The Presence of Biologically Significant Concentrations of Glucocorticoids But Little or No Cortisol Binding Globulin Within Aqueous Humor: Relevance to Immune Privilege in the Anterior Chamber of the Eye

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**Purpose.** Immunosuppressive factors in aqueous humor (AH) contribute to the immune-privileged status of the anterior chamber of the eye. One such factor is transforming growth factor-beta (TGF-β); other relevant inhibitors have not been fully identified. The authors examined AH to search for other putative inhibitors and to determine their effect on TGF-β inhibitory activity.

**Methods.** Radioimmunoassays (RIA) were used to detect the presence of hydrocortisone, corticosterone, cortisol binding globulin (CBG), and alpha-melanocyte stimulating hormone (α-MSH) in AH. The ability of these factors to inhibit murine thymocyte proliferation stimulated by phytohemagglutinin–interleukin 1 (PHA/IL-1) and proliferation of a TGF-β-sensitive cell line (CCL64) in vitro was examined. The ability of hydrocortisone to inhibit a one-way mixed lymphocyte reaction (MLR) and the ability of epidermal cells to present soluble tumor-associated antigens (TAA) for elicitation of immunity in mice in the concentration range present in AH was also examined.

**Results.** Hydrocortisone was detected in mouse, rat, and human AH (10.8 ± 1.1 ng/ml, 9.3 ± 2.1 ng/ml, and 18.0 ± 1.0 ng/ml, respectively; mean ± SEM), as was corticosterone (2.7 ± 0.9 ng/ml, 2.2 ± 0.5 ng/ml, and 0.7 ± 0.1 ng/ml, respectively). Whereas normal plasma contains a binding protein for corticosteroids (i.e., CBG), the concentration in mouse, rat, and human AH was less than the level detectable by an RIA. Hydrocortisone inhibited PHA/IL-1-stimulated murine thymocyte proliferation and CCL64 cell proliferation in the concentration range present in AH. When hydrocortisone was combined with TGF-β2 (125 pg/ml), the degree of inhibition observed was greater than with either alone. Corticosterone inhibited thymocyte costimulation only slightly at concentrations present in AH but was inhibitory for CCL64 cells. α-MSH was also detected in AH. The concentration present had only slight inhibitory effects for CCL64 cell proliferation and did not enhance TGF-β2-mediated (62 pg/ml to 250 pg/ml) inhibition of CCL64 or thymocyte proliferation. Hydrocortisone inhibited the one-way MLR in the concentration range present in AH and, at 10 ng/ml, inhibited the ability of epidermal cells to present TAA for elicitation of delayed-type hypersensitivity in tumor-immune mice.

**Conclusions.** These results show that AH contains biologically relevant concentrations of glucocorticoids and that CBG is relatively absent so that glucocorticoids present are largely free, and they suggest that regional sites take advantage of the activities of multiple factors to maintain an immune-privileged status. Invest Ophthalmol Vis Sci. 1994;35:3711–3723.

Several sites in the body normally require stringent regulation of immune and inflammatory responses to prevent potentially damaging consequences of these responses to delicate anatomic structures. These sites include the eye, the brain, and the pregnant uterus and are called immune-privileged sites. Several mechanisms to account for the suppression of such
responses have been postulated. They include depressed antigen-presenting capability, the generation of T cell-mediated suppression such as anterior chamber-associated immune deviation, the presence of regulatory cytokines, and the presence of other regulatory molecules. Aqueous humor (AH) is inhibitory for various in vitro correlates of immune function and contains transforming growth factor-beta (TGF-β). a potent immunosuppressive cytokine produced by a variety of cell types, including tissues lining the anterior chamber of the eye.

Other substances with known immunomodulatory activity include various neuropeptides and the glucocorticoids. Because we had previously determined that a substantial portion of the inhibitory activity present within AH resided in a low-molecular-weight fraction (molecular weight < 3500), particular attention was paid to molecules with this characteristic. Of interest is the neuropeptide alpha-melanocyte stimulating hormone (α-MSH; MW 1772), which has potent immunosuppressive and antiinflammatory properties, including the inhibition of interleukin 1 (IL-1) effects, neutrophil migration and activation; tumor necrosis factor-α (TNF-α) effects; and the inhibition of contact-sensitivity responses. Corticosteroids also possess recognized immunosuppressive and anti-inflammatory properties, such as the inhibition of IL-1 production and its transcriptional and posttranscriptional expression; the inhibition of prostaglandin and TNF production; and the inhibition of T cell lymphokine production. Corticosteroids also inhibit major histocompatibility complex class II antigen expression; IL-1-induced neutrophil migration; IFN-γ-induced and LPS-induced monocyte activation; and IgE-dependent cutaneous inflammation. Although a number of glucocorticoids may play a role in regulating immunity at various anatomic sites, we chose to examine the possible role of hydrocortisone and corticosterone (the major source of glucocorticoid activity in some species, including rodents) as immunoregulatory factors within AH. Hydrocortisone exists in plasma in three forms: free (approximately 6% to 10%), bound to cortisol-binding globulin (CBG, transcortin; approximately 80% to 90%), or bound to albumin (approximately 4% to 10%). Corticosterone also binds CBG with equal affinity. CBG is a plasma protein (MW 52 kd), synthesized primarily in the liver, that binds several steroid hormones and has the highest affinity for cortisol. CBG has been recognized as the primary circulating reservoir for cortisol.

Based on the observed immunosuppressive characteristics of AH and the possibility that multiple factors may function together in vivo, we investigated whether normal mouse, rat, or human AH contains hydrocortisone, corticosterone, α-MSH, or CBG in biologically relevant concentrations, and we assessed their ability to mediate inhibitory activity and to modulate the activity of TGF-β. The results show that murine AH contains α-MSH, which, at the concentration detected, failed to inhibit thymocyte proliferation and inhibited mink lung epithelial cell proliferation only slightly. AH contained hydrocortisone at a concentration that strongly inhibited these assays. Moreover, inhibition was greater when hydrocortisone was used in combination with TGF-β. Corticosterone was also found in AH; however, the concentration present was sufficient to inhibit these assays only slightly. The observation that glucocorticoids are present in AH may also help to explain a previous observation. It was recently demonstrated that AH is capable of inhibiting antigen presentation by epidermal Langerhans cells and that this activity is not due to the presence of TGF-β with AH. The concentration of hydrocortisone within AH was found to be sufficient to inhibit Langerhans cell antigen presentation. In addition, AH did not contain detectable levels of CBG, suggesting a unique restriction of this widely distributed protein from the normal fluid within the anterior chamber of the eye. Together, the results suggest that free glucocorticoids and the concurrent absence of CBG act in a significant fashion in the inhibition of immune and inflammatory processes within the anterior chamber of the eye.

MATERIALS AND METHODS

Mice

Adult female CAF1, C57BL/6, BALB/c, and C3H/HeJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used between 6 and 12 weeks of age. Adult female Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). All procedures followed the guidelines for animal care as detailed in The Guide for the Care and Use of Laboratory Animals (NIH Publication 86–23) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Reagents

α-MSH was purchased from Peninsula Laboratories (Belmont, CA), hydrocortisone and corticosterone from Sigma Chemical (St. Louis, MO), and TGF-β2 from R&D Systems (Minneapolis, MN). Hydrocortisone and corticosterone were diluted in ethanol at a concentration of 10 µg/ml as stock solutions and were diluted in the appropriate media for use in the assays described below.
Aqueous Humor

Aqueous humor was performed as described by Granstein et al. Briefly, 0.8 ml of aqueous humor was dialyzed with 3500-MW cut-off dialysis tubing (Spectra/por 7; Spectrum Medical Industries, Los Angeles, CA) against sterile distilled H2O for 24 hours. The dialysate was frozen quickly in alcohol-dry ice and lyophilized until dry. The remaining product was redissolved in sterile distilled H2O and filtered (0.20 μm) before use. Human AH was obtained from patients undergoing cataract surgery who were otherwise healthy. It was centrifuged at 8000g and stored at −70°C until use.

Radioimmunoassays for Hydrocortisone, Corticosterone, Cortisol Binding Globulin, and α-MSH

A radioimmunoassay (RIA) for the detection of rat α-MSH was performed as described by Nicholson et al. Briefly, rabbit anti-rat α-MSH was reacted with 100 μl of AH for 24 hours at 4°C, followed by a 24-hour incubation at 4°C with 125I-α-MSH. Precipitation of the complex was carried out with the addition of goat anti-rabbit γ-globulin (Calbiochem, San Diego, CA), and incubation continued for 2½ hours at 4°C. After centrifugation at 6000g for 25 minutes, the pellet was collected, and resultant radioactivity was compared to known standards.

Determinations of hydrocortisone and corticosterone concentrations were performed by initial separation using thin-layer chromatography, followed by specific RIA as described by Oka et al. Briefly, 100 μl of sample was evaporated to dryness, followed by addition of 3H-cortisol (or 3H-corticosterone) and rabbit anti-cortisol (or rabbit anti-corticosterone), each in 200 μl of buffer containing 0.1% bovine serum albumin and 0.2% bovine gamma globulin. After mixing and 2 hours of incubation at room temperature, 500 μl of saturated ammonium sulfate was added, and, after mixing and centrifugation at 1500g to 2000g, the supernatant was decanted, and radioactivity was then compared with a standard curve generated with known amounts of CBG. The lower level of sensitivity of this assay was 0.005 μg/ml for mouse and rat AH samples and 0.05 μg/ml for human samples (due to differences in dilutions).

Mink Lung Epithelial Cell (CCL64) Assay

Mink lung epithelial cell proliferation assays were performed as previously described. Cells were maintained in minimum essential medium (MEM, Gibco, Grand Island, NY) supplemented with 1 mM L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 1% penicillin-streptomycin, and 10% FCS (Gibco). 1 X 10⁵ cells in 100 μl of medium containing 3% FCS (Hyclone, Logan, UT) were placed in flat-bottomed plates (Becton Dickinson, Lincoln Park, NJ) with 100 μl of sample. Each well was pulsed with 1.0 μCi of 3H-thymidine for the last 6 hours of a 24-hour culture. Cells were harvested onto filter mats with a cell harvester (Harvester 96, Mach II; Tomtec, Orange, CT) and counted in a liquid scintillation counter (1205 Betaplate; Pharmacia LKB Nuclear, Gaithersburg, MD).

Thymocyte Costimulation Assay

A standard murine thymocyte costimulation assay was performed as described by Granstein et al. Briefly, 2.5 X 10⁴ C3H/HeJ thymocytes were incubated in 200 μl of medium (RPMI [Gibco] supplemented with 1 mM L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 0.01 M HEPES, 50 μM 2-mercaptoethanol, 1% penicillin-streptomycin, and 10% FCS [Hyclone]; “complete medium”) in flat-bottom plates. Recombinant murine IL-1α (Genzyme, Cambridge, MA) was used at a final concentration of 1.0 U/ml. PHA (Wellcome Diagnostics, Dartford, England) was used at a final concentration of 2 μg/ml. Plates were pulsed with 3H-hydroxydine for the last 6 hours of a 3-day incubation at 37°C, harvested, and counted as above.

Mixed Lymphocyte Reaction

A standard one-way mixed lymphocyte reaction (MLR) was performed. One hundred thousand x-irradiated (15 Gy) erythrocyte-free BALB/c spleen cells were cultured with 1 X 10⁵ C57BL/6 erythrocyte-free splenocytes enriched for T cell content by passage through nylon wool in 200 μl of RPMI medium supplemented with 1 mM L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 0.01 M HEPES, 50 μM 2-mercaptoethanol, 1% penicillin-streptomycin, and 2% normal mouse serum in 96-well flat-bottomed plates. The plates were pulsed with 3H-thymidine for gamma counting. Values were then compared with a standard curve generated with known amounts of CBG.
the last 20 hours of a 5-day incubation at 37°C, harvested, and counted as above.

**Elicitation of Immunity With Epidermal Cells**

Epidermal cells were prepared using a standard protocol. Briefly, truncal skins of shaved and chemically depilated (Neet; Whitehall Laboratories, New York, NY) mice were removed and depleted of subcutaneous fat and panniculus carnosus. The skins were floated dermis-side down on 0.2 U/ml dispase and 0.4% trypsin in Ca²⁺/Mg²⁺-free PBS for 30 minutes at 37°C, and epidermal sheets were collected and dissociated by incubation in 80 μg/ml DNase in Ca²⁺/Mg²⁺-free PBS for 20 minutes at 37°C under continuous gentle agitation, filtered through nylon gauze (Nitex; Tetco, Elmsford, NY), and washed. Thy 1-bearing cells were depleted by incubation in anti-Thy 1.2 monoclonal antibody solution for 30 minutes on ice, followed by washing and subsequent incubation in low-toxicity rabbit complement for 35 minutes at 37°C. Dead cells were removed by treatment with 0.05% trypsin and 80 μg/ml DNase in Ca²⁺/Mg²⁺-free PBS for 10 minutes at 37°C. Viability and percentage of I-A⁺ cells were assessed by fluorescence-activated cell sorter analysis immediately before injection into mice, and differences between groups within experiments were negligible (data not shown). The percentage of I-A⁺ cells within the epidermal cell population was usually approximately 6%.

To examine the effect of hydrocortisone on elicitation of immunity by epidermal cells in tumor-immune mice, CAF mice were immunized by subcutaneous injection 3 times at weekly intervals on each flank. The injection consisted of 0.1 ml of soluble tumor-associated antigens (TAA) derived from the murine spindel cell tumor SI509a (A/J-derived, H-2b) incubated for 2 hours at 37°C with a concentration of 2 X 10⁶ epidermal cells in 1 ml of medium containing TAA. TAA were prepared as a freeze-thaw lysate of S1509a cells suspended in complete medium at a concentration of 10⁶ per milliliter. After lysing, the supernatant was subjected to centrifugation at 6000g for 1 hour and then used as a source of TAA. Elicitation of delayed-type hypersensitivity (DTH) was performed 1 week after the last immunization as described previously by injecting a hind footpad with 5 X 10⁶ epidermal cells, cultured for 3 hours at 37°C in complete medium with or without 10 ng/ml hydrocortisone before washing, and cultured for 2 hours at 37°C in complete medium containing TAA at a concentration of 2 X 10⁶ to 5 X 10⁶ cells per milliliter. Epidermal cells were washed 4 times before footpad injection to remove soluble TAA. Footpads were measured 24 hours after elicitation using an engineer’s micrometer (Fowler, Biggsfield, Great Britain), and footpad swelling was calculated as the difference between the footpad thicknesses of the injected and the uninjected foot. The significance of differences among groups was assessed by the two-tailed Student’s t-test for independent samples.

**RESULTS**

### Levels of α-MSH, Hydrocortisone, Corticosterone, and CBG in AH

In previous reports, we have shown that normal AH from several mammalian species contains dialyzable low-molecular-weight (MW <3500) inhibitors for PHA and IL-2-stimulated or IL-1-stimulated murine thymocytes and CCL64 mink lung epithelial cells. To determine the identity of possible inhibitors present in AH, we analyzed mouse, rat, and human AH for hydrocortisone and corticosterone, and mouse and rat AH for α-MSH by sensitive RIA. Mouse and rat AH were found to contain α-MSH at a concentration similar to that previously reported by mice and 2.48 X 10⁻¹¹ M, respectively; n = 1 each). Table 1 shows that mouse, rat and human AH contain hydrocortisone and corticosterone. We have previously shown that the reconstituted, lyophilized dialysate (MW <3500) of AH contained potent immunosuppressive activity. To test whether AH prepared in this manner contained hydrocortisone or corticosterone, samples were analyzed by RIA. Again, both mouse and rat samples contained nearly the same levels of hydrocortisone and corticosterone as those found before dialysis. Because glucocorticoids can be free or protein bound, principally to CBG, samples were analyzed for the presence of CBG by RIA. Surprisingly, the concentration of CBG in four samples of mouse AH, in four samples of rat AH, and in three samples of human

<table>
<thead>
<tr>
<th>AH Sample</th>
<th>Hydrocortisone (ng/ml)</th>
<th>Corticosterone (ng/ml)</th>
<th>Cortisone Binding Globulin (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse*</td>
<td>10.8 (1.1)</td>
<td>2.7 (0.9)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Rat*</td>
<td>9.3 (2.1)</td>
<td>2.2 (0.3)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Normal AH</td>
<td>18.0 (1.0)</td>
<td>0.7 (0.1)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Dialyzed mouse†</td>
<td>6.0 (0.0)</td>
<td>2.0 (0.3)</td>
<td>—</td>
</tr>
<tr>
<td>Dialyzed rat†</td>
<td>9.5 (0.5)</td>
<td>1.6 (0.4)</td>
<td>—</td>
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</table>

Normal aqueous humor samples were analyzed for the presence of HC, corticosterone, and CBG by RIA. Following dialysis (3500 MW cutoff), lyophilization, and reconstitution, samples were analyzed for HC and corticosterone. Results represent the mean ± SEM of 3–4 samples per group or 2–3 samples per group.

**TABLE 1. Levels of Glucocorticoids and CBG in Aqueous Humor**
Thus, essentially all the glucocorticoid present in AH is free.

Effect of α-MSH, Hydrocortisone, Corticosterone, and TGF-β2 on Thymocyte and CCL64 Proliferation

In previous reports, we and others have found that TGF-β2 is the predominant TGF-β species present in AH of various mammalian species. Moreover, AH has been shown to inhibit various forms of cellular proliferation, including mitogen- and growth factor-induced thymocyte proliferation, antigen- and mitogen-induced lymphocyte proliferation, and the spontaneous proliferation in response to serum growth factors of CCL64 mink lung epithelial cells. Therefore, we used CCL64 epithelial cell proliferation and murine thymocyte proliferation stimulated by PHA/IL-1 to assess the inhibitory activity of hydrocortisone, corticosterone, and α-MSH.

Although α-MSH is known to be a potent in vivo inhibitor of inflammation, its effect on PHA/IL-1-induced thymocyte proliferation has been controversial. We were unable to demonstrate an inhibitory effect of α-MSH on thymocyte proliferation in response to PHA/IL-1 at the concentration found in AH. The inhibitory activity of glucocorticoids was evaluated next. The data in Figure 1 demonstrate that, compared to diluent alone, hydrocortisone inhibited thymocyte costimulation 26% to 57%, at a concentration

AH was less than the level of detection of the assay (Table 1). However, CBG was readily detectable in normal mouse and rat serum (3.6 mg/dl and 4.4 mg/dl, respectively), demonstrating that the antibody used in these studies was sensitive to rat and mouse CBG.
Concentration range of 5 to 15 ng/ml. Corticosterone exhibited a similar response. Note that the concentration of hydrocortisone found in AH was within the range capable of inhibiting this assay, whereas the concentration of corticosterone was less than that needed to inhibit. These results are consistent with other studies reporting the inhibitory activity of glucocorticoids on mitogen-stimulated murine thymocytes and mouse secondary allograft responses. It is possible that the separate inhibitory activities of α-MSH, hydrocortisone, or TGF-β2 masked the activity of the others, displayed additive inhibitory activity, or resulted in a synergistic level of activity. To investigate this possibility, different concentrations of α-MSH or TGF-β2 were added to hydrocortisone. α-MSH had no effect when used in combination with TGF-β2 or hydrocortisone (data not shown). In contrast, when hydrocortisone, at the concentration found in AH, and TGF-β2 were used in combination, an additive inhibitory effect was observed at lower concentrations of TGF-β2 (<250 pg/ml) (Fig. 2). The mean inhibition from five experiments of this type with 10 ng/ml of hydrocortisone and 125 pg/ml of TGF-β2 was 41.3% ± 4.9% for hydrocortisone alone, 44.5% ± 4.8% for TGF-β2 alone, and 83.8% ± 5.5% for the combination of the two. Corticosterone at the concentration found in AH demonstrated only slight augmentation of TGF-β2-mediated (50 pg/ml to 125 pg/ml) inhibition (data not shown).

CCL64 epithelial cells are sensitive to the inhibitory activity of TGF-β, the inhibitory activity of AH, and AH dialysate. We therefore used these cells to analyze the inhibitory activity of α-MSH, hydrocortisone, corticosterone, and TGF-β2. Figure 3 shows that a moderate level of inhibitory activity mediated by α-MSH is found between 10⁻¹¹ and 10⁻⁷ M; this range includes the concentration found in mouse and rat AH (approximately 2.4 × 10⁻¹¹ M). When α-MSH at the concentration found in AH was used in combination with TGF-β2 (125 pg/ml), there was no enhancement of TGF-β2-mediated inhibitory activity compared to TGF-β2 alone (data not shown). Analysis of the activity of glucocorticoids demonstrated that hydrocortisone and corticosterone inhibited proliferation in a dose-dependent manner (Fig. 4). Furthermore, inhibition mediated by TGF-β (125 pg/ml) was nearly additive for concentrations of hydrocortisone less than 2.5 ng/ml (Fig. 5) and less than additive at higher concentrations of hydrocortisone. The mean inhibition from 3 experiments of this type with 10 ng/ml of hydrocortisone and 125 pg/ml of TGF-β2 was 64.9% ± 16.8% for hydrocortisone alone, 55.5% ± 14.6% for TGF-β2 alone, and 76.5% ± 12.7% for the combination of the two. As in the thymocyte costimulation assay, α-MSH did not influence inhibition mediated
FIGURE 5. Inhibitory activity of the combination of TGF-β and hydrocortisone on the proliferation of CCL64 cells. Various dilutions of hydrocortisone were assessed alone or added to 125 pg/ml TGF-β2. Proliferation with no inhibitors is shown in the open bar labeled Media, and proliferation in the presence of TGF-β2 alone is shown in the open bar labeled TGF-β. As indicated on the x-axis, dilutions of hydrocortisone were analyzed alone (open bars) or in combination with TGF-β2 hydrocortisone (black bars). The experiment shown is representative of three separate experiments. Error bars represent SEM.

by hydrocortisone (data not shown). These results show that CCL64 cells are sensitive to the inhibitory effect of glucocorticoids, are essentially refractory to inhibition by α-MSH, and that certain combinations of factors can lead to enhanced inhibitory activity.

Effect of Hydrocortisone on the Mixed Lymphocyte Reaction

To determine whether the concentration of hydrocortisone found in AH would inhibit an antigen-driven response, we used the one-way MLR. As shown by the data in Figure 6, hydrocortisone in the concentration range present in AH significantly inhibited the MLR response.

Effect of Hydrocortisone on Epidermal Cell Antigen Presentation

To determine whether the concentration of hydrocortisone present in AH is sufficient to inhibit epidermal cell antigen-presenting function, we examined the ability of hydrocortisone to inhibit the elicitation of immunity to the S1509a tumor, a model we have used in the past. As shown by the data in Figure 7, treatment of epidermal cells with 10 ng/ml of hydrocortisone inhibits the ability of epidermal cells to elicit immunity in this system.

DISCUSSION

The results presented here show that, in addition to TGF-β (which is an important immunosuppressive agent in AH), glucocorticoids represent a significant class of immunomodulators in AH. Physiological concentrations of hydrocortisone as determined in mouse, rat, and human AH were strongly inhibitory for the proliferation of mitogen-IL-1-stimulated thymocytes and CCL64 epithelial cells. Moreover, the inhibitory activity of hydrocortisone for thymocytes was found to be nearly additive when used in combination with submaximal concentrations of active TGF-β2, suggesting that when found together, they provide a greater barrier to immune and inflammatory reactions than either does alone. Furthermore, the concentration range of hydrocortisone found in AH was sufficient to inhibit the MLR and to inhibit Langerhans cell antigen-presentation for elicitation of immunity in a model of immune response to an immunogenic tumor. This latter observation may explain, at least in part, the earlier report that a non-TGF-β factor in AH inhibits Langerhans cell antigen-presenting function. The finding that hydrocortisone inhibits the MLR in the ng/ml concentration range confirms previous results. The normal serum protein CBG, which plays a dominant role as a carrier protein for glucocorticoids, was absent or present in a concentration below...
the lower limit of detection of our assay. These results attest to the unique nature of the ocular anatomic site and lead to speculation on how various soluble factors integrate to produce an immune-privileged microenvironment. The failure of α-MSH to inhibit the assays employed herein might suggest that this peptide does not play a significant role in the immune regulation of the anterior chamber. However, the concentration of α-MSH present in AH has been shown to inhibit gamma interferon production by antigen-driven lymph node cells; thus, it may participate in immune regulation in the anterior eye.

The limited protein content of AH compared to blood has been attributed to the blood-aqueous barrier. This barrier is thought to result from ultrastructural characteristics and the activity of a layer of cells lining the ciliary body, the ciliary body epithelium. Although the amount of protein found in human AH is limited (<500 μg/ml), albumin (MW 69 kd), transferrin (MW 80 kd), and other proteins of molecular weight 140 kd or less are typically detectable. These observations indicate that the barrier is not absolute and that other routes of entry of plasma proteins to AH may exist. Interestingly, CBG with a molecular weight of approximately 52 kd is relatively excluded or is not synthesized locally, based on the data presented here representing normal mouse, rat, and human AH. In addition to its role as a carrier protein for glucocorticoids, CBG induces adenylate cyclase activity with the subsequent accumulation of cyclic adenosine monophosphate (cAMP), suggesting that CBG may have hormone-like effects. Specific CBG receptors have been identified on various cells with the receptor–ligand interaction dependent on prior protein activation by binding of hydrocortisone to CBG. One may speculate, then, that the reservoir of hydrocortisone in AH might permit rapid formation of complexes with plasma-derived CBG in the event of breakdown of the blood-aqueous barrier, leading to cAMP accumulation in cells lining the anterior chamber that express the CBG receptor. How these events might influence the local response to inflammatory stimuli after stimulation is unknown.

Hydrocortisone was previously identified as a component of normal human and rabbit AH at concentration ranges similar to those reported herein, 2.5 to 17 ng/ml and 2 to 8 ng/ml respectively. Although corticosterone is recognized as the dominant glucocorticoid in some rodents, controversy has arisen regarding the presence of hydrocortisone in mice and rats. However, cultured adrenal cortex cells from rats and mice produce hydrocortisone, and immunoreactive hydrocortisone after chromatographic separation was detected in mouse and rat AH in our studies. Because small-molecular-weight species may traverse the epithelial barrier afforded by the blood-aqueous barrier, serum is most likely the source of free glucocorticoids in AH. Besides the acknowledged intravascular pool of free glucocorticoids and glucocorticoids bound to CBG or albumin, a large amount of hydrocortisone–CBG complexes (twofold to threefold intravascular levels) are present in the extravascular space. However, several biologic fluids have low levels of CBG and high relative proportions of free hydrocortisone compared to serum. These sites are considered to be immune-privileged sites. For example, in cerebrospinal fluid, free hydrocortisone is within the range found in serum, whereas CBG levels are several hundred times lower than those in serum. Amniotic fluid showed an interesting pattern: Although hydrocortisone levels were 15% of serum levels, CBG levels...
were only 10% of serum levels. To our knowledge, CBG levels in AH have not previously been determined. Therefore, it appears that a common characteristic of AH and cerebrospinal fluid is levels of free hydrocortisone the same or somewhat lower than those of serum, with greatly reduced levels of CBG. Other comparisons between the brain and the eye show that the anatomic and functional properties of the choroid plexus, the origin of cerebrospinal fluid, are similar to those of the ciliary body epithelium. The functional studies reported here and elsewhere demonstrate that the concentration of free hydrocortisone found in AH is biologically active.

Glucocorticoids induce the production of a family of proteins, known as lipocortins, that inhibit phospholipase A2, resulting in the inhibition of arachidonic acid release and subsequent eicosanoid production. Hence, an additional functional consequence of maintaining levels of hydrocortisone within the anterior chamber of the eye may be to retain high intracellular levels of lipocortins. Indeed, when removed from the anterior chamber and cultured in vitro, mouse iris–ciliary body tissue and human ciliary body epithelium spontaneously release prostaglandins into the culture media.

Glucocorticoids within AH may also play a role in the regulation of tissue repair and fibrosis in the anterior chamber. As early as 1907, histologic observations of the human iris after iridectomy or injury noted the absence of a tissue reaction, scar tissue formation, or deposition of connective tissue. A similar observation was made in rabbits, with the additional suggestion that inhibitory influences of aqueous humor may block repair processes. The question then became, ‘What are the factors present in AH that could afford immune privilege and at the same time protect against excessive damage mediated by reparative processes?’ Although known to have potent immunosuppressive properties, TGF-β is also widely recognized to function as a stimulator of wound repair by recruiting monocytes and leukocytes, inducing angiogenesis, and stimulating the secretion of extracellular matrix proteins, all of which have potentially damaging consequences to delicate tissues. Indeed, intraocular fibrosis as a secondary complication of retinal surgery may be due to threshold increases in vitreous humor levels of TGF-β compared to eyes without a fibrotic response. Clearly, the activity of TGF-β alone in AH is not consistent with the failure to observe fibrosis after injury to the iris. By contrast, glucocorticoids arrest tissue repair and wound healing at several key cellular processes. Thus, the presence of glucocorticoids may be responsible, in part, for these observations. Collagen deposition is markedly reduced in the presence of glucocorticoids in cells isolated from various organs, such as skin, hepatocytes, fibroblasts, and granulomas. Other evidence suggests that there is a delicate balance between the activities of TGF-β and glucocorticoids. TGF-β administered at a wound incision site in glucocorticoid-treated rats, for example, reversed the wound-healing anomaly. This dichotomy is further evidenced by the observations that although TGF-β is a potent chemoattractant of neutrophils, glucocorticoids inhibited IL-1-induced and IL-1-activated neutrophil migration. Also, hydrocortisone pretreatment can substantially decrease the stimulatory effects of TGF-β on collagen synthesis in osteoblast-enriched cultures from rats. Hence, the functional balance between TGF-β and glucocorticoids in the anterior chamber may play a key role in the outcome of various ocular pathologic processes.

TGF-β and glucocorticoid effects on immune function may be complementary. It has been demonstrated that dexamethasone increases mRNA levels for example, reversed the wound-healing anomaly. This dichotomy is further evidenced by the observations that although TGF-β is a potent chemoattractant of neutrophils, glucocorticoids inhibited IL-1-induced and IL-1-activated neutrophil migration. Also, hydrocortisone pretreatment can substantially decrease the stimulatory effects of TGF-β on collagen synthesis in osteoblast-enriched cultures from rats. Hence, the functional balance between TGF-β and glucocorticoids in the anterior chamber may play a key role in the outcome of various ocular pathologic processes.
the anterior chamber of the eye. Interestingly, both cerebral spinal fluid and amniotic fluid contain TGF-β activity, and, as stated earlier, both contain hydrocortisone, suggesting that a balance between these major immune and wound-repair modulators may be of significance in these immune-privileged sites and in the eye.

The study of mechanisms used to maintain immune privilege in the eye has yielded a better understanding of how the interplay of various soluble factors and cellular interactions mediate immune suppression. These findings have implications for the immunobiology of the eye and of other immune-privileged sites, and may lead to insights into possible novel therapeutic strategies for the control of immunopathologic or inflammatory disorders. In this regard, the immunosuppressive activity of corticosteroids has been well established; however, the activity, localization, and regulation of steroid binding proteins may also contribute significantly to immunoregulatory mechanisms.

Key Words
aqueous humor, hydrocortisone, corticosterone, cortisol binding globulin, immune privilege

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