDEγ. In this study, we identified the uveitopathogenic site of PDEγ synthetic peptides and identified pivotal amino acid residues using analogue peptides.

Methods: Several synthetic peptides derived from PDEγ plus adjuvants were injected in Lewis rats. The induction of EAU was examined clinically and histologically. In addition, humoral and cellular immunity against peptides was investigated.

Results: The smallest uveitopathogenic peptide was identified as PDEγ 64-76 (ITVICPWEAFNHL), which consists of 13 amino acid residues, and the core sequence was identified as PDEγ 70-76 (WEAFNHL), which consists of 7 amino acid residues. The lowest dose of peptide to induce EAU was 0.03 nmol. The pivotal amino acid residues for eliciting EAU are at 70(W), 71(E), 73(F), and 75(H).

Conclusion: Our findings demonstrated the presence of a potent uveitopathogenic site in PDEγ whose potency in Lewis rats was comparable to that of interphotoreceptor retinoid-binding protein. **Jpn J Ophthalmol 2001;45:570–576**

Key Words: Experimental autoimmune uveoretinitis, γ-subunit of cyclic guanosine monophosphate phosphodiesterase, Lewis rat, synthetic peptides, uveitopathogenic site.

Introduction

Various causes induce human intraocular inflammatory diseases. Autoimmunity is assumed to play a major role in the pathogenesis of certain intraocular diseases that are called uveitis. Many studies have been performed to identify the antigen of human endogenous uveitis, but it is still unknown. Experimental autoimmune uveoretinitis (EAU) has been studied extensively because its clinical features resemble those of human uveitis. Several retina-specific proteins, such as S-antigen,[1,2] interphotoreceptor retinoid-binding protein (IRBP),[3] rhodopsin,[4] phosducin,[5] and recoverin,[6] are reported to induce EAU in susceptible animals.

In 1996, Ren et al[7] reported that EAU can be induced by immunization with the γ-subunit of cyclic guanosine monophosphate phosphodiesterase (PDEγ) from bovine retina. PDEγ plays an important role in the phototransduction process of rod photoreceptors, and it consists of 87 amino acids.[8–11] In the present study our goal was to determine the uveitopathogenic site of PDEγ synthetic peptides. We identified pivotal amino acid residues by immunizing with analogue peptides, substituting alanine or lysine for individual residues.

Materials and Methods

Animals

Female Lewis rats (Charles River Japan, Yokohama) aged 6–8 weeks were used as experimental

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animals. Care of the animals and experiments were performed in accordance with the Guidelines for Animal Experiments of Akita University School of Medicine and ARVO guidelines. Animals were divided into several groups for use in the studies of the overlapping segments of PDEγ. Some animals in each group were designated as controls.

Peptide

The peptides used in this study as antigens were synthesized and purified at Sawady Technology (Tokyo) according to the amino acid sequence of the PDEγ from bovine retina.[12] Five overlapping peptides consisting of about 30 amino acid residues each were synthesized (Table 1), and used to determine the uveitopathogenic site of PDEγ. After determination, the uveitopathogenic site was further divided into smaller fragments consisting of about 15 amino acids each, ie, PDEγ 45–58, PDEγ 59–76, and PDEγ 77–87 (Table 1), and used for immunization.

Immunization

Each synthetic peptide was dissolved in phosphate-buffered saline (PBS), and the solution was emulsified (1:1) in complete Freund's adjuvant containing *Mycobacterium Tuberculosis H37Ra* and injected into one hind footpad at various doses. An additional adjuvant, *Bordetella pertussis* (0.1 ml, 2×10^{10} organisms; Wako Pure Chemical, Osaka), was injected intravenously at the time of the immunization. In the control group, PBS was emulsified (1:1) in complete Freund's adjuvant and injected into one hind footpad. An additional adjuvant, *Bordetella pertussis* was also injected intravenously at the time of the immunization

Clinical and Histologic Grading of EAU

Immunized rats were examined for clinical signs of EAU by slit-lamp microscopy, and graded from 0 to 5, on the scale previously reported by Abe et al.[13] The animals were sacrificed on day 20 post-immunization, and eyes and pineal glands were obtained for histopathological examinations. The eyes and pineal glands were fixed in 2.5% buffered glutaraldehyde solution for 24 hours, followed by fixation with 10% buffered formaldehyde. Fixed preparations were embedded in paraffin and stained with hematoxylin-eosin. Histological changes were graded from 0 to 5 on the scale proposed by Merryman et al.[14]

Intracutaneous Reaction

The cellular immunological response was assessed according to the intracutaneous reaction. Antigen

Table 1. Amino Acid Sequence of PDEγ*

Segments of PDEγ	1 10 20 30 40 50 60 70 80 87
	MNLEPPKAEIRSATRVMGGPVTPRKGPPKFKQRQTRQFKSKPPKKGVQGFGDDIPGMEGLGTDITVICPWEAFNHLELHELAQYGII
Group 1 (1–29)	MNLEPPKAEIRSATRVMGGPVTPRKGPPK
Group 2 (15–44)	RVMGGPVTPRKGPPKFKQRQTRQFKSKPPK
Group 3 (30–58)	FKQRQTRQFKSKPPKKGVQGFGDDIPGME
Group 4 (45–76)†	KGVQGFGDDIPGMEGLGTDITVICPWEAFNHL
Group 5 (59–87)†	GLGTDITVICPWEAFNHLELHELAQYGII
Group 6 (45–58)	KGVQGFGDDIPGME
Group 7 (59–76)	GLGTDITVICPWEAFNHL
Group 8 (77–87)	ELHELAQYGII

†Experimental autoimmune uveitis was observed in groups 4, 5, 7 (underlined).
*γ-Subunit of cyclic guanosine monophosphate phosphodiesterase.

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peptide solution (50 μg/20 μl of PBS per animal) was injected subcutaneously into the rat auricle 18 days after immunization, and the thickness of the auricle was measured three times with a micrometer both before injection and 48 hours after injection. The thickness difference in each group of animals was calculated. Control animals in each group received an injection of only PBS. The Mann-Whitney test was used to evaluate statistical significance, and a P value of $<.05$ was regarded as significant.

Antibody Titers

The humoral immunological response was assessed by enzyme-linked immunosorbent assay (ELISA), using antibody titers from the serum.[15] Blood was collected from the hearts of the rats 20 days after immunization, and the serum was obtained after centrifugation. The sera of five normal rats were used as control. A sample of each PDEγ synthetic peptide (1.0 μg/well) was adsorbed to the surface of 96-well plates for ELISA and each well was blocked with bovine serum albumin. Diluted serum was then added in a 1:100–1:$3^7 \times 100$ dilution. The second antibody was peroxidase-conjugated goat anti-rat immunoglobulin G antibody (Zymed, South San Francisco, CA, USA), and a color reaction was obtained by adding the substrate, 2, 2-amino-bis (3-ethyl-benzothiazoline-6-sulfonic acid; Wako), in a buffer containing 0.003% hydrogen peroxide. The reaction was read at 414 nm with an ELISA autoreader (No. 2550; Bio Rad, Richmond, CA, USA). Antibody titers were determined at the initial dilution point when the optical density became flat.

Results

Uveitopathogenicity of PDEγ

Overlapping peptides consisting of about 30 amino acid residues each were tested for their uveitopathogenicity. PDEγ 1–29, PDEγ 15–44, and PDEγ 30–58 did not induce EAU even at a very high dose of 1000 μg. However, PDEγ 45–76 and PDEγ 59–87 caused EAU in immunized rats at a dose of 100 μg (Table 2).

To identify the uveitopathogenic site, PDEγ 45–76 and PDEγ 59–87 were further fragmented into PDEγ 45–58, PDEγ 59–76, and PDEγ 77–87. PDEγ 59–76 induce EAU at a dose as low as 1 μg, but the other two peptides did not cause EAU even at a dose of 100 μg (Table 2). EAU did not develop in any control animals. Pinealitis was not induced in any animals immunized with these peptides (data not shown).

A dose response study was carried out using PDEγ 45–76. EAU was induced with a dose of 0.1 μg (0.03 nmol), but not at a dose of 0.01 μg (Table 3).

Clinical and Histological Features of EAU

The clinical features of EAU induced by PDEγ 45–76 (1000 μg) are shown in Figure 1. Inflamma-

Table 2. Uveitopathogenicity of PDEγ Synthetic Peptides*

		Dose†								
		1000 μg			100μg			1 μg		
Peptide Amino Acid Sequences		Inc	Day	HS	Inc	Day	HS	Inc	Day	HS
Group 1 (1–29)	MNLEPPKAEIRSATRVMGGPVTPRKGPPK	0/5	–	0						
Group 2 (15–44)	RVMGGPVTPRKGPPKFKQRQTRQFKSKPPK	0/5	–	0						
Group 3 (30–58)	FKQRQTRQFKSKPPKKGVQGFGDDIPGME	0/5	–	0						
Group 4 (45–76)‡	KGVQGFGDDIPGMEGLGTDITVICPWEAFNHL	5/5	10.0	5.0	4/4	11.7	4.0			
Group 5 (59–87)‡	GLGTDITVICPWEAFNHLELHELAQYGII	5/5	10.0	5.0	5/5	11.4	4.9	4/5	15.0	3.5
Group 6 (45–58)	KGVQGFGDDIPGME				0/5	–	0			
Group 7 (59–76)‡	GLGTDITVICPWEAFNHL				5/5	11.6	4.9	3/5	14.3	2.1
Group 8 (77–87)	ELHELAQYGII				0/5	–	0			

*PDEγ: γ-Subunit of cyclic guanosine monophosphate phosphodiesterase.
†Inc: Experimental autoimmune uveitis (EAU) incidence, Day: onset day (mean), HS: histologic severity (mean).
‡EAU was observed in Groups 4, 5, 7 (underlined).

Table 3. Dose-Response Study to Determine Minimal Dose of Uveitopathogenicity by PDEγ Peptide 45–76*

Dose (μg)	Incidence of EAU	Mean day of EAU onset	Mean Histologic Grading
100	4/4	11.7	4.0
10	5/5	12.0	4.8
1	4/5	15.0	3.5
0.1	3/3	19.0	2.3
0.01	0/3	–	0

*PDEγ: γ-Subunit of cyclic guanosine monophosphate phosphodiesterase; EAU: experimental autoimmune uveitis.

tory debris in the anterior chamber and iris vessel dilatation were observed. In cases of severe inflammation, massive posterior chamber hypopyon and exophthalmos were observed.

The histological features of EAU induced by PDEγ 45–76 (1000 μg) are shown in Figure 2. At the beginning of the inflammation, disarrangement of the outer nuclear layer and loss of the outer segment of the photoreceptor cells were observed. In severe cases, total serous retinal detachment and infiltration of the photoreceptor cell layer, outer nuclear layer, and inner nuclear layer of the retina were observed.

Immune Response to Peptide

Cellular immune response was tested by subcutaneous injection of one of the peptides used for immunization. Intense auricular swelling was observed in rats immunized with 1000 μg of PDEγ 45–76 or PDEγ 59–87, and this reaction was significantly larger than what occurred in rats immunized with PDEγ 1–29, PDEγ 15–44, PDEγ 30–58, or in control rats ($P < .01$) (Figure 3).

Serum antibody titers were elevated in all rats immunized with any PDEγ peptide. There was no correlation between the antibody titer and the EAU incidence (Figure 4). The serum antibody titers were below 100 in all control rats (data not shown).

Identification of Uveitopathogenic Site

Various peptides consisting of about 15 residues that had a sequence shift of a few amino acid residues (PDEγ 56–70 to PDEγ 72–84) were used for immunization. PDEγ 62–76, PDEγ 63–76, and PDEγ 64–76 caused a severe and early onset of EAU even at a dose of 1 μg. However, severe EAU did not oc-

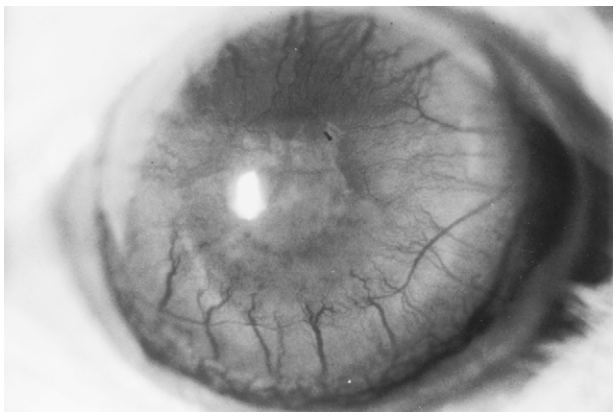

Figure 1. Anterior segment of eye of Lewis rat 12 days after immunization with 1000 μg of the γ-subunit of cyclic guanosine monophosphate phosphodiesterase 45–76. Iris vessel dilatation, inflammatory debris in anterior chamber, and posterior chamber hypopyon are observed.

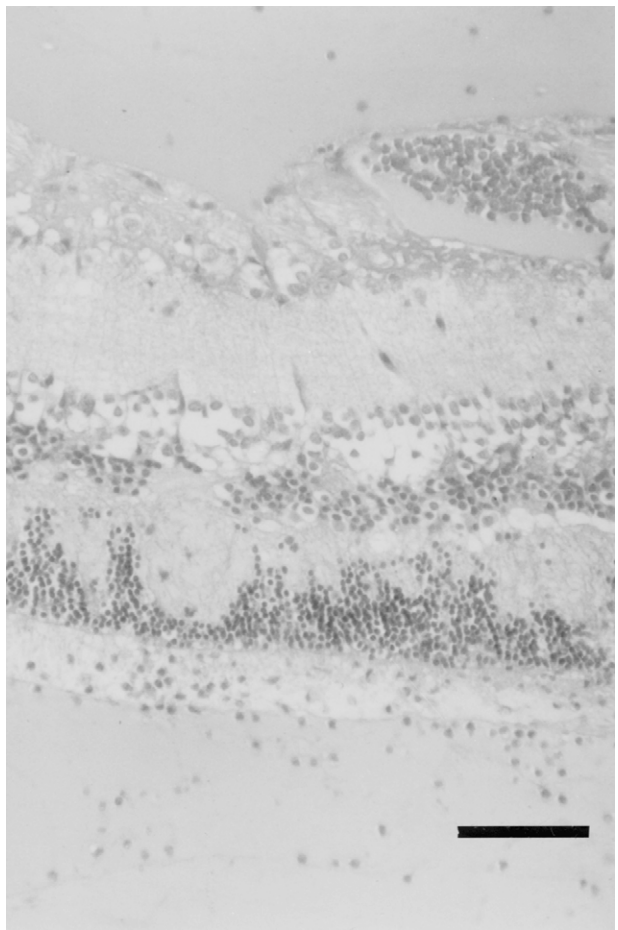

Figure 2. Histopathological changes in retina of Lewis rats immunized with 1000 μg of the γ-subunit of cyclic guanosine monophosphate phosphodiesterase (PDEγ) 45–76. Twelve days after immunization with 1000 μg of PDEγ 45–76. Massive cellular infiltration is seen in outer nuclear layer. Serous retinal detachment and disarrangement of outer nuclear layer are observed (×200). Bar = 10 μm.

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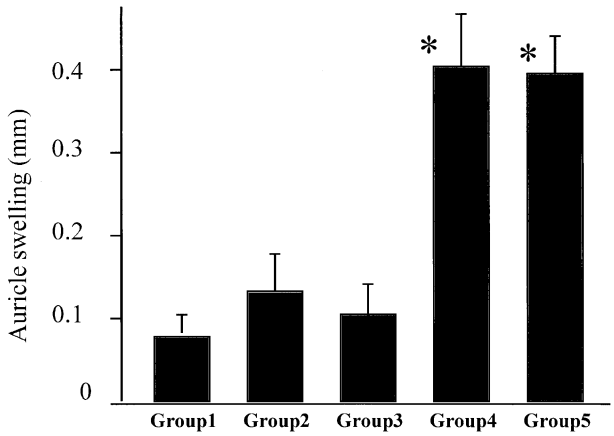

Figure 3. Intracutaneous reaction induced by peptides used in groups 1 to 5. Strong auricle swelling was observed in groups 4 and 5. $*P < .05$.

cur after immunization with PDEγ 62–75 or PDEγ 65–79. PDEγ 64–76, which consisted of 13 amino acid residues (ITVICPWEAFNHL), was identified as the smallest peptide that can elicit severe EAU with a severity corresponding to PDEγ 62–76. The core sequence was PDEγ 70–76 (WEAFNHL) which was always included within the uveitopathogenic peptides (Table 4).

Immunization with Analogue Peptides

To identify the pivotal amino acids for uveitopathogenicity, alanine was substituted for a single amino acid residue of the peptide PDEγ 63–77 (Table 5). When a peptide included alanine or an amino acid residue having the same characteristics as alanine (non-charged, hydrophobic, fatty side chain), lysine was used as a substitute. When an amino acid residue was substituted at 70(W → A), 71(E → A), 73(F → A), or 75(H → A) at a dose of 100 μg, EAU did not occur in the rats. Other analogue peptides induced EAU, but they had less uveitopathogenicity than the original peptides (DITVICPWEAFNHLE).

Auricular swelling with the immunizing analogues 70(W → A), 71(E → A), 73(F → A), or 75(H → A) was significantly weaker than with the uveitopathogenic peptide (PDEγ 63–77) (Table 5).

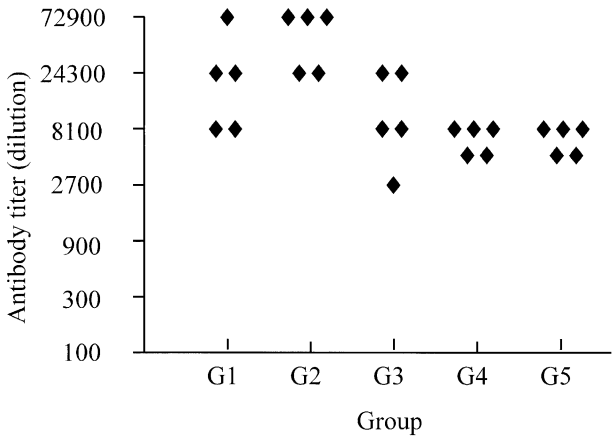

Figure 4. Antibody titrations of serum immunoglobulin G antibodies from rats immunized with peptides used in groups 1 to 5 (G1–G5). Serum antibody titers were elevated in groups 1 to 5 with 1000 μg immunization, and there was no relation to experimental autoimmune uveitis incidence. In all control group rats, serum antibody titers were below 100 (data not shown).

Discussion

This study demonstrates the uveitopathogenic site of PDEγ and the pivotal amino acid residues for eliciting EAU in Lewis rats. At the first screening test, the uveitopathogenic site of PDEγ was only one region (group 7: PDEγ 59–76). In general, antigen-presenting cells present 15 to 22-mer peptides on their MHC class II molecules.[16] We used 29 to 32-mer peptides. If these long peptides were not processed properly in antigen-presenting cells, they might have less stability for MHC class II molecules and there might have been a possibility of uveitopathogenic sites in the peptide groups 1–3.

The smallest peptide that can induce severe EAU was PDEγ 64–76 (ITVICPWEAFNHL), and the core sequence was PDEγ 70–76 (WEAFNHL). This sequence did not correspond to the uveitopathogenic site of S-antigen,[17] IRBP,[18], and phosducin,[13,19] and we could not detect other homologous peptides, using a homology search (data not shown).

Pinealitis was not observed in this study, which was in contrast to the results of Ren et al.[7] Pinealitis induced by recoverin was mild[20,21] and this result was deduced from a small quantity of recoverin in the pineal glands.[20,21] Further study is necessary to determine the quantity of PDEγ in pineal glands.

The minimal dose of PDEγ 45–76 required to induce EAU in our study was 0.1 μg (0.03 nmol). The minimal doses of uveitopathogenic peptides from other retinal specific proteins required when used in Lewis rats are: 0.015 nmol in IRBP,[22] < 0.25 nmol in S-antigen,[14] ≅ 0.24 nmol in phosducin,[13] < 2.6 nmol in recoverin,[21] < 21 nmol in rhodopsin,[23] respectively. As the minimal uveitopathogenic dose of PDEγ was 0.03 nmol, the peptide of PDEγ had almost equal uveitopathogenic potency when com-

Table 4. Determination of Uveitopathogenic Core Sequence in PDEγ*

Peptide Fragments		Dose†					
		100 μ			1 μg		
		Inc	Day	HS	Inc	Day	HS
Group 4 (45–76)	KGVQGFGDDIPGMEGLGTDITVICP*WEAFNHL*	4/4	11.7	4.0	4/5	15.0	3.5
Group 5 (59–87)	GLGTDIT VICP*WEAFNHL*ELHELAQYGII	5/5	11.4	4.9	3/5	14.3	2.1
Group 7 (59–76)	GLGTDITVICP*WEAFNHL*	5/5	11.6	4.9			
(56–70)	GMEGLGTDITVICP*W*	0/5	–	0			
(59–73)	GLGTDITVICP*WEAF*	0/5	–	0			
(62–75)	TDITVICP*WEAFNH*	0/5	–	0			
(62–76)	TDITVICP*WEAFNHL*	5/5	11.0	4.8	5/5	13.4	4.0
(63–76)	DITVICP*WEAFNHL*	5/5	11.6	4.8	5/5	12.8	4.7
(64–76)	ITVICP*WEAFNHL*	5/5	11.6	4.9	5/5	11.2	4.8
(65–79)	TVICP*WEAFNHL*ELH	5/5	12.4	4.7	2/5	17.5	1.1
(66–79)	VICP*WEAFNHL*ELH	5/5	10.8	4.9	0/5	–	0
(67–79)	ICP*WEAFNHL*ELH	5/5	10.8	5.0	1/5	20.0	0.3
(69–81)	P*WEAFNHL*ELHEL	4/5	13.3	4.3			
(70–82)	*WEAFNHL*ELHEL A	3/5	15.3	2.3			
(71–83)	*EAFNHL*ELHEL AQ	0/5	–	0			
(72–84)	*AFNHL*ELHEL AQ Y	0/5	–	0			

*PDEγ: γ-Subunit of cyclic guanosine monophosphate phosphodiesterase.
†Inc: Experimental autoimmune ureitis incidence, Day: onset day (mean), HS: histologic severity (mean).
Smallest and most potent uveitopathogenic sequence is underlined. Core sequence is italicized.

pared with the peptide of IRBP. Our data showed remarkably stronger uveitopathogenicity than that reported by Ren et al.[7] In their report,[7] 160 μg of PDEγ induced EAU in 90% of immunized rats by day 16 post-immunization and at 30 μg no EAU developed by day 20. They immunized PDEγ without *Bordetella pertussis*.[7] We suspect that injecting *Bordetella pertussis* intravenously at the time of the im-

Table 5. Uveitopathogenicity of Analogue Peptides of PDEγ 63–76*

Analogue Peptides	63 77	Inc	Day	HS	Auricle Swelling (μm)†
PDEγ 63-77	DITVICPWEAFNHLE	5/5	11.0	4.9	455 ± 43
Analogue 1, 64 (I → A)	D*A*TVICPWEAFNHLE	1/5	‡	0.4	71 ± 44§
Analogue 2, 64 (I → K)	D*K*TVICPWEAFNHLE	5/5	16.2	2.4	445 ± 80
Analogue 3, 65 (T → A)	DI*A*VICPWEAFNHLE	4/5	15.8	3.4	485 ± 43
Analogue 4, 66 (V → A)	DIT*A*ICPWEAFNHLE	3/4	16.3	2.7	505 ± 91
Analogue 5, 66 (V → K)	DIT*K*ICPWEAFNHLE	3/5	15.5	1.9	NT
Analogue 6, 67 (I → A)	DITV*A*CPWEAFNHLE	4/5	15.0	3.0	470 ± 66
Analogue 7, 67 (I → K)	DITV*K*CPWEAFNHLE	4/5	16.3	1.7	NT
Analogue 8, 68 (C → A)	DITVI*A*PWEAFNHLE	4/5	12.5	3.1	438 ± 100
Analogue 9, 69 (P → A)	DITVIC*A*WEAFNHLE	1/5	‡	0.7	252 ± 29§
Analogue 10, 70 (W → A)	DITVICP*A*EAFNHLE	0/5	–	0	220 ± 69§
Analogue 11, 71 (E → A)	DITVICPW*A*AFNHLE	0/5	–	0	256 ± 36§
Analogue 12, 72 (A → K)	DITVICPWE*K*FNHLE	2/5	12.5	2.3	NT
Analogue 13, 73 (F → A)	DITVICPWEA*A*NHLE	0/5	–	0	207 ± 86§
Analogue 14, 74 (N → A)	DITVICPWEAF*A*HLE	3/5	18.0	1.3	NT
Analogue 15, 75 (H → A)	DITVICPWEAFN*A*LE	0/5	–	0	246 ± 60§
Analogue 16, 76 (L → A)	DITVICPWEAFNH*A*E	3/5	16.0	1.3	541 ± 91
Analogue 17, 76 (L → K)	DITVICPWEAFNH*K*E	1/4	18.0	0.8	248 ± 24§
PBS		0/5	–	0	48 ± 28§

*PDEγ: γ-Subunit of cyclic guanosine monophosphate phosphodiesterase, Inc: experimental autoimmune uveitis (EAU), Day: onset day (mean), HS: histological severity (mean), NT: not tested, PBS: phosphate-buffered saline. Substituted peptides are italicized. Each peptide was administered at a dosage of 100 μg.
†Initially immunizing peptides were injected into auricular skin. Data represents mean ± standard deviation.
‡EAU was observed only histologically, not clinically.
§$P < .05$.

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munization may have been one of the causes for the severe EAU in our study.

Previous studies have indicated that cellular immunity plays a major role in the pathogenesis of EAU.[24] In using PDEγ, Ren et al[7] described that lymphocytes from the lymph nodes of diseased rats transferred EAU to naive recipients. Our data showed that the intracutaneous reaction was related to the EAU incidence, while the results of ELISA were not. These results indicate that EAU induced by PDEγ is also a T-lymphocyte- dependent disease.

EAU was not induced when alanine was substituted in analogue peptides 70(W), 71(E), 73(F), or 75(H), and the intracutaneous reactions of these peptides were weak. This result indicates that the pivotal amino acid residues for eliciting EAU are at 70(W), 71(E), 73(F), and 75(H). When 64(I) was replaced by alanine, EAU occurred weakly. When replaced by lysine, severe EAU occurred. This result indicates that the size of the side chain is more important than the charge in a 64 amino acid residue.

Singh et al[25] suggested that specific nonpathogenic analogues with a single amino acid substitution derived from pathogenic peptides have potential for the prevention and therapy of autoimmune diseases. Additional study is required to determine whether these analogue peptides also have the potential to prevent EAU.

We believe that the uveitopathogenic peptide of PDEγ is a useful tool to investigate the pathogenesis of EAU accurately. The antigen of human endogenous uveitis has not been identified, and further study will be necessary to determine whether PDEγ is involved in the pathogenicity of human uveitis.

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