flow is expected to occur when TM and the endothelial cells of the aqueous plexus shrink. Conversely, cells that swell with hyposmotic solutions would instead increase the resistance to aqueous humor flow through paracellular pathways, thus decreasing C. Although the osmolality of aqueous humor does not change under physiological conditions, there is indirect experimental evidence that volume regulation mechanisms may contribute to the adjustment of aqueous outflow resistance. Ethacrynic acid, a nonspecific blocker of the Na-K-Cl cotransporter, increases C in different species. Thus, regulatory mechanisms of cell volume in trabecular tissues, by maintaining the size and hydraulic resistance of paracellular pathways, may play a role in the determination of aqueous humor drainage.

Key Words
albumin, aqueous humor, osmolality, outflow facility, trabecular meshwork

Acknowledgments
The authors thank Prof. M. Wiederholt for his valuable comments.

References

Induction of Experimental Autoimmune Anterior Uveitis by a Self-Antigen

Melanin Complex Without Adjuvant

Nalini S. Bora, Ming-Dar Woon, Michael T. Tandhasetti, Thomas P. Cirrito, and Henry J. Kaplan

Purpose. Experimental autoimmune anterior uveitis (EAAU) is an organ-specific autoimmune disease induced by immunization with bovine melanin-associated antigen (MAA) and two adjuvants (complete Freund's adjuvant and purified pertussis toxin). This study was undertaken to explore whether an adjuvant is required in the induction of EAAU.

Methods. Insoluble MAA was extracted from the bovine iris and ciliary body. Soluble bovine MAA was derived by treatment of insoluble MAA with the proteolytic enzyme, V8 protease. Lewis rats were immunized with the insoluble or soluble antigen, with or without adjuvant (complete Freund's adjuvant and purified pertussis toxin). Adoptive transfer of CD4+ and CD8+ T cells was performed to investigate the pathogenesis of EAAU.

Results. Experimental autoimmune anterior uveitis can be induced in Lewis rats by immunization with 100 µg insoluble bovine MAA alone without the use of adjuvants. The disease can be adoptively transferred to naive syngenic rats by primed CD4+ T cells. In contrast, soluble bovine MAA was not uveitogenic unless adjuvants were employed.
TABLE 1. Induction of EAAU in Lewis Rats Without Adjuvant

<table>
<thead>
<tr>
<th>Antigen</th>
<th>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>Insoluble BMAA</td>
<td>100</td>
<td>CFA + PTX</td>
<td>24/24</td>
<td>0</td>
<td>24</td>
<td>14.0 ± 3</td>
</tr>
<tr>
<td>Insoluble BMAA</td>
<td>10</td>
<td>CFA + PTX</td>
<td>6/6</td>
<td>2</td>
<td>4</td>
<td>13.5 ± 2</td>
</tr>
<tr>
<td>Insoluble BMAA</td>
<td>1</td>
<td>CFA + PTX</td>
<td>0/6</td>
<td>0</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>Insoluble BMAA</td>
<td>100</td>
<td>—</td>
<td>50/50</td>
<td>0</td>
<td>50</td>
<td>15.0 ± 3</td>
</tr>
<tr>
<td>Insoluble BMAA</td>
<td>10</td>
<td>—</td>
<td>0/6</td>
<td>0</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>Insoluble BMAA</td>
<td>1</td>
<td>—</td>
<td>0/6</td>
<td>0</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>Soluble BMAA</td>
<td>100</td>
<td>CFA + PTX</td>
<td>28/28</td>
<td>0</td>
<td>28</td>
<td>15.5 ± 1.5</td>
</tr>
<tr>
<td>Soluble BMAA</td>
<td>100</td>
<td>—</td>
<td>0/28</td>
<td>0</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

EAAU = experimental autoimmune anterior uveitis; BMAA = bovine melanin-associated antigen.
Incidence of EAAU is given as positive/total eyes by histologic examination. Severity of inflammation on histopathologic examination was grouped as mild (1+ to 2+) or severe (3+ to 4+).

*Mean ± standard deviation.

Conclusions. The data suggest that EAAU can be induced in the Lewis rat without addition of an adjuvant. Future studies concerning the pathogenesis of EAAU can now be performed without the possible confounding effect of an adjuvant. Invest Ophthalmol Vis Sci. 1997;38;2171–2175.

Experimental autoimmune anterior uveitis (EAAU) is an organ-specific autoimmune disease of the eye mediated by CD4+ T cells. We and others have reported the induction of EAAU in the Lewis rat by a single injection of adjuvant and detergent-insoluble melanin-associated antigen (MAA) isolated from bovine retinal pigment epithelium (RPE), iris, ciliary body, and choroid.1–9 We have further shown that EAAU can be induced by adjuvant and a soluble protein fraction released by proteolytic digestion of bovine MAA with V8 protease.7 Studies to date have employed complete Freund's adjuvant (CFA) and purified pertussis toxin (PTX) as the adjuvants. In this report, we describe the unique capability of MAA to

FIGURE 1. Ocular histopathology of severe experimental autoimmune anterior uveitis was identical in Lewis rat immunized with bovine melanin-associated antigen mixed with adjuvant-complete Freund's adjuvant and purified pertussis toxin (A and B) and with bovine melanin-associated antigen alone (C and D). Iris and ciliary body were severely inflamed and were infiltrated by inflammatory cells (arrows). Mild choroiditis (arrowheads) was observed, but the retina was unaffected. Magnification, ×100.
TABLE 2. Adoptive Transfer of EAAU Induced Without Adjuvant

<table>
<thead>
<tr>
<th>Donor Immunization</th>
<th>Stimulus in Culture</th>
<th>Number of Cells (10⁶)</th>
<th>Cell Population</th>
<th>Route</th>
<th>EAAU in Recipient</th>
<th>Day of Onset*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insoluble BMAA</td>
<td>Insoluble BMAA</td>
<td>10</td>
<td>LN cells</td>
<td>IV</td>
<td>4/4</td>
<td>Severe</td>
</tr>
<tr>
<td>Insoluble BMAA</td>
<td>Insoluble BMAA</td>
<td>15</td>
<td>CD4/CD8</td>
<td>IP</td>
<td>6/6</td>
<td>Severe</td>
</tr>
<tr>
<td>Insoluble BMAA</td>
<td>Insoluble BMAA</td>
<td>8.4</td>
<td>CD4</td>
<td>IV</td>
<td>4/4</td>
<td>Severe</td>
</tr>
</tbody>
</table>

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

Incidence of EAAU is given as positive/total eyes by histological examination. Severity of inflammation on histopathological examination was grouped as mild (1+ to 2+) or severe (3+ to 4+).

*Mean ± standard deviation.

induce EAAU without the use of an adjuvant. Furthermore, the induction of EAAU without an adjuvant is a CD4+ T-cell-mediated disease.

MATERIALS AND METHODS. Animals. Pathogen-free male and female Lewis rats (5 to 6 weeks old) were obtained from Harlan Sprague–Dawley (Indianapolis, IN). The animal protocols conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Reagents. Purified pertussis toxin (PTX) was obtained from Freund's adjuvant (CFA) from Difco (Detroit, MI); and rat CD4 and CD8 immunocolumns from Biotex (Edmonton, Alberta, Canada).

Antibodies. Monoclonal antibodies obtained from Harlan Bioproducts for Science (Indianapolis, IN) were used to identify CD4 (clone W3/25) and CD8 (clone OX-8) T cells. Anti-B-cell antibodies (CD45, clone OX-8) T cells. Anti-B-cell antibodies (CD45, clone OX-8) were purchased from Pharmingen (San Diego, CA) and fluorescein isothiocyanate (FITC) conjugated goat antimouse immunoglobulin G (IgG) was supplied by Cappel (West Chester, PA). All antibodies were used at a dilution of 1:100. MOPC-21 (mouse IgG1) was obtained from Sigma.

Antigen and Immunization. Melanin-associated antigen was isolated from bovine irises and ciliary bodies, and the pathogenetic proteins were solubilized following the methods previously described.4,7,8 These antigen preparations were labeled insoluble and soluble MAA, respectively. Lewis rats were injected in the hind footpad with a single dose of the following combinations of antigen and adjuvants: insoluble MAA in phosphate-buffered saline, insoluble MAA emulsified with CFA and PTX, soluble MAA in phosphate-buffered saline, and soluble MAA mixed with CFA and PTX (1 µg per animal). Animals were examined daily between days 7 and 30 after injection for clinical signs of uveitis, and EAAU was graded, using the criteria previously described.1 Eyes were also harvested at various time points for histologic analysis, and the intensity of uveitis was histologically scored on an arbitrary scale of 0 to 4.1,7

Adaptive Transfer of Disease. Draining lymph nodes were harvested from donor Lewis rats with mild to moderate EAAU detected in clinical examination. A single-cell suspension of lymph node cells was made in Dulbecco's modified minimum essential medium, supplemented with 10% fetal calf serum, sodium pyruvate (1%), L-glutamine (0.75%), 2-mercaptoethanol (5 × 10⁻⁵ M), penicillin (1%), streptomycin (1%), HEPES (10 mM), L-arginine (0.12 mg/l), L-asparagine (0.56 mg/l), and sodium bicarbonate. Cells (20 × 10⁶) suspended in 20 ml of complete media were cultured with the insoluble MAA (40 µg/ml) for 3 days. After stimulation in vitro (5% CO₂, 100% humidity, 37°C), the cells were harvested, purified by Ficoll density centrifugation and analyzed by flow cytometry as described later. Cells were transferred by intravenous or intraperitoneal injection into naive Lewis rats. In some experiments, B cells, macrophages, and other plastic-adherent cells were depleted by a panning procedure, and T lymphocytes were further fractionated into CD4+ and CD8+ T cells, using Cellect immuno-columns (Biotex) according to the manufacturer's recommendations. These columns enrich rat T cells by a process of negative selection.

Analysis of Cell Surface Markers by Flow Cytometry. For flow cytometric analysis 10⁶ cells were incubated with primary antibodies for 45 minutes on ice. The cells were washed with Hank's balanced salt solution (HBSS), and reacted with a secondary antibody (FITC-conjugated goat antimouse IgG) at a 1:50 dilution for 45 minutes on ice. The cells were then washed twice with HBSS and were analyzed using FACScan and CELLQUEST software (Becton Dickinson, San Jose, CA). Control stains were performed by omission of the primary or secondary antibody. Additional controls consisted of staining with MOPC-21 (mouse IgG1) at concentrations similar to those of primary antibodies.

RESULTS. Induction of Disease Without Adju-
vant. Experimental autoimmune anterior uveitis was induced in Lewis rats of either sex by injection of 100 μg insoluble bovine MAA in the footpad without CFA or PTX as an adjuvant (Table 1). Clinical signs of EAAU appeared between 13 and 18 days after immunization. Severe anterior uveitis developed between days 18 and 20 and resembled EAAU induced with adjuvant clinically and histologically (Table 1, Fig. 1). Low doses of MAA alone (10 μg and 1 μg) without adjuvant were not uveitogenic; but when used with adjuvant, the 10-μg dose evoked a severe inflammatory response in five of six rats; the 1-μg dose was not uveitogenic (Table 1). Soluble bovine MAA (100 μg) with adjuvant was highly pathogenic in the Lewis rats; however, EAAU developed in none of the animals that were immunized with the soluble antigen alone (Table 1).

Adoptive Transfer of Disease. Ten million cells isolated from the draining lymph nodes of Lewis rats with mild to moderate EAAU and immunized with insoluble MAA without adjuvant transferred EAAU to naive syngenic rats (Table 2). These cells were stimulated in vitro with insoluble antigen for 3 days before transfer; cells without stimulation in culture did not induce EAAU (data not shown). The disease started as early as day 7 after transfer and remained active for 4 days. The histopathologic features observed in these animals were similar to those induced by conventional immunization. Further purification of sensitized CD4+ T cells (95% to 99.3%; Fig. 2) by panning and repeated passage through immunocolumns transferred EAAU to naive Lewis rats (Table 2).

DISCUSSION. Experimental autoimmune anterior uveitis, a model of human anterior uveitis, can be induced in Lewis rats by a single injection of insoluble MAA isolated from bovine RPE or uvea. In this report, we demonstrate that severe EAAU can be induced in Lewis rats after immunization with insoluble bovine MAA without adjuvant.

Broekhuysen et al were the first to describe EAAU. However, they used MAA extracted from bovine RPE as the pathogen and included CFA and PTX together in the original immunization protocol. In the same report, they demonstrated that a high dose of MAA (isolated from bovine RPE) with CFA alone was highly uveitogenic. In contrast, the same investigators later reported that PTX was essential for the induction of EAAU, in that 150 μg of MAA isolated from RPE did not evoke EAAU without PTX. The importance of PTX was further demonstrated by the observation that the MAA isolated from bovine choroid was weakly uveitogenic if PTX was omitted as coadjuvant—mild uveitis developed in only one of six animals. Subsequently, CFA and PTX were replaced with Hunter’s adjuvant for the induction of EAAU and evoked a more severe reaction with earlier onset. The footpads of animals were less inflamed, because Hunter’s adjuvant is composed of a blocking polymer, CRL89-41, in squalene without mycobacterium. Recently, Broekhuysen et al reported that intraperitoneally injected choroidal and iris MAA was highly pathogenic in Lewis rats when injected without the use of CFA or Hunter’s adjuvant. However, coinjection of PTX (1 μg or 2 μg) was a prerequisite for the development of uveitis.

In our study, Lewis rats were immunized with only 100 μg of insoluble MAA isolated from bovine iris and ciliary body. The clinical and histologic features of the induced anterior uveitis resembled those observed with the use of an adjuvant. Low concentrations of insoluble antigen (10 μg and 1 μg) were ineffective without adjuvant, although 10 μg of bovine MAA was pathogenic when used with CFA and PTX. Melanin contains a backbone, which is a polymerization product of enzyme generated quinones, is highly insoluble, and is often associ-
ated with proteins. We have cleaved the organ-specific ocular peptides from the melanin backbone by treatment with a proteolytic enzyme, V8 protease. The resulting soluble protein fraction was highly uveitogenic when combined with CFA and PTX. However, soluble MAA was ineffective without an adjuvant. This finding, along with our observation that a higher concentration of insoluble MAA could induce uveitis without addition of an adjuvant suggested that the backbone of uveal melanin could assume a role as a natural adjuvant in EAAU.

Broekhuys et al and Chan et al have reported that EAAU induced by immunization with bovine MAA and adjuvant is a CD4+ T-cell-mediated disease. We have also demonstrated that CD4+ T cells play an important role in the pathogenesis of EAAU, because they are the predominant inflammatory cells within the uvea. Experimental autoimmune anterior uveitis induced by bovine MAA alone without adjuvant is also a T-cell-mediated disease.

There are conflicting reports in the literature regarding the immunogenic properties of melanin. In 1992, Kaya et al demonstrated that severe intraocular inflammation occurred in the eyes of sensitized mice challenged with a combination of antigen and synthetic melanin, compared with that in eyes challenged with antigen alone. Melanin appeared to function as a slow-release depot of antigen in the presensitized host in this report.

In conclusion, we report that EAAU can be induced in Lewis rats without adjuvant when a larger dose of insoluble antigen is used. Elimination of adjuvant removes a number of variables in EAAU research, including adjuvant composition, adjuvant–antigen ratio, emulsion stability, and systemic effects of adjuvants. Moreover, the employment of an adjuvant has clouded the relation of EAAU to human disease, because there has been no evidence of an adjuvant in the human situation. Thus, induction of EAAU without the employment of an adjuvant offers a unique model of the human disease.

Key Words
adjuvant, anterior uveitis, melanin-associated antigen, rat, T cell

Acknowledgments
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References