Analysis of Immunosuppressive Properties of Iris and Ciliary Body Cells and Their Secretory Products

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The anterior chamber of the eye is an immunosuppressive microenvironment as shown experimentally by immune privilege, anterior chamber-associated immune deviation, and inability to display local delayed-type hypersensitivity responses. It recently was reported that both the aqueous humor and the cells of the iris and ciliary body (I-CB) have immune inhibitory properties in vitro, suggesting that these components of the anterior segment might contribute to the unique properties of this microenvironment. To explore the cellular sources of immunosuppressive factors in the anterior chamber, cultures of I-CB cells were established from normal eyes of BALB/c mice. Supernatants were harvested from these cultures and assayed in vitro for their ability to inhibit T-lymphocyte activation. It was found that I-CB cell-derived supernatants profoundly suppressed alloantigen-driven T-cell proliferation (mixed lymphocyte response) and interleukin-2 production by a T-cell hybridoma that responds to stimulator cells bearing I-A^d. The inhibitory activity of I-CB supernatants did not appear to be related to prostaglandins; supernatants of I-CB cells cultured with indomethacin retained their suppressive properties, as did supernatants to which neutralizing antiprostaglandin E_2 antibodies had been added. Moreover, suppression by I-CB supernatants was not relieved by antibodies specific for transforming growth factor-beta, even though this cytokine is known to be present in normal aqueous humor. Thus, the identity of the suppressive factor(s) in cultured I-CB cell supernatants remains elusive. Finally, by separating I-CB cell suspensions into bone marrow-derived (T-200-positive) and those not derived from bone marrow (parenchymal) subpopulations with a fluorescence-activated cell sorter, it was determined that the inhibitory activity of I-CB cell suspensions was produced by parenchymal, rather than hematogenous, cells. It is proposed and discussed that inhibitory factors and cytokines secreted by parenchymal I-CB cells contribute to the immunosuppressive qualities of the anterior chamber.

The anterior chamber (AC) of the mammalian eye has unusual immunologic properties. Its capacity to accept foreign tissues and grafts that would be rejected at other body sites is called immune privilege. Considerable experimental evidence generated over the past two decades revealed that immune privilege in the AC results from a dynamic process by which the quality of the systemic immune response to intraocular antigens is regulated actively. This immune response is unique because there is a selective deficiency of delayed-type hypersensitivity and a paucity of antibodies that can fix complement. At the same time, other immune effector modalities are conserved. This distinctive array of systemic immune effectors elicited by antigens placed in the AC has been termed AC-associated immune deviation (AC-AID). The selective immune deficiencies of AC-AID are mediated in part by antigen-specific suppressor T-cells. It was found that the eye itself participates in creating AC-AID. Enucleation of an eye within 4 days of intraocular injection of antigen prevents AC-AID and interferes with the generation of antigen-specific suppressor T-cells. Consequently, investigators have begun to study the cells and fluids of the anterior segment of the eye in an effort to understand the cellular and molecular basis of immune privilege. Our laboratory found that cells in the iris and ciliary body (I-CB) of normal mouse eyes can suppress T-lymphocyte proliferation in vitro in response to allogeneic stimulator cells. We also reported that normal aqueous humor is a profound inhibitor of T-cell activation in vitro. Others independently found that transforming growth factor-beta (TGF/β) is present in aqueous humor and makes an important contribution to this fluid's immunosuppressive properties.

Because aqueous humor is secreted by the epithelium of the CB and directly bathes the tissues that surround the anterior chamber (I, lens, cornea, and...
trabecular meshwork), this fluid may be viewed as a “conditioned medium” for the cells that surround this space. We examined immunoregulatory properties of supernatants harvested from I-CB cells that were cultured in vitro. Our results indicate that supernatants of I-CB cell cultures suppress T-cell activation in vitro, that the cellular sources of this suppressing activity are not bone marrow-derived cells, and that neither prostaglandins nor TGFβ accounts for the inhibitory properties of I-CB supernatants.

Materials and Methods

Experimental Animals

We bred BALB/c and C3H/HeN female mice in our domestic colony and used them for experiments when they were 6–8 weeks of age. These strains differ at the major histocompatibility complex and at numerous minor histocompatibility loci. All experimental procedures conformed to the ARVO Resolution on the Use of Animals in Research.

Preparation of Excised I-CB

The mice were killed by cervical dislocation, and their eyes were removed and microdissected to obtain the anterior half of each. The I-CB was teased carefully away from its attachment site and placed in the wash medium (RPMI-1640; GIBCO, Grand Island, NY) containing 5% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT), L-glutamine (2 mM; GIBCO), penicillin and streptomycin (100 units and 100 µg/ml, respectively; GIBCO), and HEPES (5 mM; GIBCO).

Dispersion of I-CB Cells

The excised I-CBs were transferred to another dish containing collagenase and dispase (Boehringer Mannheim, Indianapolis, IN) at 1 mg/ml in the wash medium and incubated at 37°C for 1 hr. After incubation, single-cell suspensions were obtained by trituration with a 21-gauge, followed by 23-gauge, needle attached to a syringe. The cells were washed twice in wash medium and stained with monoclonal antibodies, or resuspended in standard culture medium (SCM or RPMI-1640 containing 10% FCS, 2 × 10⁻³ M 2-mercaptoethanol, nonessential amino acids 0.1 mM, sodium pyruvate 1 mM, L-glutamine 2 mM, penicillin and streptomycin, and HEPES 5 mM; GIBCO).

Preparation of Cultured I-CB Supernatants

A single-cell suspension (15 × 10⁴ cells) containing the I-CB of five pairs of normal BALB/c eyes was resuspended in 2 ml of SCM and placed in a 35-mm tissue culture dish. The cells were incubated for 72 hr at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The supernatant was removed, and fresh SCM was added. This procedure was repeated twice weekly for 8 weeks; at this time, the cultures were abandoned. Supernatants to be tested for inhibitory activity were harvested 24 hr after the culture medium was changed last. To prepare serum-free supernatants, RPMI-1640 with penicillin and streptomycin only was added to the culture after the previous supernatant was discarded.

Staining of I-CB Cells With Monoclonal Antibodies and Sorting by Flow Cytometry

After washing with phosphate-buffered saline (PBS), a single-cell suspension containing the I-CBs of 45 pairs of freshly obtained normal BALB/c eyes was separated into three aliquots. One aliquot of 15 × 10⁶ cells was resuspended in culture medium and reserved unstained as a normal control. Another aliquot of 1 × 10⁶ cells was resuspended in 25 µl of fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ goat anti-rat immunoglobulin (GAR-FITC 1:100; Cappel, Malvern, PA) for 30 min at 4°C. The cells then were washed twice in PBS and used as a negative control for the FACS analysis as described elsewhere. The remaining cells 1.2 × 10⁶ were resuspended (50 µl) with the monoclonal antibody, T-200 (1:100; Boehringer Mannheim), and incubated for 45 min at 4°C. The cells were washed twice in PBS and resuspended in 50 µl GAR-FITC. After incubation for 30 min at 4°C, the cells were washed twice in PBS. The stained cells were analyzed and sorted with a FACS-STAR (Becton-Dickinson, Mountainview, CA) into T-200-negative and -positive populations. The number of live cells obtained was determined by trypan blue uptake. The sorted cells were resuspended in SCM and used in assays of T-cell suppression.

Preparation of Spleen and Thymus Cell Suspensions

The mice were killed by cervical dislocation, and their spleens and/or thymus were removed aseptically. Single-cell suspensions were obtained by pressing the minced tissue through a sterile 250-µm nylon mesh (Tetko, Elmsford, NY). The cells were washed twice in wash medium and resuspended in culture medium. The aliquots to be used as stimulator cells were irradiated with 2000 cGy. The spleen cells were used at a concentration of 2 × 10⁶ cells/well and the thymus cells at 4.75 × 10⁶ cells/well.

Preparation of Splenic Adherent Cells

The BALB/c spleen cell suspensions were treated with Gey’s solution to lyse the red cells. The remain-
ing cells were washed twice in wash medium and resuspended in SCM at 10 x 10^6 cells/ml. We placed 5 ml of the cell suspension in a 100-mm tissue culture dish and incubated it at 37°C for 2 hr. The medium was discarded, and the dish was swirled with warm fresh medium to remove the nonadherent cells. The adherent cells were resuspended with 10 ml of fresh medium for an additional 2 hr. The cells were washed three times with warm medium, then incubated with 5 ml of 0.6 mM ethylenediaminetetraacetic acid for 15 min. The dish was washed vigorously to remove adherent cells, and the cells were resuspended in fresh SCM before use in T-cell activation assays at 2.5 x 10^3 cells/well.

Mixed Lymphocyte Reaction (MLR)

The C3H responder spleen cells (2 x 10^5 cells/well) were cultured with irradiated stimulator cells (2 x 10^5 syngeneic spleen cells or allogeneic BALB/c spleen cells) in round-bottomed 96-well plates. The cultures were incubated at 37°C and, on day 4, pulsed with tritiated thymidine (Amersham, Arlington Heights, IL) at a concentration of 0.5 µCi/well. The cells were harvested on day 5, and the thymidine incorporation was measured.

Assay of T-Cell Hybridoma Activation (DG11 Assay)

The tumor DG11 is a T-cell hybridoma that secretes interleukin-2 (IL-2) in the presence of stimulator cells bearing the I-A\(^d\) alloantigen. The A20.1-11 (American Tissue Culture Collection, Rockville, MD) is a BALB/c-derived B-cell lymphoma that expresses I-A\(^d\) on its cell surface. The DG11 and A20 cells were irradiated with 2000 cGy (X-A20). The DG11 cells (2 x 10^5/well) were cultured with medium alone (final volume, 100 µl/well) were placed in 96-well flat-bottomed plates. The I-CB cell supernatants and/or various antisera were added to the DG11/A20 cultures with a total volume of 200 µl maintained. Triplicate wells were plated for each experimental situation and incubated for 24 hr at 37°C. The supernatants from these cultures were harvested and assayed for the presence of IL-2 using the CTLL assay.

CTLL Assay for IL-2 Production

Serial twofold dilutions of each supernatant with culture medium (final volume, 100 µl/well) were placed in 96-well flat-bottomed plates. The IL-2 was assayed as described previously. Briefly, 100 µl of CTLL cells (1 x 10^4) with the blocking antibody (αIL-4) was added to each well. Six cultures of CTLL cells and medium alone were used as negative controls. Positive controls of CTLL cells with serial dilutions of mouse recombinant IL-2 (Genzyme, Cambridge, MA) were included in all experiments. The CTLL cultures were incubated for 20 hr at 37°C, after which tritiated thymidine was added to the cultures at a concentration of 0.5 µCi/well. The cultures were incubated for an additional 4 hr before harvesting the cells and measuring the thymidine incorporation.

Neutralizing Antisera

Three neutralizing antisera were used. The antibody αTGFβ (R & D Systems, Minneapolis, MN) neutralizes both TGFβ\(_1\) and TGFβ\(_2\), and αTGFβ\(_2\) neutralizes only TGFβ\(_2\). Anti-prostaglandin E\(_1\), E\(_2\), and A antibodies (Sigma, St. Louis, MO) were used at a concentration of 0.1 µg/ml in the well. These antibodies neutralize approximately 50-100 ng/ml of their respective prostaglandin activity, as measured by inhibition in the DG11 assay.

Inhibition of Prostaglandin Synthesis

Indomethacin (1 µg/ml; Sigma) was used in several ways as follows: (1) DG11 cells were cultured with X-A20 cells in SCM alone or with indomethacin added; (2) in the presence of cultured I-CB cell supernatant, indomethacin was added to DG11/A20 cultures; and (3) I-CB cells were cultured for 24 hr in medium containing indomethacin and the resulting supernatant added to DG11/A20 cultures. At concentrations of 1 µg/ml indomethacin prevents prostaglandin synthesis and release from single-cell suspensions of murine keratinocytes cultured at 2 x 10^4/cell (unpublished observations).

Results

The first experiments were designed to demonstrate that supernatants harvested from cultures of I-CB cells had immunosuppressive properties in vitro and that a simple assay of antigen-driven T-cell activation, using a well-characterized T-cell hybridoma, could be used to measure the immunosuppressive activity.

Immunosuppressive Properties of Supernatants of Cultured I-CB Cells

After determining that cell suspensions prepared from I-CB of normal mouse eyes suppress T-lymphocyte proliferation in vitro in response to alloantigenic stimulator cells, we first wished to determine whether a secretory product from I-CB cells might be responsi-
ble. Single-cell suspensions from freshly isolated BALB/c I-CB were cultured for 7–8 days in SCM to which no exogenous growth factor was added. At the end of the culture interval, the supernatant was removed and replaced with fresh medium. Twenty-four hours later, this supernatant was removed and progressively diluted (twofold) with SCM; undiluted and progressive dilutions of supernatant then were added at the initiation of mixed lymphocyte cultures containing C3H responder spleen cells and irradiated (2000 cGy) BALB/c stimulator spleen cells (total volume/well, 200 µl). The results of a typical experiment are presented in Figure 1 and indicate that the supernatant from cultured I-CB cells profoundly inhibited allostimulation of T-cells. Even when the supernatant was diluted sevenfold (1:128) and added to MLR cultures, the proliferative response of C3H T-cells was only 50% of that obtained with MLRs to which no I-CB supernatant had been added. Thus, I-CB cells when placed in vitro appear to release into the medium, without any further provocation, factor(s) that inhibit T-cell activation by allogeneic stimulator cells.

Suppression of T-Cell Hybridoma Activation by I-CB Cells

Because our goal was to identify the cellular source and suppressive factor(s) found in the previous experiment, we wished to use a more reliable assay of T-cell activation than a MLR, one that was less labor intensive and would permit the study of a large number of different samples. To that end, we selected, for a responder cell, the T-cell hybridoma DG11 that secretes IL-2 only when confronted by stimulator cells bearing I-A<sup>d</sup> alloantigens. For a stimulator cell, A20, a B-cell lymphoma adapted to continuous in vitro growth and that expresses I-A<sup>d</sup>, was selected. Preliminary studies confirmed that DG11 cells cultured with X-A20 cells secreted large amounts of IL-2, as measured in the CTLL bioassay. Using the DG11/A20 model culture system, we tested whether BALB/c I-CB cells added to these cultures could suppress directly the activation of DG11. Freshly obtained I-CB cells (5 × 10<sup>4</sup>/well) were added to cultures containing DG11 cells (2 × 10<sup>5</sup>/well) and X-A20 cells (2 × 10<sup>5</sup>/well). Positive control cultures contained responders and stimulators without I-CB cells. To control for potential cell crowding in the cultures containing I-CB cells, additional control cultures containing DG11 and X-A20 cells were set up with 2.5 × 10<sup>3</sup> BALB/c splenic adherent cells (equivalent to the number of I<sup>a</sup> cells in a suspension of 5 × 10<sup>4</sup> I-CB cells) and 4.75 × 10<sup>4</sup> BALB/c thymocytes (equivalent to the number of I<sup>a</sup> cells in a suspension of 5 × 10<sup>4</sup> I-CB cells). The supernatants of these cultures were collected 24 hr later and added directly (and with progressive twofold dilutions) to indicator CTLL cells. Then 3<sup>H</sup>-thymidine was added 20 hr later, and the amount of radioactivity incorporated after an additional 4 hr was assessed. The results of a representative experiment are presented in Figure 2. Supernatants from DG11 cells cultured alone with X-A20 cells stimulated CTLL cells vigorously, indicating that IL-2 was present. Similarly, supernatants from DG11 cells co-cultured with splenic adherent cells and thymocytes also secreted large amounts of IL-2. However, in the presence of
Effect of Iris and Ciliary Body Cells on IL-2 Production by DG11 Exposed to A20

Fig. 2. I-CB cells ($5 \times 10^5$) were added to cultures containing DG11 responders ($2 \times 10^5$) and irradiated (2000 cGy) A20 stimulators ($2 \times 10^5$). Control cultures contained DG11 and X-A20 cells alone, or these cells were mixed with BALB/c thymocytes ($4.75 \times 10^5$) plus BALB/c splenic adherent cells ($2.5 \times 10^5$) instead of I-CB cells. Supernatants collected after 24 hr were assayed for IL-2 activity on CTLL cells, presented as mean counts per minute of $^3$H-thymidine (±SEM) incorporated during the terminal 4 hr of a 24-hr culture.

I-CB cells, the supernatants of DG11 cells exposed to X-A20 cells did not induce proliferation of CTLL cells. Thus, I-CB cells were capable of inhibiting alloantigen-driven activation of DG11 cells; the supernatants of these cultures did not cause CTLL cells to proliferate. Because the DG11 assay appeared to be a sensitive indicator of these inhibitory properties, it was used in the subsequent experiments.

Effect of I-CB Cell Supernatants on Activation of T-Cell Hybridoma

We next wished to determine whether supernatants from I-CB cell cultures could suppress antigen-driven activation of DG11 cells. Supernatants from cultured I-CB cells were added to cultures containing DG11 cells as responders and X-A20 cells as stimulators. In some assays, supernatants harvested from cultures of I cells only were used; in other assays, supernatants harvested from cultures of CB cells only were added. The results (Fig. 3) indicate that T-cell activation—as revealed by alloantigen-triggered IL-2 production—was inhibited strongly by supernatants harvested from cultured I-CB cells and from cultures established with I-CB cells alone. In each instance, IL-2 production was reduced to less than 5% of the amount produced by DG11 cells without I-CB supernatants.

In the studies described to this point, the supernatants were harvested from I-CB cells cultured in SCM containing 10% FCS. To determine whether the serum supplement was important in the suppressive properties displayed by the supernatants, I-CB cells were cultured for 7 days in SCM containing serum. At this time, the supernatant was removed and replaced with serum-free medium. After 24 hr, this supernatant was removed and tested for suppressive activity in the DG11 assay. A representative experiment is shown in Figure 4. These results indicate that the presence of serum in the medium had no effect on the suppressive properties of I-CB supernatants; serum-containing and serum-free supernatants had comparable inhibitory effects on T-cell activation.

Because the CTLL cells used as indicators to measure IL-2 were T-cell lines, it became important to
determine whether I–CB supernatants had any inhibitory effect on the ability of CTLL to proliferate in response to IL-2. Supernatants obtained from I–CB cell cultures were added to wells containing CTLL cells (1 × 10^4/well) to which graded amounts of recombinant murine IL-2 were then added. When the proliferation of CTLL cells was assayed with ³H-thymidine incorporation, no inhibitory effect of I–CB supernatants was observed (Fig. 5). Thus, the suppressive properties of factor(s) released in vitro from I–CB cells did not interfere with our bioassay for IL-2 (the ability of IL-2 to cause proliferation of CTLL cells.) Therefore, we concluded that the inability of supernatants from DG11 cells exposed to X-A20 cells in the presence of I–CB cell culture supernatants to induce CTLL proliferation is a result of suppressive factor(s) secreted by I–CB cells.

In other experiments, I–CB supernatants were placed in cultures with DG11 cells alone and unirradiated A20 cells alone. When ³H-thymidine incorporation by these cells was measured, no inhibition was observed (data not shown). Thus, I–CB supernatants can inhibit activation but not proliferation of the DG11 hybridoma cells.

Role of Prostaglandins in Immunosuppressive Properties of I–CB Cell Supernatants

Prostaglandins are potent inhibitors of aspects of T-cell function. Moreover, they are known to be secreted, under certain conditions, by cells in the eye.

These experiments examined the possibility that prostaglandins secreted by cultured I–CB cells might be responsible for suppression of T-cell activation by I–CB supernatants. First, it was necessary to determine whether DG11 responses to X-A20 cells were susceptible to prostaglandin inhibition. A dose–response assay was conducted in which the amount of prostaglandin E₂ added to cultures containing DG11 responders and X-A20 stimulators ranged from 10 µg to 6 ng/ml. Virtually complete suppression of DG11 IL-2 production was achieved at 10 and 1 µg/ml prostaglandin E₂, and partial inhibition was still evident when as little as 12.5 ng/ml was added (data not shown). Thus, if prostaglandin E₂ were present in I–CB supernatants at concentrations in excess of 12.5 ng/ml, the DG11 assay should be able to detect it. Next, we determined whether indomethacin, an inhibitor of prostaglandin synthesis, interfered with the DG11 assay. The DG11 cells were cultured with X-A20 cells in SCM alone, or with indomethacin (using concentrations of 1, 5 and 10 µg/ml). After 24 hr, the supernatants of these cultures were assayed on CTLL cells and found to contain virtually identical amounts of IL-2 (cultures A and D, Fig. 6). Although only data from cultures in which indomethacin at 1 µg/ml was used are presented in this figure, these results were fully representative of those using higher concentrations of indomethacin. Thus, if indomethacin was present in the
culture medium, it did not interfere with the DG11 assay. Next, I-CB cells were cultured for 24 hr in SCM alone or with indomethacin (1 μg/ml). The supernatants were harvested and added to DG11 cells exposed to X-A20 cells. As the results in Figure 6 indicate, supernatants from I-CB cells cultured with indomethacin (culture C) and supernatants from I-CB cells cultured without indomethacin (culture B) both suppressed IL-2 production by DG11 cells exposed to X-A20 cells. These results suggest that prostaglandins are not involved in the suppressive activities of I-CB cell supernatants. This suggestion was corroborated by the results of a companion experiment. The I-CB supernatants from conventional cultures were added to cultures of DG11 and X-A20 cells. Simultaneously, neutralizing antibodies directed at prostaglandin E1, E2, and A were added to these cultures. As the data presented in Figure 6 (compare culture E with culture B) indicate, suppression of DG11 by I-CB cell supernatants was comparable whether α-prostaglandin E2 antibodies were present in the culture or not. Identical results were obtained if the antibodies added to the cultures were directed at prostaglandin E1 or A (data not shown). Finally, indomethacin (1, 5, or 10 μg/ml) was added to cultures of DG11 and X-A20 cells in the presence of I-CB cell supernatants. Again, indomethacin did not abrogate the T-cell suppression mediated by I-CB cell supernatants (data not shown). We concluded that prostaglandins played no detectable role in the immunosuppressive activities of supernatants derived from cultured I-CB cells.

### Effect of TGFβ on IL-2 Secretion by DG11 Exposed to A20

![Graph showing the effect of TGFβ on IL-2 secretion by DG11 exposed to A20](image)

**Fig. 7.** Transforming growth factor-beta, or TGFβ, was added at 10 and 1 ng/ml directly to cultures containing DG11 responders and X-A20 stimulators. IL-2 activity in the supernatants was assessed on CTLL cells as described in legend to Figure 2.

antibodies to the DG11 assay. As described earlier, one antiserum neutralizes TGFβ1 exclusively, and the other antiserum neutralizes both TGFβ1 and TGFβ2.

In preliminary experiments, it was found that the ability of exogenous TGFβ (10 ng/ml) to inhibit IL-2 secretion by DG11 cells was reversed completely in the presence of the neutralizing antisera (data not shown). Accordingly, each antiserum was added to separate cultures containing DG11 responders, X-A20 stimulators, and I-CB supernatants. As the results of Figure 8 reveal, in neither case was the suppressive property of the supernatant abolished (compare cultures C and D with culture B). Moreover, in critical controls (cultures E and F), it was determined that αTGFβ antibodies did not affect the secretion of IL-2 by DG11 cells exposed to X-A20 cells (Fig. 8). Thus, TGFβ does not appear to be responsible for the immunosuppressive properties of I-CB cell supernatants.

### Evidence That I-CB Cells Not Derived From Bone Marrow Secrete Immunosuppressive Factor(s)

Recently, our laboratory and others characterized partially the bone marrow-derived cells that comprise a minor population in the stroma of the normal I-CB. Because our studies were designed to explore the immunologic properties of cells in the anterior segment of the eye, we suspected that stromal bone marrow-derived cells might be responsible for producing the suppressive activity found in supernatants of cultured I-CB cells. To address this issue formally, single-cell suspensions were prepared from I-CBs harvested from 45 pairs of normal BALB/c eyes. A portion of the suspended cells was stained with rat anti-mouse T-200 antibody (this detects a marker on
Role of TGFβ in Inhibitory Properties of Iris and Ciliary Body Supernatants

Fig. 8. Control cultures (A) of DG11 responders and X-A20 stimulators were modified by addition of (B) I-CB culture supernatant alone, (C) I-CB culture supernatant plus anti-TGFβ1,2, (D) I-CB culture supernatant plus anti-TGFβ2, or (E) anti-TGFβ1,2 alone, or (F) anti-TGFβ1 alone. IL-2 activity in the supernatants was assessed on CTLL cells as described in legend to Figure 2.

![Graph showing role of TGFβ in inhibitory properties of iris and ciliary body supernatants.](image)

Discussion

The AC of the eye is an immunologically privileged site where grafts of foreign tissue and cells may survive
The phenomenon of privilege is mediated in part by a deviant systemic immune response elicited by antigenic materials placed in the AC (ACAID). Not only is the systemic immune response to ocular antigens deviant, but recent studies show that expression of conventional immunity in the AC also is aberrant. Especially in mouse eyes, graft-versus-host reactions are difficult to elicit, and the normal AC is incapable of displaying delayed-type hypersensitivity reactions. In an effort to understand the physiologic basis for these restrictions on immunity in the eye, we began to investigate the tissues and fluids of the eye itself. We previously showed that normal aqueous humor interferes with antigen and mitogen-driven T-cell activation. In addition, others found that the normal I-CB do not activate allogeneic T-cells, despite the presence of bone marrow-derived cells bearing class II antigens encoded by the major histocompatibility complex. In the latter experiments, cells from the I-CB were found unexpectedly to suppress alloreactive T-cells in vitro. Our studies explored this immune inhibitory phenomenon in further detail.

We found that supernatants harvested from cultured I-CB cells profoundly suppress T-cell activation in vitro, whether expressed as T-cell proliferation (MLR) or as IL-2 production by alloantigen-activated T-cell hybridomas. Although TGFβ is an important inhibitor in normal aqueous humor, our results indicate that TGFβ probably is not responsible for the suppression mediated by I-CB cell supernatants in our studies. Moreover, the suppression did not appear to be caused by prostaglandins.

Our efforts to identify the cellular source of the suppressive activity were only partially successful. It was found that supernatants from either I or CB cells cultured alone were comparably suppressive, indicating that both tissues possess this property. More importantly, I-CB cells depleted by sorting out bone marrow-derived cells on a flow cytometer produced supernatants with powerful suppressive properties; a suspension of I-CB cells enriched for T-200 cells had no similar effect. Having thus excluded lymphoreticular cells as the source of the suppressing activity, we were eager to identify the constitutive cell(s) of the I-CB that produced this activity. Epithelial cells were prime candidates for this role, although neural elements, endothelial cells, and smooth muscle cells also should be considered. It recently was reported that porcine iris and ciliary epithelial cells contain immuregulatory proteins. This finding was consistent with our expectation that epithelial cells are the cellular source of suppressing activity in supernatants of I-CB cultures. Others recently found that cells harvested from rat CB can be induced by interferon-γ to express class II major histocompatibility complex antigens. Despite upregulation of these molecules, the cells did not function as effective antigen presenters to T-cells. Instead, the interferon-γ-treated ciliary epithelial...

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**Table 1. Allostimulatory capacity of T200-enriched I-CB cells**

<table>
<thead>
<tr>
<th>Responders (2 X 10⁵/well)</th>
<th>Stimulators (8/well)</th>
<th>³H-thymidine incorporation (cpm)</th>
<th>Stimulation index*</th>
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<tr>
<td>C3H</td>
<td>None</td>
<td>933 ± 45</td>
<td>—</td>
</tr>
<tr>
<td>C3H</td>
<td>X-C3H spleen cells (2 X 10⁵)</td>
<td>1021 ± 95</td>
<td>1</td>
</tr>
<tr>
<td>C3H</td>
<td>X-BALB/c spleen cells (2 X 10⁵)</td>
<td>11,566 ± 201</td>
<td>11</td>
</tr>
<tr>
<td>C3H</td>
<td>T200+ I-CB cells† (2 X 10⁵)</td>
<td>1131 ± 72</td>
<td>1</td>
</tr>
</tbody>
</table>

* Stimulation index = cpm allogeneic stimulation divided by cpm stimulation of C3H responders by X-C3H stimulators.
† Responders were splenic T cells from C3H mice.
‡ I/CB cell suspension contained 64% T200+ cells.

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ial cells inhibited T-cell activation by at least two mechanisms, one of which appeared to be indomethacin sensitive. Thus, it would appear that interferon-γ-activated cells from the ciliary body can produce prostaglandins and that this may account for some of their immunoinhibitory properties. This report did not describe the immunomodulatory properties of ciliary epithelial cells that were not treated with interferon-γ. Because we have not examined the properties of I-CB cells after exposure to interferon-γ, it is not possible to reconcile our failure to find prostaglandin-dependent suppression with I-CB supernatant with the findings of these authors. It may be that unstimulated I-CB cells create an immunosuppressive environment that is prostaglandin independent, but that interferon-γ stimulation may force the cells to suppress by an alternate mechanism that involves prostaglandins. Another intraocular cell with immunosuppressive properties is the Muller cell of the retina. Isolated rat Muller cells did not act as antigen-presenting cells in vitro, even when the cells were first treated with interferon-γ. The interferon-γ-treated Muller cells exerted their immunosuppressive properties by direct cell contact, rather than through elaboration of a soluble factor.

The capacity of the I-CB supernatants to suppress activation of DG11 cells suggests that there may be an in vivo role for this activity in the failure of the AC to display delayed-type hypersensitivity. The DG11 cells are T-cell hybridomas, derived by fusion of a T-cell lymphoma with sensitized T-cells that react with I-A<sup>4</sup> alloantigen. In this sense, DG11 cells resemble sensitized T-cells. We speculated that the I-CB-derived suppressing factor(s) may act in vivo by preventing delayed-type hypersensitivity T-cells (T<sub>DH</sub>) that enter the AC from responding to their relevant antigen in situ. An alternative possibility is that the suppressor factor(s) act on antigen-presenting cells and impair their capacity to activate T<sub>DH</sub> cells. In our DG11 assay, A20 cells (stimulators) would be the analogous target of suppression. Although I-CB supernatants do not interfere with proliferation of either DG11 or A20 cells, we have preliminary evidence to indicate that pretreatment of either DG11 or A20 cells with I-CB supernatants reduces their subsequent capacity to be activated (DG11) or to act as stimulators (A20) (manuscript in preparation). Thus, the secretory products of I-CB cells may be central to the inability of the normal AC to display conventional delayed-type hypersensitivity. The aqueous humor also can inhibit antigen-driven T-cell activation, and therefore, the normal AC offers a formidable barrier to expression of cell-mediated immunity.

The ability of I-CB cells to secrete suppressive factors also may be pertinent to the phenomenon of ACAID. Wilbanks and Streilein recently discovered that creation of the antigenic signal responsible for ACAID induction is initiated in the AC itself. Antigen injected into this site appears to be processed by macrophages of the I-CB that bear the marker F4/80. In fact, F4/80-positive macrophages from blood or peritoneal cavity acquire ACAID-inducing properties when they are pulsed with antigen in vitro and injected into the anterior chamber. Based on the results of studies of I-CB supernatants and aqueous humor, we believe that the unique immunosuppressive microenvironment of the AC is factor mediated and confers ACAID-inducing properties on F4/80 cells in this site. To determine the molecular nature of the suppressive factors in I-CB supernatants and aqueous humor and to understand their mechanism of action on putative intraocular antigen-presenting cells and T-cells are major goals of our laboratory.

Key words: anterior chamber, iris and ciliary body, T-cell activation, immunosuppressive factors

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