Experimental Autoimmune Anterior Uveitis
Induction With Melanin-Associated Antigen From the Iris and Ciliary Body

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Purpose. This study was designed to investigate an animal model of uveitis that resembles anterior uveitis in humans after immunization with iris-ciliary body antigen.

Methods. Male Lewis rats 6 to 8 weeks of age were immunized with the buffer- and detergent-insoluble bovine iris-ciliary body antigen mixed with complete Freund's adjuvant and pertussis toxin. Antigen was digested with various proteolytic enzymes and tested in different rodent strains for a uveitogenic response.

Results. Acute iridocyclitis developed in both eyes of the Lewis rat during the second week after immunization, and the pattern of inflammation was similar to acute anterior uveitis in humans, with sudden onset, localization to the anterior uvea, and spontaneous resolution. Among the strains tested, F344 rats were susceptible to experimental autoimmune anterior uveitis but Long-Evans rats were not. Experimental autoimmune anterior uveitis did not develop in any of the mice studied, nor was it induced by immunization with synthetic melanin, amelanotic bovine tissues, pigmented bovine skin, or pigmented rat and rabbit iris-ciliary body. A soluble fraction derived from bovine melanin-associated antigen (BMAA) after digestion with the proteolytic enzyme V8 protease resulted in a disease similar to that observed with intact BMAA.

Conclusions. A model of anterior uveitis has been induced in the Lewis rat after immunization with bovine uveal antigen, and it resembles the acute iridocyclitis observed in humans. These results suggest that the pathogenic antigen is a melanin-associated protein(s) present within the iris-ciliary body. Invest Ophthalmol Vis Sci. 1995; 36:1056-1066.

Acute anterior uveitis (AAU) is a form of intraocular inflammation that includes iritis, iridocyclitis, or both. It is the most common form of uveitis and accounts for approximately 75% of cases. Inflammation occurs in the iris, the ciliary body, and the anterior choroid with a spillover of inflammatory cells into the vitreous space behind the lens. Retinal involvement is not a component of anterior uveitis.

A single episode of anterior uveitis does not cause permanent visual loss. It is uncomfortable for the patient but rarely results in significant visual damage. However, it is the recurrent nature of the disease that ultimately results in the loss of vision from cataract, cystoid macular edema, or glaucoma.

In the past, investigators induced experimental autoimmune uveitis (EAU) in inbred rodents with various proteins, such as the retinal soluble antigen (S-Ag), the (IRBP), rhodopsin, and, most recently, phosphoducin.

Unfortunately, EAU induced by soluble retinal proteins does not have the clinical characteristics of human AAU. Although the severity of EAU can be altered by the dose of retinal protein used for sensitization, as well as the accompanying adjuvant, the inflammation produced is primarily confined to the posterior segment of the eye. The disease in rats is characterized by the appearance of optic disk edema.
and retinal vasculitis, followed by focal infiltrates in the deep retinal layers, with the subsequent development of necrosis of the photoreceptor layer and the pigment epithelium. In the rat, EAU resembles some of the clinical features of idiopathic retinal vasculitis in humans. In the nonhuman primate, the disease more closely resembles the clinical entities of sympathetic ophthalmia, birdshot choroidopathy, and possibly Vogt–Koyanagi–Harada syndrome. In no other species does the disease have the characteristic features of human AAU.

In 1991, Broekhuyse and colleagues described experimental autoimmune anterior uveitis (EAAU), which was induced by a detergent-insoluble fraction of the retinal pigment epithelium (RPE). Fresh RPE cells were isolated and detergent treated. A pathogenic fraction derived from the RPE (termed PEP-X) evoked the inflammatory response. It was noted in the report that EAAU differed from the other known forms of EAU induced by photoreceptor proteins because the inflammation remained exclusively anterior and the photoreceptor cells and the pineal gland were not affected. A T-cell dependency of this disease was implicated as a consequence of inhibition by cyclosporine treatment.

In 1999, this model was explored further in studies that showed a dose-dependent induction of EAAU. Furthermore, it was thought that the pathogenic RPE protein, PEP-X, is antigen bound to melanin. At this time, it was demonstrated that the disease could be transferred adoptively from a sensitized host to a naive recipient—the Lewis rat—by in vitro-stimulated CD4+ T cells. Furthermore, EAAU could not be transferred by serum.

Recently, Broekhuyse et al., Chan et al., and we independently reported an animal model of anterior uveitis developed in Lewis rats using an insoluble antigen solely derived from bovine iris, ciliary body, and uveitis developed in Lewis rats using an insoluble antigen solely derived from bovine iris, ciliary body, and photoreceptor cells and the pineal gland were not affected. A T-cell dependency of this disease was implicated as a consequence of inhibition by cyclosporine treatment.

Reagents

Purified pertussis toxin, ponceau S, synthetic melamin, and Staphyloccocus aureus V8 protease were obtained from Sigma Chemical (St. Louis, MO). Complete Freund’s adjuvant (CFA) was obtained from Difco (Detroit, MI), and 125I protein A was obtained from Amersham Life Sciences (Arlington Heights, IL). Proteinase K was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Antigens

Monoclonal antibodies against S-antigen (F4C1, C10C10, B11A11, and D952), IRBP (H3B5), and phosphodiusc (4D4) were kindly provided by Dr. Larry A. Donoso, Wills Eye Hospital (Philadelphia, PA). Monoclonal antibodies to rhodopsin (K16-107C and B6-90N) were a gift of Dr. G. Adamus, R.S. Dow Neurological Sciences Institute (Portland, OR).

MATERIALS AND METHODS

Animals

Pathogen-free male rats and mice of the following strains were purchased from Harlan–Sprague–Dawley (Indianapolis, IN): rats—Lewis, Long–Evans, and Fischer 344 (F344); mice—Balb/c, B10.A, and SJL/J. Albino (New Zealand white) and pigmented-eye rabbits were obtained from Boswell Farms (St. Louis, MO). Animals were housed under conventional conditions, given water and chow (Purina, St. Louis, MO) ad libitum, and used at 6 to 8 weeks of age. Animal protocol conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
hydrolyze surface proteins. After HCl treatment, MAA was washed three times with PBS and stored at 20°C.

**Proteolytic Enzyme Treatment**

Ten milligrams of MAA was digested with 100 U of S. aureus V8 protease. The reaction was allowed to proceed in 75 mM potassium phosphate buffer (pH 7.8) at 37°C for 30 minutes to 1 hour in the presence of 4 M urea. Forty micrograms of proteinase K was used to digest 0.5 mg of MAA in a buffer containing 10 mM Tris HCl (pH 8.0), 0.5 mM EDTA, and 10 mM NaCl. Two different incubation conditions were used for proteinase K digestion: 37°C for 30 minutes and 65°C for 1 hour in the presence of 1% SDS. After digestion with these enzymes, samples were centrifuged at 1.2 X 10^5 g for 30 minutes at 4°C. The supernatant was dialyzed against water for 48 hours, lyophilized, and redissolved in PBS. Protein concentration in these samples was determined according to the method of Lowry et al., and samples were analyzed on SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

**SDS-PAGE and Immunoblotting**

SDS-PAGE was performed as described using a 10% slab gel. Proteins were transferred to nitrocellulose essentially as described by Towbin et al. After transfer, the nitrocellulose membrane was stained with ponceau S to monitor transfer efficiency. Unbound sites on the membrane were blocked with 1% ovalbumin or 3% nonfat milk. Diluted sera were incubated with the nitrocellulose for 3 hours at room temperature. Dilutions of antibodies used were 1:500 and 1:1000 (S-Ag, IRBP, and phosducin) and 1:100 and 1:200 (rhodopsin). This incubation was followed by successive washes with PBS—twice with PBS containing 0.05% Tween 20 and again with PBS to remove unbound antibody. Bound antibody was traced by protein A and unbound 125I removed by washing as described. Bound antibodies were visualized by exposure of air-dried nitrocellulose to Kodak X-OMAT AR films (Eastman Kodak, Rochester, NY) at −80°C for 2 to 3 days.

**Immunization and Assessment of EAAU**

Animals were immunized with the antigen using a single-dose induction protocol in the hind foot pad. To immunize the animals, 100 μl of stable emulsion containing 100 μg of the antigen, prepared by mixing equal volumes of CFA and the antigen, was used. Purified pertussis toxin at a concentration of 1 μg per animal was used as an additional adjuvant. Control animals were injected with the mixture of CFA and purified pertussis toxin only.

Starting from day 7 after immunization, the animals were examined daily by a masked observer using slit lamp biomicroscopy to determine disease onset and to grade iritis using the criteria previously reported. The experiments were terminated 30 days after immunization or at other time points for histologic analysis. Animals were killed, and their eyes and pineal glands were harvested for histologic analysis to assess the development and severity of the inflammation. Freshly enucleated eyes and pineal glands were fixed immediately for 24 hours in 2.5% buffered gluteraldehyde and transferred into 10% neutral formalin. Fixed and dehydrated tissues were then embedded in paraffin. Six-micron sections were stained with hematoxylin and eosin and examined by light microscopy. In some experiments, liver and kidney also were harvested and processed for paraffin sectioning. Intensity of uveitis (iritis) was scored in a masked fashion on the arbitrary scale of 0 to 4, as follows: 0 = normal; 1 = dilated iris vessels and thickened iris stroma; 2 = moderate infiltration of inflammatory cells in the anterior chamber with protein, a few scattered inflammatory cells, or both; 3 = heavy infiltration of inflammatory cells within the anterior chamber; 4 = heavy exudation of cells with dense protein aggregation in the anterior chamber; inflammatory cell deposits on the corneal endothelium.

The minimal criterion for scoring an animal as positive by histopathology was the presence of inflammatorycell infiltration of the iris, ciliary body, and anterior chamber.

**RESULTS**

**Model of EAAU**

The insoluble fraction, as well as the soluble fraction obtained after buffer, Triton, and SDS extraction of the bovine iris and ciliary body, was used to immunize male Lewis rats. One hundred micrograms of the antigen was injected in the hind footpad using a single-dose induction protocol. Uveitis did not develop in any of the animals immunized with the soluble protein fraction. However, the insoluble antigen fraction was pathogenic in all recipients and the disease, EAAU, caused by this antigen resembled AAU in humans. The disease did not develop in control animals injected with a mixture of CFA and purified pertussis toxin only (Table 1).

The clinical pattern of uveitis observed in EAAU is shown in Figure 1. Acute iridocyclitis developed around day 14 after injection. Maximum inflammation occurred between days 18 to 20, and by day 22 disease was subsiding and only mild inflammation was noted. Fourteen days after immunization, mononu-
TABLE 1. Pathogenicity of Different Antigens in Lewis Rats

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Eyes With EAAU</th>
<th>Days of Onset*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antigen Dose (µg)</td>
<td>Incidence</td>
</tr>
<tr>
<td>Bovine iris/ciliary body (soluble antigen)</td>
<td>100</td>
<td>0/12</td>
</tr>
<tr>
<td>Bovine iris/ciliary body MAA</td>
<td>100</td>
<td>120/120</td>
</tr>
<tr>
<td>Bovine conjunctiva (insoluble antigen)</td>
<td>100</td>
<td>0/12</td>
</tr>
<tr>
<td>Bovine gut (insoluble antigen)</td>
<td>100</td>
<td>0/6</td>
</tr>
<tr>
<td>Synthetic melanin</td>
<td>100</td>
<td>0/12</td>
</tr>
<tr>
<td>Synthetic melanin</td>
<td>400</td>
<td>1/12†</td>
</tr>
<tr>
<td>Deproteinized bovine iris/ciliary body MAA</td>
<td>100</td>
<td>0/16</td>
</tr>
<tr>
<td>Bovine skin MAA</td>
<td>100</td>
<td>0/24</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0/120</td>
</tr>
</tbody>
</table>

Incidence of EAAU given as positive/total eyes by histopathologic examination. MAA = melanin-associated antigen; EAAU = experimental autoimmune anterior uveitis.

* Mean ± SD. Severity of inflammation on histopathologic examination was grouped as mild (1+ to 2+) or severe (3+ to 4+).
† Six rats were immunized with bovine iris/CB MAA. Disease in both eyes developed in all animals.
Six rats were immunized with 400 µg of synthetic melanin; EAAU developed in one eye of one rat.

Clear cells started to infiltrate the center of the ciliary body stroma, with a few cells in the iris and anterior chamber (Fig. 2B). In the severe stage of disease, the ciliary body and iris became swollen and heavily infiltrated with inflammatory cells (mainly mononuclear cells, with only very few polymorphonuclear cells). An exudate of proteinaceous material with inflammatory cells appeared in the anterior chamber, with cellular precipitates on the corneal endothelium and lens surface and spillover into the anterior vitreous (Figs. 2C, 3). The posterior segment of the eye was minimally affected with mild choroiditis (Fig. 4). The cornea (Fig. 5A), retina (Fig. 4), pineal gland (Fig. 5), liver, and kidney were not affected in these animals.

In additional experiments, the iris and ciliary body obtained from the RCS (pigmented) and Lewis (non-pigmented) rat were used as a source of MAA. Insoluble and soluble fractions obtained after buffer and detergent extraction were used to immunize Lewis rats, but none of these fractions were pathogenic (not shown). Similarly, soluble and insoluble fractions of pigmented and albino rabbit iris–ciliary body did not induce uveitis (not shown). Based on these results, the insoluble antigen obtained from the bovine iris–ciliary body was used exclusively in the subsequent experiments and is abbreviated as BMAA (bovine melanin-associated antigen).

Reinduction of EAAU

Acute anterior uveitis spontaneously subsided by week 4, after the induction of EAAU, in Lewis rats with BMAA mixed with CFA and purified pertussis toxin. The reinduction of EAAU in these animals was then studied by the injection of BMAA 30 days after immunization. These animals were injected subcutaneously in the tail with a mixture of BMAA and incomplete Freund’s adjuvant (IFA). The results are presented in Table 2. EAAU recurred in all animals, with mild to severe disease in both eyes; onset of disease was delayed until day 20. Recurrent uveitis did not develop either clinically or histologically in control animals immunized with a mixture of IFA and saline.

Species Specificity and Genetic Susceptibility to EAAU

Various mouse and rat strains were screened for a uveitogenic response to BMAA. We used two common mouse strains, Balb/C(H-2b) and SJL/J(H-2b), and one recombinant strain, B10.A(H-2ª). Uveitis did not develop in any of the mice in our study (Table 3).
FIGURE 2. Histopathologic changes in the eye of Lewis rats after immunization with bovine melanin-associated antigen from iris–ciliary body. Normal ciliary body and iris 7 days after injection (A). At 14 days after injection (B), inflammatory cell infiltration is observed mainly in the basal central part of ciliary body stroma (arrow). At day 18 (C), severe inflammation of iris and ciliary body is observed with spillover into the anterior vitreous (arrow). Original magnification, ×100.

FIGURE 3. Severe experimental autoimmune anterior uveitis induced by immunization of Lewis rats with bovine melanin-associated antigen. At the peak of inflammation, severe iritis is present. The anterior chamber and iris are infiltrated by many cells (arrow), with dense protein aggregation (A). The ciliary body is also severely inflamed (arrow), but the retina is not involved (B). Original magnification, ×200.
that an antigen bound to melanin may be the pathogen. Furthermore, melanin containing the insoluble antigen obtained from bovine skin was used to immunize Lewis rats and also was not pathogenic. These results suggest that the pathogenic antigen in EAAU is bound to melanin, which is localized to the eye.

Chemical Nature of the Antigen. To study the chemical nature of the antigen, BMAA was deproteinized. For this purpose, BMAA was treated with 6N HCl at 110°C for 24 hours, and the deproteinized antigen was tested for pathogenicity. Results shown in Table 1 indicate that the deproteinized BMAA is not uveitogenic. Thus, these results imply that uveitogenic activity is related to the protein(s) bound to the melanin granules. Because the uveitogenic pathogen in BMAA appears to be an ocular protein bound to melanin, we attempted to solubilize the insoluble protein antigen.

Solubilization of BMAA. To solubilize the insoluble protein antigen, BMAA was digested with the proteo-

Among the rat strains tested, F344 was susceptible to EAAU (Table 4), as was the Lewis rat; the Long-Evans rat was resistant. The onset of disease in the F344 rat was slightly delayed compared to onset in the Lewis rat.

Pathogenic Antigen in EAAU

Association With Ocular Melanin. The association of insoluble bovine antigen with melanin was studied by using amelanotic bovine tissues, such as conjunctiva and gut. These tissues were treated in the same way as bovine iris-ciliary body, and the detergent insoluble fraction was used to immunize Lewis rats. None of the animals immunized with insoluble antigen derived from conjunctiva and gut developed EAAU (Table 1), suggesting that the antigen is probably associated with melanin or may be melanin itself. To test the second possibility, synthetic melanin (Sigma) was used to immunize Lewis rats in the standard manner. As shown in Table 1, melanin was not pathogenic, suggesting
TABLE 2. Reinduction of EAAU in Lewis Rats

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antigen Dose (μg)</th>
<th>Adjuvant</th>
<th>Incidence</th>
<th>Mild</th>
<th>Severe</th>
<th>Day of Onset*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMAA</td>
<td>100</td>
<td>IFA</td>
<td>6/6</td>
<td>2/6</td>
<td>4/6</td>
<td>20 ± 0</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>IFA</td>
<td>0/6</td>
<td>0</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

Incidence of EAAU given as positive/total animals (both eyes involved). BMAA = bovine melanin-associated antigen from the iris-ciliary body; IFA = incomplete Freund’s adjuvant; EAAU = experimental autoimmune anterior uveitis.

* Mean ± SD.

Severity of inflammation on histopathologic examination was grouped as mild (1+ to 2+) or severe (3+ to 4+), with the score in each eye averaged.

lytic enzyme, V8 protease or proteinase K. After dialysis and lyophilization, 100 μg of the soluble antigen was used to immunize Lewis rats using the standard immunization protocol. The proteinase K-digested soluble fraction was not pathogenic; however, EAAU developed in all Lewis rats immunized with the V8 protease-digested soluble antigen (Table 5). Disease severity and histopathologic features were similar to those observed with insoluble BMAA. The enzyme-digested soluble fractions were analyzed on 10% SDS-PAGE, and the gel was stained with Coomassie blue. Approximately 18 to 20 well-resolved protein bands were observed in the V8 protease digested fraction, whereas only one darkly stained band was observed in the proteinase K digested soluble fraction (Fig. 6).

Absence of Retinal Antigens in V8 Protease-Digested Soluble Fraction. The possible presence of a pathogenic soluble retinal protein (S-Ag, IRBP, rhodopsin, and phosducin) in the soluble fraction derived from the V8 protease digestion was investigated by immunoblotting using monoclonal antibodies. Buffer soluble crude retinal extract was used as the positive control for S-Ag, IRBP, and phosducin, whereas a Triton X-100 soluble crude retinal extract was used as the positive control for rhodopsin. Forty micrograms of the protein was analyzed on SDS-PAGE, and the proteins were transferred to the nitrocellulose membrane by electroblotting. Results presented in Figure 7 show the inability to detect S-Ag and IRBP. Rhodopsin and phosducin also were not detected in our soluble antigen fraction derived from the V8 protease digestion of BMAA (not shown).

DISCUSSION

Human AAU is the most common form of intraocular inflammation. We recently developed a clinically relevant animal model of anterior uveitis. Essential features of the disease have been reproduced in the Lewis rat by immunization with the insoluble fraction of bovine iris-ciliary body. The importance of studying this model is its resemblance to human AAU. Previous models of EAU induced with retinal protein(s), with endotoxin, or in the nude mouse have important differences with AAU. The present study describes our model and provides partial identification of the putative pathogenic antigen.

Crude uveal antigen was isolated from the iris and ciliary body of the pigmented cow eye. Pigmented tissue was subjected to buffer and detergent extraction, and detergent-soluble and detergent-insoluble fractions were used to immunize male Lewis rats. Acute iridocyclitis developed in all animals immunized with insoluble melanin containing the antigen BMAA after the second week following immunization. Clinically and histologically, the disease observed in these animals resembled human AAU with its sudden onset, localization to the anterior uvea, and spontaneous resolution. Mild choroiditis was present, suggesting that a similar or cross-reacting antigen is present in the choroid. However, inflammation was not observed in the retina or pineal gland. Detergent-soluble fractions were not pathogenic in our experimental system, nor was iris and ciliary body obtained from rats (pigmented and nonpigmented) or rabbits (pigmented and nonpigmented).

After the spontaneous resolution of EAAU, during week 4 after immunization, we were able to reinduce the disease in these animals by reinjecting antigen and incomplete Freund’s adjuvant; purified pertussis toxin was not used. The disease observed after the second challenge with the antigen was clinically and histologi-
The clinical and histologic features observed in our model of EAAU resemble those reported by Broekhuyse and colleagues. These investigators originally described a new experimental model of anterior uveitis induced by immunization with detergent-insoluble antigen isolated from bovine RPE. More recently, induction of the disease with insoluble antigen extracted from the choroid and iris has been reported. Study of genetic susceptibility to the induction of EAAU by BMAA revealed two sensitive rat strains, Lewis and F344. Lewis was the more susceptible, with slightly earlier onset and increased severity. In contrast, the Long–Evans strain was resistant to EAAU. Studies performed in rodents with EAU indicated a dual regulation of susceptibility by major histocompatibility complex (MHC) and non-MHC genes. Susceptible MHC is required for the induction of EAU; however, in strains having a susceptible haplotype, the final expression of the disease is strongly modulated by the genetic background (i.e., non-MHC genes) that controls such factors as interferon gamma production, mast cell density in the uvea, and corticosteroid response to physiologic stress. The Lewis and F344 strains share the same MHC class II (RT1B1); however, the Lewis is highly susceptible to EAU induced by immunization with S-Ag or IRBP, whereas F344 is a low responder. In Lewis rats, a defective hypothalamic–pituitary–adrenal axis, resulting in a reduced ability to mount a corticosteroid stress response and a higher number of mast cells in uveal tissue, has been implicated in the sensitivity of Lewis rats to autoimmune disease. These same factors might account for the higher sensitivity of Lewis rats to EAAU.

Attempts to induce EAAU in the mouse were unsuccessful. Two mouse strains (SJL/J and B10.A) were chosen on the basis of known susceptibility to other organ-specific autoimmune diseases. SJL/J (H-2) mice are susceptible to experimental autoimmune encephalomyelitis, and B10.A (H-2k) are susceptible to S-Ag and IRBP-induced EAU. However, neither BALB/c (H-2d), SJL/J, nor B10.A mice were susceptible to EAAU. These results could be due to several factors, including the nature of the antigen. S-Ag and IRBP are soluble proteins, whereas our antigen is buffer- and detergent-insoluble. In anterior chamber-associated immune deviation (ACAID), an animal model of ocular immunity, different immune mechanisms have been proposed for soluble and insoluble proteins. Ferguson and coworkers have demonstrated that soluble factor(s) are responsible for ACAID with insoluble proteins, such as TNP-coupled spleen cells, whereas cell-borne factor(s) have been implicated with albumin.

Our study reports the localization and the partial identification of the putative uveitogenic antigen in BMAA used to induce EAAU. In our system, the crude melanin-associated insoluble antigen isolated from bovine skin was not pathogenic, indicating that the antigen is localized to the eye and is tissue specific. These

### Table 4. Uveitogenic Response of Rats to BMAA

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rats With EAAU/Total</th>
<th>Day of Onset*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lewis</td>
<td>4/4</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>Fischer 344</td>
<td>4/4</td>
<td>17 ± 0</td>
</tr>
<tr>
<td>Long Evans</td>
<td>0/4</td>
<td>—</td>
</tr>
</tbody>
</table>

* Mean ± SD.

BMAA = bovine melanin-associated antigen from iris–ciliary body; EAAU = experimental autoimmune anterior uveitis.

### Table 5. Pathogenicity of Proteolytic Enzyme Digested BMAA in Lewis Rats

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antigen Dose (µg)</th>
<th>Incidence</th>
<th>Mild</th>
<th>Severe</th>
<th>Day of Onset*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insoluble BMAA</td>
<td>100</td>
<td>20/20</td>
<td>0</td>
<td>20</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>Proteinase K digested</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>soluble fraction</td>
<td>100</td>
<td>0/10</td>
<td>0</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>V8 protease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>digested soluble fraction</td>
<td>100</td>
<td>24/24</td>
<td>2/24</td>
<td>22/24</td>
<td>15.5 ± 1.5</td>
</tr>
</tbody>
</table>

Incidence of EAAU given as positive/total animals.

BMAA = bovine melanin-associated antigen; EAAU = experimental autoimmune anterior uveitis.

* Mean ± SD.

Severity of inflammation on histopathologic examination was grouped as mild (1+ to 2+) or severe (3+ to 4+), with the score in each eye averaged.
Melanin is the main pigment found in mammalian skin, hair, and eye and is synthesized in the melanosomes of melanocytes. Melanin is a complex insoluble polymer composed mainly of oxidized derivatives of tyrosine. Natural melamins are composed of two distinct portions—a chromophoric backbone and a protein. Little is known about the bonding between these components. It is suggested that the proteins are covalently bound to the chromophoric component. The biologic function of melanin is not fully understood; it is relatively inert metabolically, with a low turnover. It may provide protection against ultraviolet radiation and may serve as a scavenger for free radicals and metal ions.

The pathogenic antigen in EAAU is a melanin-associated protein because buffer- and detergent-

**FIGURE 6.** SDS-PAGE (10% reducing) analysis of the soluble fractions obtained after digestion of bovine melanin-associated antigen with proteinase K (1) and V8 protease (2). Molecular weight markers in kilodaltons are indicated on the left margin. The gel was stained with Coomassie blue.

findings are in contrast to those reported by Broekhuysen and coworkers, who have reported that bovine skin melanin is pathogenic; however, uveitogenicity of this preparation is much lower than that of bovine ocular melamins. The differences between our results and those reported by Broekhuysen and coworkers could be due to the fact that higher antigen dose and more purified pertussis toxin (2 µm/animal) were used in their study for immunization with skin-derived melanin antigen.

Melanin is the main pigment found in mammalian skin, hair, and eye and is synthesized in the melanosomes of melanocytes. Melanin is a complex insoluble polymer composed mainly of oxidized deriv-
treated amelanotic bovine tissues, synthetic melanin, and HCl-treated deproteinized insoluble BMAA are not pathogenic. Although melanin granules are known to absorb proteins to their surface, the pathogenic activity associated with our antigen is not caused by adsorbed protein. Our method of preparation of the crude antigen involves extensive treatment with PBS, Triton X-100, and SDS, which insures that melanin will be virtually free of all absorbed proteins.

Our uveitogenic protein(s) can be solubilized and cleaved from melanin by proteolytic enzyme treatment. The soluble fraction obtained after V8 protease digestion has uveitogenic activity, whereas that obtained after protease K treatment is not pathogenic. On SDS—PAGE, more than 20 protein bands could be resolved in the V8 protease-digested soluble fraction; however, in the protease K-treated soluble fraction, only one well-resolved band was observed. The uveitogenic activity associated with the V8 protease-digested soluble fraction was probably not caused by the presence of the known pathogenic soluble retinal proteins—such as S-Ag, IRBP, rhodopsin and phosducin—because no retinitis was observed. Additionally, we were unable to detect these proteins using monoclonal antibodies specific for these proteins, although the restricted epitope specificity of monoclonal antibodies severely limited their usefulness for this purpose.

Our results demonstrate that the uveitogenic antigen in EAAU is a protein(s) associated with bovine melanin from the iris and ciliary body. The Lewis rat eye contains melanocytes with premelanosomes; however, no melanin was present in these premelanosomes. Nevertheless, we postulate that the pathogenic antigen in EAAU is present in the iris, ciliary body, and choroid of this amelanotic rat strain. Studies on the further characterization of the pathogenic antigen(s) are underway in our laboratory that should help us to gain insights into the pathogenesis of this disease.

Key Words
uveitis, rat, iris, ciliary body, melanin

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