Experimental Autoimmune Uveoretinitis Induced by the γ-Subunit of Cyclic Guanosine Monophosphate Phosphodiesterase in Rats

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Purpose. To investigate the capacity of the recombinant γ-subunit (Py) of cyclic guanosine monophosphate phosphodiesterase to induce experimental autoimmune uveoretinitis in Lewis rats.

Methods. Bovine Py was expressed in Escherichia coli cells and purified by fast protein liquid chromatography. Lewis rats were immunized by a single footpad injection of Py emulsified in complete Freund's adjuvant. Clinical and histopathologic changes in the eye and pineal gland were examined. Lymphocytes were prepared from the lymph nodes of rats with uveitis and transferred by intraperitoneal injection to naïve recipient rats.

Results. Immunization of rats with Py induced panuveitis and pinealitis with clinical and histopathologic changes similar to those induced by S-antigen. Lymphocytes from the lymph nodes of diseased rats transferred uveitis to naïve recipients.

Conclusions. Py, a retina-specific protein of molecular weight less than 10,000 kDa, is capable of inducing uveoretinitis in Lewis rats. The disease can be transferred adoptively to naïve rats by injection of lymphocytes from donor rats with experimental autoimmune uveoretinitis.

Cyclic guanosine monophosphate (cGMP) phosphodiesterase (PDE) regulates cGMP levels in vertebrate photoreceptors. The enzyme consists of two identical inhibitory subunits (Py) and the catalytic subunits (Paβ). Although the release of Py from Paβ is a prerequisite for PDE activation, the process that involves interaction with transducin is regulated in a complex manner. Recent studies showed that Py associates with transducin in unphosphorylated form and dissociates from it on phosphorylation. It was also shown that Py release from Paβ at high cGMP and calcium concentrations results in PDE activation, whereas, at low cGMP and calcium concentrations, Py release does not activate PDE but rather facilitates dissociation of cGMP from noncatalytic sites of PDE. Thus, Py plays a critical role in the enzymatic mechanism of visual transduction.

Several retina-specific proteins such as S-antigen, interphotoreceptor retinoid-binding protein, rhodopsin, phosducin, and recoverin were found to induce experimental autoimmune uveoretinitis (EAU) in laboratory animals. Animal models of EAU have been studied extensively because their clinical features resemble those of human uveitis. The primary structure of Py deduced from the sequence analysis of complementary DNA (cDNA) clones indicates that the subunit protein has a unique sequence, and Py is believed to be a retina-specific protein. In this work, therefore, we tested the capacity of the Py subunit of bovine PDE to induce EAU in Lewis rats. To ascertain that the protein antigen used for immunization was free of other uveitogenic retina proteins, recombinant Py was prepared by expressing Py protein in Escherichia coli cells in which a bovine Py cDNA had been incorporated.

MATERIALS AND METHODS. Preparation of Py. All DNA manipulations were carried out by standard procedures. Two oligonucleotides (Up-5'-GCCAACCTGGCATATGAGCCGC-3' and Down-5'-GGGTCCGATCTAGATGATGCCATACTG-3') were used in a PCR reaction to introduce Nde I and Bam HI sites of a bovine Py cDNA. The Nde I-Bam HI fragment was cloned into the Nde I-Bam HI-digested pET 11A (Novogene, Madison, WI). The vector was transferred to E. coli BL21 (DE3) (Novogene) for expression of Py. When the absorbance at 600 nm of bacterial cell suspension reached approximately 0.6, protein expression was induced by the addition of 1 mM (final) isopropyl β-D-thiogalactopyranoside to the cells and incubation for 4 hours. The cells were harvested, sonicated, and centrifuged. The supernatant was loaded on an SP Sepharose Fast Flow column pre-equilibrated with buffer (50 mM Tris-HCl, pH 7.5, 2 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 50 mM sodium chloride), washed, and eluted with a gradient of 0.2 to 0.5 M sodium chloride in buffer. Fractions containing PDE inhib-

Immunization of Rats. Ten female Lewis rats (110 g body weight; Harlan Sprague-Dawley, Indianapolis, IN) were immunized with a single footpad injection of 0.1 ml Py (160 μg/rat) per complete Freund's adjuvant (CFA) supplemented with heat-killed mycobacterium (7 mg/ml adjuvant). Four control animals received complete Freund's adjuvant supplemented with mycobacterium only. The high dose of Py was used to ascertain the development of EAU. Lower doses were tested in later experiments. All procedures involving animals were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animals were immunized on the same day (day 0).

Evaluation of Experimental Autoimmune Uveoretinitis. The severity of EAU was evaluated on the basis of clinical and histopathologic observations. Severity index for a given group of rats was calculated by dividing the sum of EAU grades by total number of EAU eyes. For histologic analysis, eyes were fixed in 2% glutaraldehyde, dehydrated in increasing concentrations of ethanol, embedded in paraffin, and sectioned (3-μm thick) and stained with hematoxylin–eosin.

Adoptive Transfer of Lymphocytes. Lymph nodes (from 16 donors) were dispersed and centrifuged in a Ficoll-Hypaque gradient (Pharmacia) to separate lymphocytes. The cells were washed in phosphate-buffered saline, suspended in RPMI 1640 medium containing 10% fetal calf serum and Py (20 μg/ml), and incubated for 72 hours. For transfer, the cells (7 × 10^6 per recipient rat) were harvested, washed, suspended in phosphate-buffered saline, and injected intraperitoneally into seven recipients. The high number of cells was injected so as to ascertain the transfer of disease.

RESULTS. Separation and Purity of Recombinant Py. A reverse-phase fast protein liquid chromatography chromatogram of recombinant Py and its purity is shown in Figure 1. Py was eluted at 42% acetonitrile and migrated as a single band (arrowhead, molecular mass of 13 kDa) in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Thus, the purity of the protein was greater than 95%. Gel mobility appeared to give an overestimate of molecular mass. The molecular weight of Py calculated from the amino acid sequence is 9.7 kDa.

Induction of Experimental Autoimmune Uveoretinitis. Experiments were repeated three times (6, 8, and 10 rats for immunization with 160 μg Py per rat). Figure 2 shows typical results on the development of EAU and disease severity in 10 Lewis rats immunized with recombinant bovine Py. By day 16 postimmunization, 90% of the eyes of immunized rats developed
FIGURE 3. Histopathologic changes of the eye and pineal gland of rat immunized with Py. (A to C) Sections from control eye. (D to F) Sections from experimental autoimmune uveoretinitis eye (day 14). (A,D) Retina. (B,E) Anterior tissues. (C,F) Pineal gland. os = outer segment; on = outer nuclear layer; in = inner nuclear layer; cb = ciliary body; i = iris; p = pinealocyte; l = lymphocyte. Magnification, ×200 (A,B,D,E), ×400 (C,F).
Days After Transfer

**FIGURE 4.** Adoptive transfer of Py-induced experimental autoimmune uveoretinitis. Day 0 is day of transfer of lymphocytes.

EAU. None of control rats had EAU. The histopathologic index (mean ± standard deviation for n = 10) at the peak of inflammation was 2.7 ± 0.8. Clinical and histopathologic features of Py-induced EAU were similar to those of S-antigen-induced EAU. Clinical changes at the peak of inflammation included vasodilation, serous and cellular infiltration of the anterior and posterior chambers, and hypopyon. Histopathologic changes are shown in Figure 3. Compared with tissue sections from control eyes (Figs. 3A, 3B, 3C), tissue damage in EAU eyes was evident (Figs. 3D, 3E, 3F). On day 14, inflammatory cells infiltrated the ciliary body and iris (Fig. 3E). Ciliary epithelial infoldings were destroyed and the iris became plump. In the retina, the photoreceptor cell layer was destroyed, and the outer nuclear layer became disorganized and thinner (Fig. 3D). Py immunization induced pinealitis, pathologic features of which also resembled those observed in S-antigen-immunized rats. Infiltrated inflammatory cells (lymphocytes) were found as dense masses (Fig. 3F) and primarily in the peripheral or subcapsular regions of the pineal gland. We also tested lower doses of Py. At 10 and 30 µg per rat, no disease developed by day 20. At 50 µg, 20% of immunized rats showed bilateral EAU by day 20 with the clinical severity index (mean ± standard deviation for n = 4) of 1.0 ± 0.2. Because 10 to 30 µg bovine S-Ag can induce EAU in Lewis rats, Py appears to be a moderate uveitogen compared with S-Ag.

**Adoptive Transfer of Uveitogenic Cells.** Adoptive transfer of lymph node lymphocytes (7 X 10^8) from rats with Py-induced EAU resulted in the development of EAU (clinical severity index of 1.6) in 70% of recipient rats on day 8 after cell transfer (Fig. 4). The histopathologic index (mean ± standard deviation for n = 6) was 2.0 ± 0.7. Thus, clinical and histopathologic features of transferred EAU were similar to those of Py-induced disease.

**DISCUSSION.** The result of this study shows that Py, a soluble bovine retina protein with a molecular mass of less than 10,000 Da, is uveitogenic in Lewis rats. The recombinant Py used in this experiment consists of a single polypeptide chain not modified post-translationally. It is known that phosphorylation modifies the function of Py. For example, phosphorylated Py has a higher inhibitory activity toward cGMP PDE than did nonphosphorylated Py, and the affinity of Py for transducin is greater in unmodified form than in phosphorylated form. It is not known at the present whether the uveitogenicity of Py is affected by phosphorylation.

Although lymph node lymphocytes from Py-immunized donor rats were able to transfer EAU to recipient animals, it remains to be determined whether T cells or B cells are involved. Autoantigens have not been identified unequivocally for any type of human uveitis of presumed autoimmunity. Future studies will determine whether Py is involved in the pathogenicity of human uveitis.

Induction of pinealitis in Py-immunized rats suggests that Py is not only localized to the retina but also to the pineal gland. Pinealitis could be induced by immunization with other retina-specific proteins such as S-antigen, interphotoreceptor retinoid-binding protein, and recoverin. The pineal gland, therefore, appears to possess a complete replica of metabolic machinery of the retina. It is of interest to investigate in what signal transduction pathway in the pineal gland Py is involved and what role Py plays in the pathway.

**Key Words**

adoptive transfer of disease, experimental autoimmune uveoretinitis and pinealitis, Lewis rat, Py-subunit of bovine cyclic guanosine monophosphate phosphodiesterase, recombinant protein

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References


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