Effects of Time of Storage, Albumin, and Osmolality Changes on Outflow Facility (C) of Bovine Anterior Segment In Vitro

Arcadi Gual,* Artur Llobet,* Rosa Gilabert,† Miguel Borràs,* Jordi Palès,* Michael V. W. Bergamini,‡ and Carlos Belmonte§

Purpose. To analyze the influence of time of storage, the presence of albumin at physiological concentrations, and the perfusion with anisosmotic media on the aqueous humor outflow facility (C) of isolated bovine anterior segments (AS).

Methods. Anterior segments dissected from cow eyes were perfused at a constant pressure of 10 mm Hg with Dulbecco’s modified Eagle’s medium (DMEM; osmolality 300 mOsm/kg), with hypotonic media (150, 210, and 270 mOsm/kg), or with hyperosmotic media (360, 420, and 480 mOsm/kg). Outflow facility was calculated every 5 seconds as the ratio between average inflow from the reservoir (in microliters per minute) and the perfusion pressure (in millimeters of mercury). Three groups were studied: a 0-hour group, with AS perfused with DMEM 1 to 3 hours after enucleation; a 0-hour albumin group, with AS perfused with DMEM plus 0.1 mg/ml albumin 1 to 3 hours after enucleation; and a 24-hour group, with AS perfused after storage for 24 hours in DMEM. In the 0-hour groups, perfusion with increasingly hypotonic or hyperosmotic media was also made in 30-minute steps, followed by a return to isosmotic medium for 90 minutes.

Results. Perfusion of AS with DMEM for 9 hours caused a progressive increase in C that was statistically significant at 225 minutes in the 0-hour group perfused with DMEM and at 195 minutes in the 24-hour group perfused with DMEM. The 0-hour albumin group perfused with DMEM did not show changes in C throughout the 9-hour perfusion period. Perfusion with increasingly hypotonic or hyperosmotic media resulted in a progressive increase in C that did not recover on return to isotonic medium. Hyperosmotic media caused a progressive increase in C that returned to control values when isotonic medium was again perfused.

Conclusions. Preservation of tissue for C measurements is best achieved with short storage time (1 to 3 hours). Physiological concentrations of albumin (0.1 mg/ml) prevent development of washout, suggesting that albumin or an albumin-bound factor in aqueous humor may play a role in the maintenance of outflow resistance. Outflow facility may also be influenced by volume changes in the trabecular meshwork. Invest Ophthalmol Vis Sci. 1997;38:2165–2171.

Perfusion of the anterior chambers has been used to measure aqueous humor outflow facility (C) and to assess the influence of drugs on aqueous humor drainage. Erickson–Lamy et al described a preparation of the isolated anterior segment (AS) that allows the measurement of C through conventional pathways, thus excluding the contribution to outflow resistance of uveoscleral routes. A problem encountered during perfusion through the conventional aqueous outflow pathways is the development of washout. Washout is defined as a progressive decrease in outflow resistance as time elapses. It has been suggested that the phenomenon is mainly caused by the washing out of proteins from the trabecular meshwork (TM), thus supporting the hypothesis that some chemical constituents of these structures play a role in the release of aqueous outflow resistance. Recently, it has been reported that serum or a serum component may contribute to outflow resistance. Results of studies in vitro suggest that the volume changes of TM cells may play a role in varying tissue permeability. However, the possible functional role of volume variation of the TM cells in the definition of C has not been established.

METHODS. Eyes were obtained in a local abattoir from calves 3 to 6 months old and were enucleated 1 to 3 hours after death. Eyes were immediately submerged in phosphate-buffered saline with antibiotics (described later). Enucleated eyes were bisected along the equator to isolate the anterior segment and were placed in a perfusion chamber. Mean volume of the artificial anterior chamber, formed by the AS and the polysulfone plastic chamber, was 1854 ± 64 μl (mean ± SEM; n = 52). Perfusion was made with Dulbecco’s modified Eagle’s medium (DMEM; Bio-Whitaker, Barcelona, Spain), osmolality 300 mOsm/kg, containing 4.5 mg/ml glucose, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 5 μg/ml amphotericin B. All solutions were prepared under sterile conditions in a laminar-flow hood. Three experimental groups were stud-
FIGURE 1. Effects of 9 hours of perfusion on outflow facility (C) in bovine anterior segments. •, perfusion with DMEM 1 to 3 hours after enucleation (0-hour DMEM group, n = 15); ▲, perfusion with DMEM plus 0.1 mg/ml albumin 1 to 3 hours after enucleation (0-hour alb-DMEM group, n = 9); ■, perfusion with DMEM after 24 hours of storage at 4°C (24-hour DMEM group, n = 9). Data are expressed as mean values ± SEM (n = number of eyes) of the ratio of C at each time (Co) to the baseline C (Co). Statistical significance (repeated measurements analysis of variance with Bonferroni correction) of paired comparisons between C at each time and baseline Co for each treatment group is indicated for the period between the arrows. DMEM = Dulbecco’s modified Eagle’s medium.

Hyposmotic solutions were prepared by adding distilled water to the control media to final osmolality values of 270 mOsm/kg (90% of control value), 210 mOsm/kg (70% of control value), and 150 mOsm/kg (50% of control value). Hyperosmotic solutions were prepared by adding D-sorbitol to the control media to final osmolality values of 360 mOsm/kg (120% of control value), 420 mOsm/kg (140% of control value), and 480 mOsm/kg (160% of control value). Osmolality values were checked in an osmometer before and after each experiment. Perfusion with hypo- or hyperosmotic solutions were performed in separate experiments. In either case, after 90 minutes of perfusion with the control medium (300 mOsm/kg, baseline facility Co), solutions of decreasing or of increasing osmolality were introduced successively for 30-minute periods. At the end of a stepwise decrease or increase in osmolality, perfusion with the control medium (300 mOsm/kg) was resumed for another 90-minute period (post osmolality changes period, Cp).

Outflow facility C was calculated as the ratio between inflow of perfusion medium into the eye (in microliters per minute) and perfusion pressure (in milliliters of mercury) and was expressed as microliters per minute per milliliters of mercury. C values were calculated and recorded every 5 seconds, using specially designed computer software. Additionally, C values were averaged during successive 15-minute intervals for statistical analysis and for graphic representation. As a result, each of these 15-minute intervals, hereinafter described as 15-minute subperiods, was the mean of 180 C values. To obtain the baseline (Co), six initial 15-minute subperiods were averaged for 90 minutes (from minute 0 to minute 90). To determine the validity of the experiment, two criteria were established during the baseline period (initial 90 minutes): the C value of each of these six 15-minute subperiods should be between 0.4 and 1.3 microliters per minute per millimeter of mercury, and the differences among the C ratios calculated during the 15-minute subperiod making up (Co) should be less than 10%. Changes of the perfusion medium were made by rapidly replacing the contents of the anterior chamber with new medium by opening the exit needle until 200% of the artificial anterior chamber volume had been exchanged; this exchange was always made at a pressure below 10 mm Hg. Recording of C measurements started after stabilization of flow.

The influence of elapsed time on C was evaluated by measuring the ratio between C (obtained at different times) and Co. Osmolality effects were expressed as the ratio between facilities at the end
### TABLE 1. Effect of Time of Perfusion and Albumin on Outflow Facility (C) in Isolated Anterior Segments

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Co</th>
<th>Cmin 15</th>
<th>Cmin 30</th>
<th>Cmin 45</th>
<th>Cmin 60</th>
<th>Cmin 75</th>
<th>Cmin 90</th>
<th>Cmin 105</th>
<th>Cmin 120</th>
<th>Cmin 125</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h-DMEM</td>
<td>15</td>
<td>0.73 ± 0.05</td>
<td>0.69 ± 0.05</td>
<td>0.74 ± 0.05</td>
<td>0.75 ± 0.06</td>
<td>0.74 ± 0.06</td>
<td>0.73 ± 0.05</td>
<td>0.72 ± 0.06</td>
<td>0.73 ± 0.06</td>
<td>0.74 ± 0.06</td>
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<td></td>
<td></td>
<td>(94%)</td>
<td>(102%)</td>
<td>(102%)</td>
<td>(99%)</td>
<td>(102%)</td>
<td>(101%)</td>
<td>(99%)</td>
<td>(100%)</td>
<td>(102%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24h-DMEM</td>
<td>0.50 ± 0.05</td>
<td>0.47 ± 0.03</td>
<td>0.50 ± 0.03</td>
<td>0.47 ± 0.04</td>
<td>0.49 ± 0.04</td>
<td>0.49 ± 0.04</td>
<td>0.50 ± 0.05</td>
<td>0.49 ± 0.04</td>
<td>0.46 ± 0.04</td>
<td>0.46 ± 0.04</td>
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<tr>
<td></td>
<td>9</td>
<td>(100%)</td>
<td>(103%)</td>
<td>(97%)</td>
<td>(101%)</td>
<td>(100%)</td>
<td>(103%)</td>
<td>(99%)</td>
<td>(100%)</td>
<td>(94%)</td>
<td>(95%)</td>
</tr>
<tr>
<td></td>
<td>24h-DMEM</td>
<td>0.51 ± 0.05</td>
<td>0.57 ± 0.05</td>
<td>0.55 ± 0.05</td>
<td>0.54 ± 0.05</td>
<td>0.52 ± 0.05</td>
<td>0.51 ± 0.05</td>
<td>0.53 ± 0.05</td>
<td>0.54 ± 0.04</td>
<td>0.56 ± 0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>(100%)</td>
<td>(106%)</td>
<td>(103%)</td>
<td>(101%)</td>
<td>(99%)</td>
<td>(94%)</td>
<td>(99%)</td>
<td>(102%)</td>
<td>(104%)</td>
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</tbody>
</table>

C₀ = baseline facility; Cmin = facility at each time in minutes; 0h-DMEM = anterior segments perfused immediately after enucleation with DMEM; 0h-Alb-DMEM = anterior segments perfused immediately after enucleation with DMEM plus 0.1 mg/ml albumin; 24h-DMEM = anterior segments perfused after 24 hours of storage in DMEM at 4°C.

Data are mean ± SEM; n = number of eyes. Average of the relation Cmin/Co is shown in parentheses. For the three groups, analysis of variance with Bonferroni correction was performed between C₀ and C obtained every 15 minutes.

* P < 0.001.
† P < 0.01.
‡ P < 0.001.
FIGURE 2. Effects of different hyposmotic media on outflow facility (C) in perfused bovine anterior segments. The perfusion was divided into three periods of 90 minutes each. The first and last periods were performed with 300 mOsm kg DMEM. The second period was performed with three steps of 30 minutes each of hyposmotic DMEM, 270, 210, and 150 mOsm/kg, respectively (open symbols). For comparison, every plot includes in filled symbols each respective control (Table 1, Fig. 1). The plots also include, expressed as a solid line, the difference in C values (algebraic subtraction) between every hyposmotic group (open symbols) and their control counterparts (filled symbols). (A) Perfusion with DMEM 1 to 3 hours after enucleation (J, n = 8). (B) Perfusion with DMEM plus 0.1 mg/ml albumin 1 to 3 hours after enucleation (Δ, n = 10). (C) Perfusion with DMEM after 24 hours of storage at 4°C (Ξ, n = 9). Data are expressed as mean values ± SEM (n = number of eyes) of the ratio of C at each time (Ci) to the baseline C (C0). The statistical significances of unpaired comparisons between C in each hyposmotic group and C in their respective control groups at the same times are indicated by asterisks. Ordinary analysis of variance was used. DMEM = Dulbecco’s modified Eagle’s medium.

RESULTS

Effect of Albumin on Changes in Outflow Facility With Elapsed Time. Figure 1 presents the C values of AS from freshly collected eyes perfused in vitro for 9 hours without (0-hour DMEM group, n = 15) and with albumin (0-hour alb–DMEM group, n = 9). Figure 1 also shows C values of AS that were stored at 4°C for 24 hours in DMEM, and then perfused with DMEM (24-hour DMEM group, n = 9). Perfused anterior segments in the 0-hour DMEM group and in the 24-hour DMEM group displayed a progressive increase in C with elapsed perfusion time. The difference between C and C0 was significant at minute 225 (P < 0.05) in the 0-hour DMEM group, whereas in the 24-hour DMEM group, the difference was significant at minute 195 (P < 0.05). In contrast, the 0-hour alb–DMEM group AS did not show any significant difference between C and C0 throughout the 9-hour perfusion (Table 1).

Influence of Osmolality on Outflow Facility. Hyposmotic Solutions. Anterior segments perfused immediately after enucleation without (0-hour HYPO group, n = 8) and with albumin (0-hour alb–HYPO group, n = 10) showed a progressive decline of C when the osmolality of the perfusion fluid was reduced to 90%, 70%, or 50% of that in controls (270, 210, 150 mOsm/kg; Figs. 2A, 2B). Comparison of mean C values in these experiments (Table 2) with the corresponding C values in control experiments (Table 1) shows that C decreases after reductions in osmolality were statistically significant from minute 150 until minute 270 (P < 0.001) for the 0-hour HYPO group and from minute 165 until minute 270 (P < 0.01) for the 0-hour alb–HYPO group. The profile of the decline in
### TABLE 2. Effect of Osmolality Changes of Perfusion Medium on Outflow Facility (C) in Isolated Anterior Segments

<table>
<thead>
<tr>
<th>Hypo-osmotic groups (n)</th>
<th>C₀</th>
<th>C₀, 270</th>
<th>C₀, 210</th>
<th>C₀, 150</th>
<th>C₀, (min 270)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h-Hypo (n = 8)</td>
<td>0.59 ± 0.11</td>
<td>0.50 ± 0.11</td>
<td>0.46 ± 0.09</td>
<td>0.44 ± 0.08</td>
<td>0.50 ± 0.12</td>
</tr>
<tr>
<td>0h-Alb-Hypo (n = 10)</td>
<td>0.66 ± 0.10</td>
<td>0.61 ± 0.09</td>
<td>0.52 ± 0.07</td>
<td>0.46 ± 0.06</td>
<td>0.51 ± 0.09</td>
</tr>
<tr>
<td>24h-Hypo (n = 9)</td>
<td>0.65 ± 0.09</td>
<td>0.58 ± 0.08</td>
<td>0.52 ± 0.07</td>
<td>0.43 ± 0.06</td>
<td>0.40 ± 0.07</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hyperosmotic groups (n)</th>
<th>C₀</th>
<th>C₀, 270</th>
<th>C₀, 210</th>
<th>C₀, 150</th>
<th>C₀, (min 270)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h-Hyper (n = 7)</td>
<td>0.49 ± 0.06</td>
<td>0.56 ± 0.08</td>
<td>0.62 ± 0.07</td>
<td>0.68 ± 0.11</td>
<td>0.52 ± 0.09</td>
</tr>
<tr>
<td>0h-Alb-Hyper (n = 7)</td>
<td>0.55 ± 0.09</td>
<td>0.60 ± 0.12</td>
<td>0.68 ± 0.12</td>
<td>0.73 ± 0.11</td>
<td>0.62 ± 0.09</td>
</tr>
<tr>
<td>24h-Hyper (n = 7)</td>
<td>0.64 ± 0.12</td>
<td>0.71 ± 0.15</td>
<td>0.76 ± 0.15</td>
<td>0.81 ± 0.16</td>
<td>0.74 ± 0.11</td>
</tr>
</tbody>
</table>

Mean of outflow facility (C) values expressed for the groups treated with the hypoosmotic or hyperosmotic protocol. Average of the relation Cti/Co obtained in each experiment is shown in parentheses. Analysis of variance was performed in every group pooling the values of the experimental groups with their respective controls. C₀ = average of C during baseline period; C₀, 270, C₀, 210, and C₀, 150 = C values obtained after treatment with hypoosmotic solutions of 270, 210, and 150 mOsm/kg, respectively; C₀, 360, C₀, 420, and C₀, 480 = C values obtained after treatment with hyperosmotic solutions of 360, 420, and 480 mOsm/kg respectively; C₀, (min 270) = C at the end of the experiments (min 270).

* P < 0.05.
† P < 0.01.
‡ P < 0.001.

C throughout the 270 minutes was similar in both groups (compare Figs. 2A and 2B). Anterior segments stored for 24 hours in DMEM and perfused with hypoosmotic solutions (24-hour HYPO group, n = 9) showed a progressive decrease in C that continued when reperfusion with isosmotic medium was resumed (Table 2, Fig. 2C). The C decreases were statistically significant from minute 150 through minute 270 (P < 0.001) in comparison with the equivalent C values in the 24-hour DMEM group at the same times.

**Hyperosmotic Solutions.** Anterior segments perfused immediately after enucleation with (0-hour alb–HYPER group, n = 7) and without albumin (0-hour HYPER group, n = 7) and subjected to stepwise increases in osmolality showed a progressive increase in C when the osmolality of the perfusion fluid was increased to 120%, 140%, and 160% of control osmolality (360, 420, and 480 mOsm/kg respectively; Figs. 3A, 3B). Comparison with control values at similar times showed that the C increases were statistically significant from minute 135 (P < 0.001) in the 0-hour HYPER and 0-hour alb–HYPER groups. The C values recovered gradually after the onset of reperfusion with isosmotic medium, returning approximately to the initial values at minute 270 (Figs. 3A, 3B). No differences in the response to the various hyperosmotic solutions were observed between 0-hour HYPER and 0-hour alb–HYPER groups. Table 2 presents the pooled data of C throughout the complete perfusion cycle with increasingly hyperosmotic solutions. Anterior segments stored for 24 hours in DMEM and perfused with hyperosmotic media (24-hour HYPER group, n = 7) showed a progressive increase in C that appeared to be reversed by reperfusion with isosmotic medium (Table 2, Fig. 3C). The C increases seen in the 24-hour HYPER group did not differ significantly from those obtained in the control group (24-hour DMEM group), in which a gradual rise of C was observed during long-lasting perfusion with control medium. The apparent reversal after reperfusion with isosmotic media did not reach statistical significance during the 90-minute period; however, by minute 270, C was substantially (24%) lower than that in the corresponding control C270.

**DISCUSSION.** In isolated AS, it has been reported that prolonged anterior chamber perfusion causes a time-dependent increase in C that is known as the washout effect. Similarly, in our experiments, AS in the 24-hour DMEM group showed significant increases in C that appeared after 3 hours of perfusion and were similar to values of washout reported by...
FIGURE 3. Effects of different hyperosmotic media on outflow facility (C) in perfused bovine anterior segments. The perfusion was divided into three periods of 90 minutes each; the first and the last periods were performed with 300 mOsm/kg DMEM. The second period was performed with three steps of 30 minutes each of hyperosmotic DMEM, 360, 420, and 480 mOsm/kg, respectively (open symbols). For comparison, every plot includes in filled symbols each respective control (Table 1, Fig. 1). The plots also include, expressed as a solid line, the mean difference in C values (algebraic subtraction) between every hyperosmotic group (open symbols) and their control (filled symbols). (A) Perfusion with DMEM 1 to 3 hours after enucleation (J, n = 7). (B) Perfusion with DMEM plus 0.1 mg/ml albumin 1 to 3 hours after enucleation (Δ, n = 7). (C) Perfusion with DMEM after 24 hours of storage at 4°C (□, n = 7). Data are expressed as mean values ± SEM (n = number of eyes) of the ratio of C at each time (Ct) to the baseline C (C0). The statistical significances of unpaired comparisons between Ct in each hyperosmotic group and C in their respective control groups at the same times are indicated by asterisks. Ordinary analysis of variance was used. DMEM = Dulbecco's modified Eagle's medium.

other authors. In comparison, AS perfused immediately after dissection showed a smaller and more delayed C elevation during equivalent perfusion periods. The differences in response between AS in the 0-hour DMEM group and those in the 24-hour DMEM group stresses the importance of using fresh ocular tissues for C measurements in vitro. A short interval between enucleation and the onset of perfusion (1 to 3 hours) partially prevented the development of the washout effect. Nevertheless, a 9-hour perfusion period in the presence of 0.1 mg/ml albumin (0-hour alb–DMEM group) completely prevented the development of the washout effect. Johnson et al. and Kee et al. prevented the development of the washout adding different concentrations of serum to the perfusion media. In many species, the total protein concentration in aqueous humor is low, 1/500 of the plasma proteins levels. The albumin represents approximately one half of the total protein amount in aqueous humor and reaches concentrations of 0.05 to 0.07 mg/ml. In our experiments, we used 0.1 mg/ml albumin, a concentration that can be considered to be within the physiological range. In nonhuman species, the washout effect has been associated with a progressive depletion of plasma-derived proteins entering the TM rather than with a decrease of glycosaminoglycans in the aqueous humor pathway. In our experiments, the addition of albumin to the perfusion medium successfully counteracted the increase in C caused by prolonged perfusion; thus albumin at a physiological concentration of 0.1 mg/ml prevents washout during at least 9 hours of perfusion. This observation lends support to the hypothesis that plasma proteins may contribute to the resistance of the TM to the passage of aqueous humor. Nevertheless, the findings in our experiments do not clarify whether this effect is related to albumin or to an albumin-bound factor, or whether the effect of plasma proteins is mediated by an action on the extracellular matrix or by an action on cell membrane receptors.

The possibility that outflow resistance was modified by volume changes in TM tissues was also explored by changing the osmolality of the perfusion medium. Hyposmotic solutions induced a pronounced decrease in C, with no recovery of C values after reperfusion with isosmotic medium; conversely, hyperosmotic solutions elicited a marked and progressive increase in C that was reversed when isosmotic medium was again perfused. It is conceivable that these changes in outflow resistance were caused by volume modifications of TM. Under physiological circumstances, the participation of volume regulation mechanisms in the control of C is presumably insignificant. Nevertheless, the possibility that cell volume changes contribute to the final outflow resistance in some pathologic conditions should be considered. The available information about the effects in vitro of anisosmotic media on TM cells in culture do not allow conclusions to be made about the correlation between C and perfusate osmolality. However, because para-cellular pathways participate in the passage of aqueous humor through the trabecular tissue, an increase of
flow is expected to occur when TM and the endothelial cells of the aqueous plexus shrink. Conversely, cells that swell with hyposmotic solutions would 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 Cin 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

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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.