Blood-Borne Signals That Induce Anterior Chamber-Associated Immune Deviation After Intracameral Injection of Antigen

J. Wayne Streilein, Shigeki Okamoto, Yoshiyuki Hara, Michele Kosiewicz, and Bruce Ksander

**Purpose.** Anterior chamber–associated immune deviation (ACAID) is elicited by an antigen-specific signal that escapes the antigen-containing eye and travels through the blood to the spleen. Two types of ACAID-inducing signals have been described: those associated with blood-borne monocytes, and a soluble factor found in serum. The authors sought to understand the basis for the existence of two distinct types of ACAID-inducing signals.

**Methods.** Different kinds of antigens (soluble, cell associated, particulate) were injected into the anterior chamber (AC) of normal, presensitized, and immunodeficient mice. In addition, peritoneal exudate cells were pulsed in vitro with different kinds of antigen in the presence of transforming growth factor beta and then evaluated for the ability to induce ACAID in naive (nonsensitized) as well as T- and B-cell–deficient recipients.

**Results.** Among antigens injected into the AC, inert particulate antigens could not induce ACAID, but soluble and cell-associated (minor histocompatibility) antigens generated cell-associated ACAID-inducing signals. In contrast, antigens injected into the AC of presensitized mice generated ACAID-inducing signals that were soluble and located in the plasma fraction of blood. All ACAID-inducing signals created in vitro with soluble, particulate, or cell-associated antigens induced ACAID in vivo.

**Conclusions.** Cell-associated ACAID-inducing signals are generated in naive mice regardless of the kind of antigen, and these signals arise from mobile intraocular antigen-presenting cells. However, when antigen is injected into the AC of presensitized mice, a soluble signal emerges, perhaps derived from T cells that enter the antigen-containing eye. Together, these signals dictate that subsequent exposures to ocular antigen will not evoke immunogenic inflammation. Invest Ophthalmol Vis Sci. 1997;38:2245–2254.

Injection of antigenic material into the anterior chamber (AC) of the eyes of mice, rats, and primates induces a deviant systemic immune response termed anterior chamber–associated immune deviation (ACAID). This stereotypic systemic immune response is elicited by a diversity of antigens, ranging from soluble heterologous proteins to cell-associated antigens, and can be evoked in immunologically naive animals as well as in animals previously sensitized to intracameral injected antigen. Because antigens injected into the AC are dispersed in aqueous humor, and because aqueous humor drains directly into the bloodstream through the trabecular meshwork, it is not surprising that large amounts of intraocularly injected antigenic material enter the bloodstream.

However, recent evidence indicates that intraocularly injected soluble antigen that reaches the bloodstream is not responsible in its native form for ACAID induction. Wilbanks and Streilein showed that intracameral injection of a soluble heterologous protein antigen, such as bovine serum albumin or interphotoreceptor retinol binding protein, produced a blood-borne antigen-specific signal that was responsible for ACAID induction in naive mice. This signal was not native antigen but was associated with leukocytes that...
express F4/80, a surface molecule restricted to certain monocytes, macrophages, and dendritic cells. However, Ferguson et al\textsuperscript{10} have reported that when trinitrophenol (TPN)-derivatized syngeneic spleen cells were injected into the AC, the blood-borne antigen-specific ACAID-inducing signal generated was a soluble serum protein, perhaps of T-cell origin. The latter investigators hypothesized that two different types of ACAID-inducing signals were generated in these separate experimental protocols because soluble antigens elicit cell-associated ACAID-inducing signals,\textsuperscript{11} whereas insoluble or particulate antigens elicit soluble ACAID-inducing signals. The logic for such a dichotomy remains obscure.

To explore this issue further, we studied the capacity of cell-associated and particulate antigens to generate ACAID-inducing signals in vivo and in vitro. After examining the induction of ACAID in naïve and specifically sensitized mice, we concluded that intracameral injections of antigens give rise to cell-associated signals in naïve mice and soluble signals in pre-sensitized mice. Moreover, particulate antigens injected intracameraly appear to be incapable of inducing ACAID.

**MATERIALS AND METHODS**

**Mice**

BALB/c and B10.D2 mice and SCID mice (8 to 12 weeks old) were obtained from our domestic breeding facility or purchased from Jackson Laboratories (Bar Harbor, ME). Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Antigens and Reagents**

Bovine serum albumin (BSA; Sigma Chemical, St. Louis, MO) was used as soluble protein antigen. DBA/2-derived P815 mastocytoma cells and the hypoxanthine–aminopterin–thymine (HAT)-sensitive mutant cell line,\textsuperscript{12} P511, were used as cell-associated antigens (minor histocompatibility antigens when placed in BALB/c recipients). For particulate antigen, mycobacterial antigen was prepared by ultrasonication (×20 minutes) lyophilized H37RA (Difco Laboratories, Detroit, MI) in 10 mg/ml phosphate-buffered saline. Transforming growth factor beta (TGF/β) was purchased from R & D Systems (Minneapolis, MN). Supernatants of cultured cells of iris and ciliary body of mouse eyes were prepared as described previously.\textsuperscript{15}

**Tumor Cell Cultures**

In some experiments, blood from recipients of AC-injected P511 cells were tested for ACAID induction. P511 cells are a HAT-sensitive derivative of P815 cells originally developed by Dr. T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium).\textsuperscript{12} P511 cells were used in these experiments to eliminate tumor cells from the blood selectively. This was necessary because the blood of AC recipients of P815 cells contains viable tumor cells.

The following preliminary experiments were performed to confirm that HAT-containing medium had eliminated contaminating P511 cells from the blood. Blood was recovered from groups of mice 48 hours after AC inoculations of P511 cells and was cultured for 1 month in conventional culture medium or HAT-containing medium. Blood from AC recipients of P511 cells was found to possess viable tumor cells that grew in cultures containing conventional medium. In contrast, no viable tumor cells were detected in cultures of blood from mice inoculated with P511 cells and cultured in HAT-containing medium. Subsequent experiments determined that blood cultured in HAT medium for 24 hours eliminated all contaminating tumor cells. Thus, in experiments that tested blood for the presence of an ACAID-inducing signal, blood cells were harvested 48 hours after AC inoculation of tumor cells and were cultured for 24 hours in HAT-containing medium. To confirm that these cell preparations did not contain tumor cells, aliquots of cells were cultured in conventional medium for 30 days; no tumor cells grew out (data not shown). However, this does not exclude the possibility that blood cultured in HAT-containing medium contains nonviable P511 cells or membrane fragments.

**In Vitro Preparation of ACAID-Inducing Signal**

Peritoneal exudate cells (PECs) were obtained as described previously from naïve BALB/c and B10.D2 mice that had received 3 ml of a 3% thioglycolate solution intraperitoneally 4 days earlier.\textsuperscript{16} The cells were washed and resuspended into a single cell suspension with culture media (RPMI 1640, GIBCO, Grand Island, NY) containing 1% normal mouse serum (Cedarlane Laboratories, Hornby, Ontario, Canada), 2 × 10⁻⁵ M 2-ME, minimum essential medium nonessential amino acids (0.1 mM, GIBCO), L-glutamine (2 mM, GIBCO), penicillin and streptomycin (100 U/ml and 100 mg/ml, respectively, GIBCO), and Hepes (5 mM, GIBCO), 2 × 10⁵ cells in each well in a 96-well round-bottom plate. TGFβ (0.1 ng/well) or supernatant of iris and ciliary body cultures (50 μl/well) was then added directly to B10.D2 PEC, 2 × 10⁵ cells/cell (cell-associated antigens), or to BALB/c PEC that were then pulsed with particulate (sonicated H37RA, 10 μg/ml) antigen. We have shown previously\textsuperscript{19} that the ACAID-promoting capacity of supernatants of cultured iris and ciliary body cells is due to the presence of TGFβ. Normal iris and ciliary body culture supernatants contain 3 to 4 ng/ml TGFβ.
Moreover, anti-TGF-β antibodies abolish the ability of these supernatants to create ACAID-inducing signals in vitro. After overnight incubation at 37°C, the PEC were cooled for 30 minutes at 4°C, dislodged from the culture plate by vigorous pipetting, washed twice, resuspended, and infused intravenously into naïve syngeneic recipients.

**Preparation of ACAID-Inducing Signals From Blood**

BSA (50 μg/2 μl), P815 or P511 cells (2 × 10⁵), or sonicated H37RA (10 to 500 μg) was injected into the AC of mice that had been splenectomized 7 days previously. After 2 days, blood was obtained by cardiac puncture, heparinized, and separated into plasma and cellular fractions as described previously. The latter fraction was washed extensively with phosphate-buffered saline. Plasma or washed cells (from one donor equivalent) were then infused intravenously into naïve syngeneic recipients.

**Assay for ACAID**

Recipients of (1) AC injection of antigen, (2) plasma or blood cells from AC recipients of antigen, and (3) PEC incubated overnight with TGF-β and antigen, were rested 7 days and then immunized subcutaneously with relevant antigen in complete Freund’s adjuvant as described previously. Positive controls received subcutaneous immunization but no prior exposure to the antigen. Delayed hypersensitivity (DH) was determined 1 week later by intrapinna injection of relevant antigen. Ear swelling was assessed 24 and 48 hours after challenge by using a micrometer. Each experimental panel consisted of five mice. Ear swelling responses were read in a masked fashion such that the person assessing the swelling was unaware of the experimental manipulations of the panel under examination. All experiments were repeated at least twice with similar results.

**Splenectomy**

Spleens were removed from anesthetized mice through a lateral abdominal incision. Sham-treated mice had abdominal incisions, but the spleens were not removed.

**Statistical Evaluation**

Data were analyzed by using Student’s t-test or analysis of variance with Scheffe’s test. Means were considered to be significantly different from controls when P < 0.05.

**RESULTS**

In the following experiments, three different types of antigens were used: cell-associated antigens (minor histocompatibility antigens expressed by P815 mastocytoma cells when injected into BALB/c mice), particulate antigens (sonicated mycobacterial organisms), and soluble antigens (the heterologous protein BSA). After these antigens were injected intracamerally into naïve or specifically sensitized mice, the blood was examined to determine whether an ACAID-inducing signal was present and, if so, whether it was associated with the leukocytic fraction or with plasma or serum. In addition, these antigens were incubated in vitro with PECs in the presence of TGF-β, after which the cells were tested for their ability to induce ACAID when injected intravenously into naïve or immune animals.

**ACAID-Inducing Potential of Blood From Recipients of AC-Injected Tumor Cells**

Our first goals were to determine whether P815 cells could generate a blood-borne ACAID-inducing signal and whether that signal was associated with leukocytes or plasma. Normal adult BALB/c mice had their spleens surgically excised. Seven days later, these mice received an injection in the AC of the right eye of 2 × 10⁵ P815 tumor cells. The mice were killed 48 hours later by exsanguination from cardiac puncture. The blood was collected in the presence of heparin to avoid coagulation and was centrifuged, enabling the plasma fraction to be separated from the cellular fraction. The latter was washed three times with medium. Panels of naïve BALB/c mice received intravenous infusions (one donor equivalent) of either plasma or washed cells from donor blood. Seven days later, recipients of these infusions were immunized by a subcutaneous injection of P815 cells (10⁶). Positive controls were BALB/c mice that received an intravenous injection of whole blood from splenectomized but not AC-injected BALB/c mice. ACAID controls were represented by normal BALB/c mice that received an AC injection of P815 cells (2 × 10⁵). The injected eyes were enucleated 7 days later, and the mice were ear challenged with irradiated P815 cells (2 × 10⁶) 3 days thereafter. Experimental mice were similarly ear challenged 7 days after subcutaneous immunization with tumor cells.

The results of a representative experiment (of three) are displayed in Figure 1. As anticipated, P815 cells injected into the AC of normal mice elicited ACAID; the ears of these mice displayed significantly reduced swelling compared with positive controls. BALB/c mice that received an intravenous infusion of plasma obtained from mice with AC P815 injections displayed intense ear swelling responses, comparable to positive controls; this indicates that ACAID was not present. In contrast, the ears of recipients of intravenously infused blood cells from donor mice with P815 in the AC showed feeble ear-swelling responses, indic-
ACAID-inducing potential. When subcutaneous injections of P815 cells (10^6) were made in vivo, we suspected that the cellular fraction of blood might function as ACAID-inducing signals. The minor histocompatibility antigens displayed by P815 cells to BALB/c recipients are cell associated with hematopoietic cells, not tumor cells. Therefore, panels of splenectomized BALB/c mice received AC injections of P511 tumor cells (2 × 10^6). Two days later, blood was obtained by cardiac puncture, the plasma was removed, and the cells were resuspended in HAT medium and cultured at 37°C for 24 hours. Thereafter, the cells were collected. One aliquot was placed in conventional tissue culture medium to determine if any P511 tumor cells survived HAT exposure. The remaining blood cells were injected intravenously into naive BALB/c mice. ACAID control mice received P511 cells into the AC, and positive control and experimental mice were immunized by subcutaneous injection of P511 cells (10^6). Ear challenge of these mice with irradiated P511 cells was conducted as described above.

As the results presented in Figure 2 reveal, HAT medium–treated blood cells obtained from P511-injected donors induced ACAID in recipient mice. Ear-swelling responses of these mice were slight and similar to those of the ACAID controls. In addition, no tumor cells grew out of the cultured aliquots of HAT medium–treated blood cells during the 4-week observation interval (data not shown). Based on these results, we made two conclusions: first, tumor cells that might escape from the tumor-containing eye and appear in the blood cannot be responsible for ACAID induction, and second, the ACAID-inducing signal found in the blood of AC tumor recipients is associated with hematopoietic cells, not tumor cells.

**ACAID-Inducing Potential of Blood-Borne Tumor Cells**

Because P815 cells are highly mobile and metastatic when injected in vivo, we suspected that the cellular fraction of blood used in the previous experiments contained viable P815 cells. If tumor cells were present in the cellular fraction, then the tumor cells themselves might function as ACAID-inducing signals. The following experiments were designed to evaluate this possibility.

Aliquots were removed from the cellular fraction of blood obtained from mice in which P815 cells had been injected into the AC 48 hours previously. When these aliquots were placed in tissue culture, P815 cells grew out within 3 to 4 weeks (data not shown), indicating that tumor cells were present in the blood 48 hours after AC injection of P815 cells. The presence of tumor cells in the cellular fraction of blood made it necessary to determine whether viable tumor cells were essential to the ACAID-inducing potential of the blood. To accomplish this, we turned to P511, a variant of P815 that is sensitive to HAT medium. When placed in HAT medium in vitro, P511 cells die within a short time. Thus, we reasoned that by incubating blood containing the putative ACAID-inducing signal in HAT medium, P511 cells should be eliminated. It was previously demonstrated that the growth pattern of P511 tumor cells in the AC of BALB/c eyes is similar to that of P815, and that P511 induces ACAID when injected into the AC of normal BALB/c mice. Therefore, panels of splenectomized BALB/c mice received AC injections of P511 tumor cells (2 × 10^6). Two days later, blood was obtained by cardiac puncture, the plasma was removed, and the cells were resuspended in HAT medium and cultured at 37°C for 24 hours. Thereafter, the cells were collected. One aliquot was placed in conventional tissue culture medium to determine if any P511 tumor cells survived HAT exposure. The remaining blood cells were injected intravenously into naive BALB/c mice. ACAID control mice received P511 cells into the AC, and positive control and experimental mice were immunized by subcutaneous injection of P511 cells (10^6). Ear challenge of these mice with irradiated P511 cells was conducted as described above.

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**ACAID-Inducing Potential of Particulate Antigen**

The minor histocompatibility antigens displayed by P815 cells to BALB/c recipients are cell associated but are not, strictly speaking, particulate. Therefore, similar experiments were conducted with killed, lyophilized, and ultrasonicated mycobacterial tubercu-
Nature of ACAID-Inducing Signals

FIGURE 2. Localization in blood of anterior chamber-associated immune deviation (ACAID)-inducing signal generated by injection of P511 cells into the anterior chamber of BALB/c mice. Two panels of mice splenectomized 7 days previously received $2 \times 10^5$ P511 cells into the anterior chamber of one eye. One panel was rested for 7 days; from the other panel, blood was removed 48 hours later by cardiac puncture and separated into cell and plasma fractions. The cell fraction was washed and cultured for 24 hours in hypoxanthine–amethopterin–thymine medium. Thereafter, the plasma fraction or the cultured cell fraction (one donor equivalent per recipient) was injected intravenously into naïve syngeneic mice. Recipients of blood obtained from normal mice served as positive controls. Five days later, recipients of blood fractions received subcutaneous injections of $2 \times 10^6$ P511 cells; another panel received no immunization. Ear swelling was assessed as described in Figure 1. Asterisk, mean swelling responses significantly less than positive controls ($P < 0.001$).

losa (MT) antigen. Several doses of MT antigen were injected into the AC of BALB/c mice: 10 and 50 μg (which induce intraocular inflammation when injected into the AC), and 200 and 500 ng (which are subinflammatory when injected into the AC). Seven days after AC injection of MT antigen, the mice were immunized subcutaneously with MT antigen (1 mg in 100 μl of mineral oil). The ears of these mice were challenged with an intrapininal injection of MT antigen (200 ng/10 μl) 1 week later.

Results of a representative experiment are displayed in Figure 3. Impaired DH was not observed in mice pretreated in the AC with 10 or 50 μg MT antigen (which induce intraocular inflammation when injected into the AC), and 200 and 500 ng (data not shown). In companion experiments, mouse erythrocytes to which BSA had been attached covalently were injected intracameraly, and the mice were immunized subsequently with BSA in complete Freund’s adjuvant (CFA). As with MT antigen, BALB/c mice pretreated with BSA-derivatized BALB/c erythrocytes mounted intense DH reactions when challenged with BSA (data not shown). These findings contrast sharply with repeated observations that soluble BSA injected intracameraly induces ACAID in BALB/c mice, and they strongly suggest that particulate antigens cannot induce ACAID.

Generation of Blood-Borne ACAID-Inducing Signal in Presensitized Recipients

Antigen injected into the AC has been found to suppress DH, even if the recipient has been presensitized to the antigen. Thus, BALB/c mice that have rejected subcutaneous P815 tumors and display DBA/2-specific DH are susceptible to ACAID induction by a subsequent AC injection of P815 cells. When the ears of these mice were tested 1 week after the AC injection of P815 cells, only insignificant ear swelling was observed. We wondered whether the blood of these mice contained an ACAID-inducing signal and, if so, whether the signal was plasma or cell associated. Accordingly, a large number of BALB/c mice were subjected to splenectomy. One week later, one panel of these mice was immunized by subcutaneous injection of $2 \times 10^5$ P815 cells; another panel received no immunization. Seven days later, both panels received an AC injection of P815 cells. After 48 hours, blood was removed by cardiac puncture and separated into cellular and plasma fractions. These fractions were then injected intravenously into naïve BALB/c mice. One week later, each recipient received a subcutaneous immunization with P815 cells ($10^6$). The ears of the mice were challenged 7 days later with irradiated P815 cells.

FIGURE 3. Induction of anterior chamber-associated immune deviation with particulate antigen. Mycobacterial tuberculosis (MT) antigen (ultrasonicated, lyophilized H37RA, 10 or 50 μg) was injected into the anterior chamber of one eye of normal BALB/c mice. Seven days later these mice, plus a panel of normal mice (positive controls), were immunized subcutaneously with MT antigen (1 mg) in mineral oil (100 μl). After 1 week, the ears of these mice (plus naïve mice, negative controls) were challenged with intrapininal injection of MT antigen (200 ng/10 μl). Ear swelling was assessed as described in the legend to Figure 1.
In preliminary experiments, the intensity of ear swelling displayed by the positive controls (P815 cells injected into the AC of naïve mice) was no greater than the intensity of responses observed when P815 cells were injected into the AC of mice that had received 1 week previously an intravenous injection of either plasma harvested from naïve mice 48 hours after AC injection of P815, or blood cell fractions harvested from previously primed mice 48 hours after AC injection of P815 cells (data not shown; Fig. 4). Mice that received blood cells from P815-immune recipients of AC P815 cells displayed intense DH, whereas mice that received plasma from the same donors displayed a reduced level of DH. As described above, recipients of blood cells from nonimmune donors with P815 cells in the AC also developed only weak DH responses. Recipients of plasma from the same nonimmune donors mounted intense DH responses.

These findings indicate that two different ACAID-inducing signals can be found in the blood of mice that receive AC injections of P815 tumor cells. If the mice have not been sensitized previously to the tumor alloantigens, the ACAID-inducing signal is associated with blood cells—presumably monocytes.8 However, if the mice have already been immunized to the alloantigens at the time tumor cells were injected into the AC, a soluble ACAID-inducing signal is generated that localizes to the plasma fraction of blood.

**Generation of Blood-Borne ACAID-Inducing Signal in SCID Mice**

Circumstantial evidence has been reported that suggests that the ACAID-inducing signal present in the blood after AC injection of hapten-derivatized spleen cells is of T-cell origin.15 We wished to know whether a similar origin might apply to ACAID-inducing signals generated by soluble protein antigens. We selected SCID mice for these studies because a genetic defect robs these mice of T cells, the putative source of ACAID-inducing signals of the soluble type.

Accordingly, BSA (50 μg) was injected into the AC of eyes of SCID mice that had been splenectomized 7 days previously. In control experiments, BSA was injected into the AC of normal BALB/c mice whose spleens had been extirpated 7 days previously. Blood was obtained from AC-injected mice 48 hours after AC injection and was injected intravenously into naïve BALB/c mice to determine whether an ACAID-inducing signal had been generated. As before, mice that received blood from AC-injected donors were immunized 7 days later with a subcutaneous injection of BSA in CFA and then were ear challenged with BSA 1 week thereafter.

The results are presented in Figure 5. No ACAID-inducing signal was detected in the plasma or among the blood cells harvested from nonsplenectomized SCID mice that received an AC injection of BSA 48 hours before. In contrast, blood cells, but not plasma, of AC-injected SCID mice contained an ACAID-inducing signal. These results indicate that SCID mice resemble normal BALB/c mice in that after an AC injection of soluble protein antigen, blood-borne cells, rather than plasma, carry an ACAID-inducing signal. Thus, SCID mice can generate blood-borne ACAID-inducing signals. Because these mice lack T lymphocytes, we conclude that T lymphocytes are not essential to the generation of ACAID-inducing signals after AC injection of soluble protein antigens.

**In Vitro Generation of ACAID-Inducing Signal With Particulate and Cell-Associated Antigens**

It has been reported that peritoneal exudate cells that are pulsed with antigen in vitro in the presence of TGFβ acquire ACAID-inducing properties.13 Although PEC suspensions are heterogeneous, containing macrophages, dendritic cells, and T and B lymphocytes, the ACAID-promoting cells are F4/80 positive.
FIGURE 5. Localization in blood of anterior chamber-associated immune deviation-inducing signal generated by injection of bovine serum albumin (BSA) into the anterior chamber of SCID mice. One panel of SCID mice was subjected to splenectomy. Seven days later these mice, plus sham-operated mice, received BSA (50 μg) into the anterior chamber of one eye. Forty-eight hours later, blood was removed by cardiac puncture and separated into cell and plasma fractions. These fractions were injected (one donor equivalent per recipient) intravenously into naive BALB/c mice. One week later these mice were immunized subcutaneously with BSA (200 μg) in complete Freund’s adjuvant. After 1 week the ears of these mice, plus naive BALB/c mice, were challenged with BSA (200 μg/10 μl). Ear swelling was assessed as described in the legend to Figure 1. Asterisk, ear swellings significantly greater than naive controls (P < 0.01).

positive and do not include T cells. In previously published experiments,7,16 soluble antigens (BSA, ovalbumin, interphotoreceptor retinoid binding protein) were used to generate ACAID-inducing signals in vitro. To explore the possibility suggested by Ferguson and Herndon11 that soluble versus cell-associated ACAID-inducing signals might be related to the solubility of antigen, we performed the following experiments.

In the first, spleen and lymph node cells were harvested from B10.D2 mice; tissues from these mice display numerous minor histocompatibility antigens that can be recognized by immune cells of BALB/c mice. BALB/c PECs were mixed with B10.D2 lymphoid cells and incubated overnight in the presence of supernatants of cultured iris and ciliary body cells. The next day, the nonadherent cells (including B10.D2 lymphoid cells) were washed away, and the adherent cells were harvested and injected intravenously (2 × 10⁴ per recipient) into naive BALB/c mice. One week later these mice received an immunizing dose of B10.D2 spleen cells (10 × 10⁶) subcutaneously. These animals were ear challenged 1 week later with irradiated B10.D2 spleen cells (10⁶/20 μl).

In the second, BALB/c PECs were placed in culture with a sonicated mycobacterial antigen preparation (10 μg/ml) in the presence or absence of TGFβ. After overnight incubation the PECs were washed and injected intravenously (2 × 10⁴) into naive syngeneic mice. One week later these recipients were immunized with a subcutaneous injection of CFA. These animals were ear challenged with MT antigen (250 ng/2 μl) 7 days later.

The results of representative experiments are presented in Figure 6. Mice that received B10.D2 PECs treated with TGFβ failed to develop DH when immunized subsequently with B10.D2 cells. Similarly, recipients of mycobacterial antigen–pulsed PEC developed only feeble DH responses when immunized subsequently with CFA. These findings reveal that a cell-associated ACAID-inducing signal, similar to that found in the blood after AC injection of antigen into unprimed mice, is created in vitro when PEC are incubated with particulate or cell-bound antigens in the presence of TGFβ. Thus, in the presence of TGFβ, PECs pulsed with soluble, particulate, and cell-bound antigens all give rise to ACAID-inducing signals. Because PECs are derived exclusively from adherent cells, T cells are not present; therefore, T cells appear to be irrelevant to the outcome.

DISCUSSION

It is remarkable that AC injection of antigenic material induces ACAID, a unique, systemic form of immune deviation. Animals with ACAID fail to mount DH responses to the relevant antigen, even after specific attempts to immunize them with immunogenic adjuvants. It is even more remarkable that within 48 hours of AC injection of antigen, the blood contains an antigen-specific ACAID-inducing signal, and that this signal is not native antigen itself.

Two laboratories have produced contradictory evidence concerning the nature of the blood-borne ACAID-inducing signal. Our laboratory, working primarily with soluble protein antigens, has found the signal to be associated with leukocytes bearing the F4/80 marker (i.e., monocytes).8 Ferguson et al,10 working primarily with hapten-derivatized syngeneic spleen cells, have found the signal to be soluble and present within the plasma fraction of blood. The latter investigators have interpreted these data to mean that soluble antigens give rise to cell-associated ACAID-inducing signals,11 whereas particulate antigens give rise to soluble signals.

The findings in this report suggest a different interpretation. We found that soluble ACAID-inducing signals were detected in the blood of splenectomized
mice that were presensitized at the time allogeneic tumor cells or BSA was injected into the AC. In contrast—and in confirmation of our previous findings with soluble protein antigens injected into the AC of naive (unsensitized) mice— injection of alloantigen-bearing tumor cells into the AC of naive mice generated cell-associated ACAID-inducing signals in the blood. Therefore, we propose that the recipient’s state of prior sensitization is the critical factor that dictates whether the ACAID-inducing signal is soluble (occurs in presensitized recipients) or cell associated (occurs in naive recipients).

Corroboration for this proposal has been forthcoming from experiments conducted in association with Cone and collaborators, in which an in vitro assay was used to detect soluble, antigen-specific factors in the serum of animals after AC injection of the soluble protein antigens BSA and ovalbumin. This assay detects T-cell–associated binding molecules, which are believed to be derived from T cells and to possess antigen specificity. After the injection of TNP-modified spleen cells, BSA, or ovalbumin into the AC of naive BALB/c mice, Cone et al. reported that no T-cell–associated binding molecules were detected in the serum. In contrast, T-cell–associated binding molecules were readily detected in the sera of mice that received an AC injection of TNP-modified spleen cells or ovalbumin after they had been previously sensitized with these antigens in immunogenic form. Thus, soluble factors of the T-cell–associated binding molecule type appear to be produced by re-exposure to antigen in the AC in sensitized mice, but not by initial exposure to antigen in the AC of naive mice.

Griffith et al. have proposed that T cells that enter, or are injected into, the eye are exposed to constitutive expression of Fas ligand on parenchymal cells. They have demonstrated that Fas+ T cells placed in the eye are induced to undergo apoptosis, and they further propose that Fas ligand–triggered cells may release soluble factors related molecularly to the T-
cell receptor for antigen. These putative factors may be similar to the plasma-borne factor Ferguson et al. have described in the blood of mice that received an AC injection of TNP-modified spleen cells.

Direct evidence makes it unlikely that the solubility of the inducing antigen can dictate the type of ACAID-inducing signal. We were unable to induce ACAID by AC injection of truly particulate, nonviable antigens (sonicated MT antigen and BSA-derivatized mouse erythrocytes). We do not have a ready explanation for this failure, but it may relate to the extraordinary ability of the parenchymal cells of the trabecular meshwork to phagocytize particulate material placed in the AC. Although P815 tumor cells or TNP-derivatized spleen cells may be described as particulate, in fact they are viable entities that can secrete or release soluble molecules into their environment, some of which may be related to antigen. Although we could not induce ACAID by direct AC injection of particulate antigen, we generated an ACAID-inducing signal in vitro by incubating P815 cells with mycobacterium in the presence of TGFβ. When cells treated in this manner were injected intravenously into naive recipients, they induced mycobacterial antigen-specific ACAID. Indirectly, this result confirms that particulate antigens do not necessarily give rise to "soluble" ACAID-inducing signals, for in this case the signal was a leukocyte from the peritoneal cavity.

Our ability to identify an ACAID-inducing signal in the blood of SCID mice 48 hours after an AC injection of soluble antigen indicates that T cells are not necessary for the generation of all ACAID-inducing signals. We anticipated this finding because we had previously reported that the ACAID-inducing signal in the blood of normal mice that received an AC injection of soluble protein antigens is a bone marrow-derived cell that lacks markers for either T or B cells. Instead, the relevant ACAID-inducing cell obtained from the blood of AC-injected, unsensitized mice was demonstrated to be a monocyte of the macrophage/dendritic cell lineage bearing the surface marker identified by monoclonal antibody F4/80. Current evidence indicates that the F4/80+ cells that carry ACAID-inducing signals in the blood migrate preferentially to the spleen and that they function as the proximate antigen-presenting cells for the novel regulatory T cells that are activated in the spleens of naive mice destined to acquire ACAID.

Once we had confirmed that a soluble ACAID-inducing signal existed in the blood after AC injection of antigen into presensitized mice, we were perplexed to find that the blood of these mice did not contain a cell-associated ACAID-inducing signal. We cannot explain why a cell-associated ACAID-inducing signal is missing from the blood of sensitized animals. One possibility is that the antigen-specific soluble factors produced by presensitized mice may alter the migratory properties of eye-derived cells that carry ACAID signals to the spleen. Another possibility is that splenectomy of donor mice before initial sensitization (a necessary condition of our experiments) may have the unexpected effect of preventing the generation of cells capable of carrying ACAID signals from the eye to the spleen.

In summary, we propose that two types of ACAID-inducing signals can be found in the blood of recipients of AC injections of antigen. One type is cell associated and correlates with F4/80+ leukocytes that migrate to the spleen as the initial antigen-presenting cells of ACAID in immunologically naive mice. The other type is soluble and may be found among plasma proteins of mice already immunized to the AC-injected antigen. We are confident that bone marrow-derived cells within the eye give rise to the cell-associated ACAID-inducing signal, and there is good reason to believe that the soluble ACAID-inducing signal is produced by antigen-specific T cells activated by prior immunologic experience.

Key Words
ACAID-inducing signals, anterior chamber-associated immune deviation, cell-associated antigens, delayed hypersensitivity, soluble antigens

References
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