Anterior Chamber-Associated Immune Deviation
Induced by Soluble Antigens

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Immune responses to cellular antigens placed in the anterior chamber of the eye are deviant: antibodies and cytotoxic T cells are generated, but delayed hypersensitivity is impaired. To determine whether a similar pattern of unusual reactivity would be induced by soluble antigens placed in this privileged site, we have examined the systemic immune responses of mice to anterior chamber injections of bovine serum albumin and bovine retinal S antigen—both soluble molecules. Recipients of intraocular injections of these antigens without adjuvant developed no detectable systemic immune response. When BSA was mixed with complete or incomplete Freund’s adjuvant and injected into the anterior chamber, recipients produced serum specific antibodies; however, they displayed impaired delayed hypersensitivity. Anterior chamber recipients of soluble antigens subsequently proved refractory to the development of delayed hypersensitivity when immunogenic doses of the same antigens were placed subcutaneously. Moreover, the inability to mount delayed hypersensitivity could be adoptively transferred with spleen cells from animals that had previously received intraocular injections of bovine albumin or S antigen. It is concluded that soluble antigens, as well as surface membrane-bound antigens, are capable of inducing anterior chamber-associated immune deviation (ACAID). The possibility is discussed that the capacity of soluble retinal S antigen to induce ACAID may be pertinent to the maintenance of self-tolerance to this autologous, intraocular molecule. Invest Ophthalmol Vis Sci 30:1112-1119, 1989

Systemic immune response to intraocular antigens has been a subject that this laboratory has studied for the past decade. Results of our experiments, as well as those of others, have indicated that antigenic materials injected into the anterior chamber (AC) of the eye elicit a deviant form of immunity which has been termed anterior chamber-associated immune deviation (ACAID). Intraocular injections of allogeneic tumor cells, immunogenic tumors bearing tumorspecific antigens, hapten-derivatized syngeneic spleen cells and herpes simplex virus type 1 produce a unique spectrum of immune effectors, including specific antibodies, and selective suppression of delayed hypersensitivity (DH). Because some histoincompatible tumor cells placed in the anterior chamber of mouse eyes enjoy a privileged existence (they grow progressively without evidence of host rejection) and simultaneously induce ACAID, the idea has been advanced that immune privilege results from ACAID, which selectively interferes with cell-mediated immunity, and thereby protects the tumor from rejection.

Although a wide variety of intracamerally injected antigens have now been documented to induce ACAID, they all share the property of being cell surface molecules or of being covalently bound to cell surface molecules. Therefore, it has not been demonstrated that ACAID can be induced by a soluble antigen. This is not an idle consideration. At least two soluble molecules obtained from the retina—retinal S antigen (S Ag), and the interphotoreceptor retinoid binding protein (IRBP)—have been used to induce experimental autoimmune uveitis in laboratory animals. Moreover, it has been reported that antibodies to retinal S antigen are present in the sera of patients with certain types of presumed autoimmune uveitis in man. Since experimental evidence strongly suggests that the T cells that mediate DH are the proximate effectors of laboratory-induced autoimmune uveitis, it is important to determine whether soluble antigens placed intracamerally are capable of inducing ACAID, and therefore of down-regulating DH reactions. Successful induction of ACAID by soluble antigens would raise the possibility that therapeutic strategies might be developed that could interfere with the induction of autoimmune diseases that are directed at soluble molecules. The experiments that
form the basis of this communication test the hypothesis that soluble antigens can induce ACAID.

Materials and Methods

Animals

BALB/c female mice (8-10-week-old) (Taconic Farms, Germantown, NY, The Jackson Laboratory, Bar Harbor, ME, and our breeding colony) were used in these experiments. All experimental procedures were performed under sodium pentobarbital anesthesia (0.6 mg/10 g, Barber Veterinary Supply Co., Richmond, VA). Animals were treated according to the ARVO Resolution on the Use of Animals in Research.

Soluble Antigens

Bovine serum albumin (BSA) and retinal soluble antigen (S Ag) were used in these experiments. BSA was purchased from Sigma Chemical Company (St. Louis, MO). S Ag was prepared from bovine retinas by a modification of the methods of Wacker et al. Both antigens were injected intracamerally (IC) or subcutaneously (SC), alone or incorporated (1:1) into complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA) (Difco Laboratories, Detroit, MI).

Antigen Inoculations

Anterior chamber (AC) injections were carried out as described previously. The AC of the right eye of each mouse received either 50–100 μg of BSA or S Ag with or without CFA, contained in 5 μl. Subcutaneous injections of the same doses of either antigen were given in equal volume at the base of tail.

Measurement of Delayed Hypersensitivity

For assay of DH, ear thickness before injection was measured by an engineer's micrometer (Mitutoyo, Tokyo, Japan). The DH response was assayed by injecting 10 μl containing either 200 μg of BSA or 5 μg of S Ag in physiological salt solution (PSS) into the ventral aspect of the right ear using a 100 μl syringe (Hamilton, Reno, NE) and a 30 gauge needle. The other ear received 10 μl of PSS as a negative control. Both ears were measured immediately prior to injection and at 24 hr and 48 hr later. The maximum difference between experimental and control ears (peak ear swelling response) of each animal in a panel was used as the measure of specific reactivity. From these individual values, a mean value ± 2 standard errors of the mean was calculated. Statistical significance of difference was determined with a student t-test.

Assay for Anti-BSA Antibodies

Blood samples were removed from the retro-orbital plexus just before ear challenge for DH, and the serum was separated and tested for antibodies by passive hemagglutination, using the chromic chloride method for sensitization of sheep red blood cells with BSA.

Adoptive Transfer of ACAID

Spleens from donor mice were collected aseptically 7 days after intracameral injection of antigen in CFA, and mononuclear suspensions were prepared by pressing whole spleens through nylon mesh (Nitex, 250 μm, TETKO, Inc., Elmsford, NY). Cells were washed twice in Hank's balanced salt solution (HBSS) and resuspended in HBSS. Each mouse received 10^8 spleen cells into the tail vein, and 24 hr later all spleen cell recipients received a footpad injection of 100 μg BSA in CFA. DH reactivity was measured 7 days later as described above.

Results

Bovine serum albumin is a molecule that has been used classically by immunologists to examine both cell mediated and antibody mediated immune responses. When incorporated into complete Freund's adjuvant and injected SC or into the footpad of adult BALB/c mice, BSA induces vigorous DH as well as circulating anti-BSA antibodies. In our hands, 10 μg BSA in CFA evoked DH in some, but not all, BALB/c mice. However, at a dose of 50–100 μg BSA in CFA injected into the hind footpads, all BALB/c mice developed vigorous antibody as well as cell-mediated immune responses (data not shown). Thus, for the intracameral injection experiments to be described, the 50–100 μg dose range of BSA was chosen as being optimally immunogenic.

Immune Response to Intracameral Injection of BSA

Injections of 100 μg BSA/5 μl in PSS were placed into the anterior chamber of one eye of adult female BALB/c mice. Groups of five animals each were tested 7 days later for evidence of BSA specific DH by challenging their ears with 200 μg BSA in PSS and measuring the ear swelling responses 24 and 48 hr later. Serum was also removed and tested for anti-BSA antibodies. Control animals received 100 μg BSA in PSS SC. Positive control mice received 100 μg BSA in CFA in their hind footpads. The results are presented in Figure 1. Animals receiving IC BSA (Group A) developed no evidence of BSA-specific DH (21% of positive control, Group C) or circulating anti-BSA antibodies. Similarly, recipients of 100 μg
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Fig. 1. Five BALB/c mice received into the anterior chamber of one eye injections of 100 μg BSA alone (A). Control animals (3) received 100 μg BSA alone subcutaneously (B). Positive control (5) mice received 100 μg BSA in complete Freud's adjuvant subcutaneously (C). The ears of these animals were challenged 7 days later with 200 μg BSA. Ear swelling responses (hatched bars) were measured at 24 and 48 hr with a low pressure engineer's micrometer. Serum was also removed and tested for anti-BSA antibody activity (open bars) in a hemagglutination assay using BSA-derivatized sheep erythrocytes as indicators. Mean values ± 1 standard error of the mean (SEM) for peak ear swelling responses are presented. Values significantly different from positive control (P < 0.01) are indicated by an *.

BSA in PSS SC (Group B) developed no detectable DH responses nor anti-BSA antibodies when tested on day 7 post-inoculation. As expected, mice immunized with 100 μg BSA in CFA injected into the hind footpads developed vigorous DH responses and their serum contained high titer anti-BSA antibodies. Additional panels of mice that received IC injections of 100 ng BSA were ear-challenged with BSA 14 or 21 days later. None of these animals developed DH, nor did their serum contain detectable amounts of specific antibody. Thus, both IC and SC injections of soluble BSA in this dose range seem to have no effect upon immune responsiveness as measured by these assays. The next experiments examined the possibility that down-regulation of immunity to BSA might have occurred following IC and/or SC injection of antigen.

Effect of Intracameral BSA Injections on Response to Subsequent Immunization with BSA Injected Subcutaneously

A hallmark of ACAID is that IC injection of antigen results in the induction of suppression of DH. One approach to determining whether an IC injection of BSA can induce suppression is to pretreat mice with soluble BSA injections into the AC, followed within several days by an immunogenic regimen of BSA in CFA injected SC. Accordingly, panels of adult BALB/c mice received IC injections of 100 μg BSA/μl on day 0. Control mice received 100 μg BSA in PSS SC. Seven days later all mice, including a panel of positive control mice that had received no pretreatment, received 100 μg BSA in CFA in the hind footpads. The ears of these animals were challenged with BSA 7 days later. As the results presented in Figure 2 reveal, mice that received pretreatments with BSA in the AC (Panel A) were able to mount only feeble DH responses (30% of positive control, Group C). By contrast, animals pretreated with similar doses of BSA SC (Group B) mounted vigorous BSA-specific DH responses, equivalent to those of the positive controls. Sera from all groups of mice displayed anti-BSA antibody activity of comparable magnitude. Thus, pretreatment of mice with soluble antigen injected into the AC appears to interfere with the development subsequently of DH when these mice receive immunizing doses of the same antigen. However, the capacity of AC-pretreated animals to produce antibodies to the intraocular antigen was unimpaired. This result represents circumstantial evidence in favor of the hypothesis that soluble antigens such as BSA can induce ACAID when injected IC. Importantly, mice pretreated with BSA SC were not impaired in their capacity to develop DH when subsequently immunized with BSA in CFA SC. Thus, ACAID uniquely follows AC injections of soluble antigens.

Immune Response to Intracameral Injection of BSA in CFA

Soluble molecules of the size of BSA would be expected to be washed quickly out of the AC after being injected there. Previous experiments with allogeneic tumor cells indicated that optimal induction of
ACAID required that antigen be released chronically over 3–4 days, as though the eye was acting as an antigen depot. In the next experiments, BSA was incorporated into CFA and injected IC (50 μg/5 μl CFA containing killed M. tuberculosis at a concentration of 20 mg/ml) in an effort to create a depot of antigen. Seven days later the sera of these mice were tested for anti-BSA activity and their ears were challenged for BSA-specific DH. The results are presented in Figure 3. Although recipients of BSA in CFA injected SC (Group B) responded with both circulating anti-BSA antibodies and readily detectable DH, the animals that received BSA in CFA IC (Group A) displayed lesser amounts of circulating anti-BSA antibodies (titer was 2^2 as opposed to 2^3 for Group B). Moreover, they failed to develop DH responses to challenge with BSA (6% of responses of positive control, Group B). Thus, although soluble BSA injected into the anterior chamber of the eye fails to induce DH or antibody responses, incorporating the BSA in adjuvant transforms the immunogenic stimulus into one that elicits circulating antibodies. However, these animals remain DH-unresponsive.

In the next experiment, mice received IC injections of either BSA in CFA, or BSA alone. Seven days later they received immunogenic BSA in CFA by the footpad route. They were then ear-challenged 7 days later with BSA and their sera were tested for anti-BSA activity (see Fig. 4). It should first be noted that the sera of all animals contained easily detectable levels of anti-BSA antibodies. However, the animals that were pretreated with BSA IC (Groups A and B) developed DH responses that were significantly lower than both the positive control and the SC-injected control animals. Moreover, the impairment of DH expression in the mice that were pretreated IC with BSA in CFA (Group B) seemed slightly greater than that displayed by the IC recipients of BSA alone. We interpret this result to mean that soluble molecules are capable of inducing ACAID (induction of antibody formation and impairment of cell-mediated immunity). In the case of BSA, this property appears to be promoted if the antigen is mixed with adjuvant.

Adoptive Transfer of BSA-Specific ACAID

The most stringent criterion for the presence of ACAID is the capacity to transfer the suppression to an immunologically naive recipient. To satisfy this criterion, panels of BALB/c mice received IC injections of BSA alone, or admixed with IFA into the AC. Seven days later they were immunized subcutaneously with BSA in CFA. One week later their ears were challenged with BSA. The results displayed in Figure 5 reveal that BSA mixed with IFA leads to the induction of ACAID. Thus, it would appear that mycobacteria are not relevant to the capacity of adjuvant to promote ACAID induction to soluble antigens.
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Fig. 5. Five BALB/c mice received anterior chamber injections of 100 μg BSA in incomplete Freund's adjuvant (A). Control animals (4) received 100 μg BSA in IFA SC (B). Seven days later these panels, plus a positive control (4) (C) received 100 ng BSA in CFA into the hind footpads. DH was assayed 7 days later. The data are presented and analyzed statistically in a manner similar to that displayed in Figure 1.

Fig. 6. Panels of BALB/c mice received anterior chamber injections of 50 μg BSA in CFA. Control mice received similar injections SC. Seven days later these animals served as donors of spleen cell suspensions which were then injected (100 × 10⁶) intravenously (IV) into syngeneic BALB/c recipients. One day later these recipients were immunized via footpad with 100 μg BSA in CFA. When their DH responses were assayed 7 days later (see Fig. 6), the peak ear swelling responses of the recipients of lymphoid cells from anterior chamber injected donors (5) (A), from subcutaneously injected donors (4) (B) and from donors with no pretreatment (3) (C) were measured 48 hr later. The ear swelling was also measured in animals that received only footpad injections of BSA in CFA (3) (D) and negative control mice (E). Data are presented as described in Figure 1. Panel A is significantly smaller than Panels B, C and D (P < 0.05).

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Fig. 7. Panels of four BALB/c mice each received into the anterior chamber of one eye injections of 50 μg S Ag alone (A), or 50 μg S Ag in CFA (B). Positive control mice (3) received 50 μg S Ag in CFA subcutaneously (C). Seven days later the ears of these animals were challenged with 5 μg S Ag in the pinna. A negative control panel (D) received only intrapinna injections of S Ag. Peak ear swelling responses were measured 24 and 48 hr later. Data are presented as described in Figure 1.

Immune Response to Retinal S Antigen in Mice and Effect of Intracameral Injection

Retinal S antigen prepared from bovine retinas is a highly immunogenic molecule. It has been used to induce experimental autoimmune uveitis in guinea pigs, rats and monkeys.14-16 The T cells responsible for mediating these autoimmune reactions are of the T_{DH} variety. It is of importance to determine whether ACAID can be induced by S Ag. In order to examine this possibility, it was first necessary to know whether mice can be immunized with this bovine ocular molecule. BALB/c mice received IC injections of 50 ng S Ag alone, or mixed with CFA. Control mice received 50 ng S Ag in CFA injected SC. Seven days later the ears of these mice were challenged with S Ag; they developed vigorous ear swelling responses, indicative of DH (data not shown). This result permitted us to extend our study of ACAID to S Ag. Adult BALB/c mice received IC injections of 50 μg S Ag alone, or mixed with CFA. Control mice received 50 μg S Ag in CFA injected SC. Seven days later the ears of these mice were challenged with S Ag (see Fig. 7). Neither set of mice receiving IC injections of S Ag (Groups A and B)
mounted DH responses, whereas positive control mice (Group C) developed vigorous DH. In order to determine whether initial presentation of antigen via AC inoculation altered the subsequent capacity of mice to respond to immunogenic S Ag, panels of BALB/c mice received 50 μg S Ag alone, or mixed with CFA/10 μl IC on day 0. Four days later, each mouse received 50 μg S Ag in CFA into the hind footpads. The ears of these mice were challenged with S Ag. As the results displayed in Figure 8 reveal, pretreatment of mice with IC S Ag (Groups A and B) prevents these mice from developing vigorous DH when they receive an immunogenic dose of S Ag.

The capacity to demonstrate ACAID induction by two different soluble antigens, BSA and S Ag, made it possible for us to determine whether ACAID was antigen-specific. A panel of BALB/c mice received 50 μg S Ag intracamerally. Seven days later these mice received subcutaneous immunizations with BSA in CFA. When their ears were challenged with BSA 7 days later, they mounted vigorous DH responses (data not shown). This result indicates that the suppression of DH elicited by S Ag is specific and does not lead to suppression of reactivity to an unrelated antigen, BSA.

To determine whether the impaired expression of DH to S Ag was associated with induction of active suppression, an adoptive transfer experiment was performed. A panel of BALB/c mice received an intracameral injection of 50 μg S Ag in CFA. A control panel received a similar inoculum injected subcutaneously. Seven days later, the spleens from these animals were harvested, rendered into single cell suspensions, and injected (100 x 10^6 recipient) IV into syngeneic recipients. Within 2 hr, these recipients were each given an immunogenic dose of S Ag (50 μg S Ag in CFA injected into the hind footpads). Seven days later the ears of these animals were challenged with S Ag and the ear swelling responses measured 24 and 48 hr later. The results, displayed in Figure 9, indicate that recipients of spleen cells from AC-injected donors (Panel A) mounted feeble DH responses (45 swelling units) when compared to recipients of spleen cells from SC-injected donors (Panel B, 85 swelling units). The responses of the latter were indistinguishable from positive control mice (Panel C), and thus these results indicate that the spleens of animals that received S Ag intracameraly contain suppressor cells that are revealed in adoptive transfer. Since the immune response to IC injected S Ag resembles that evoked by IC injected BSA, we conclude that induction of ACAID by soluble proteins extends to ocular molecules that are known to be able to produce autoimmune eye disease under appropriate experimental circumstances.

Discussion

The inoculation of foreign antigens into the anterior chamber of the eye induces an unusual spectrum of immune effector responses termed anterior chamber-associated immune deviation. Until the current studies, all antigens used to induce ACAID in mice have been insoluble: cell surface alloantigens, hapten-derivatized cell surface molecules, or virus-encoded surface antigens. With the current results, soluble antigens, such as BSA and S Ag, can now be added to the list. Both of these soluble proteins, when
injected IC, produced no apparent effect upon the host's immune system—recipient mice displayed no antigen-specific DH. However, the absence of an effect was more apparent than real; when recipients of IC-injected soluble antigens were subsequently challenged with the same antigens in immunogenic form (mixed with CFA and injected into the footpad), they still failed to display DH, indicating that the pretreatment had rendered them unresponsive. However, IC-treated animals did make circulating anti-BSA antibodies, a feature that is typical of animals with ACAID. More importantly, the unresponsive state induced by IC injection of soluble antigen was transferred adaptively to naive recipients with spleen cells, indicating that a typical ACAID-like suppressor cell population was present.

It is of interest that not only antigen in soluble form, but antigen admixed with CFA, injected into the AC induced ACAID. We had originally expected that the inclusion of CFA in the intraocular inoculum might induce a local inflammatory response that could drastically alter the microanatomy and physiology of the anterior segment of the eye, and therefore mitigate against ACAID induction. However, although a brisk inflammatory response was observed in these eyes, the recipients nonetheless developed ACAID. We infer from this result that the capacity of adjuvant to form micelles with soluble antigen and thereby act as an antigen depot, releasing small amounts continually over time, may be an important factor. Earlier studies on ACAID induction by alloantigenic tumor cells had revealed that enucleation of the inoculum-containing eye within 4 days of inoculation prevented ACAID, suggesting that the intact eye was required during this period of time, as though the eye was acting as an antigen depot. It is relevant that soluble antigen mixed with incomplete Freund's adjuvant also induced ACAID. This further emphasizes the depot property of adjuvant, rather than its mycobacterial component, as important in ACAID induction.

The fact that pretreatment of mice with soluble antigen injected SC did not induce ACAID suggests that solubility of antigen alone is not the critical feature. This result focuses attention on the route of antigen administration as being important. The contents of the anterior chamber drain from the eye through the trabecular meshwork directly into a system of collecting veins and therefore directly into the blood. The simplest explanation for the ability of IC-injected soluble antigen to induce ACAID is that an AC inoculation is equivalent to an IV one. However, it has been previously shown that the ability of allogeneic tumor cells to induce ACAID after IC injection is lost when these tumor cells are injected IV. Thus, we believe that the fact that an IC injection of antigen leads eventually to the intravenous dissemination of the antigen is insufficient to account for the spectrum of unusual effects on the immune system. At present, we are considering two alternative explanations. In the first, we postulate that antigen injected into the anterior chamber is processed or modified locally such that an altered form of antigen is produced—one that is particularly efficient at activating suppressor cells. In the second, we propose that recognition of antigen by circulating immunocompetent lymphocytes takes place within the anterior chamber before antigen has had time to escape systemically, and that the subsequent functional properties of lymphocytes that are activated in this unique environment are altered such that the cells activate suppressor networks. This idea has recently been advanced by Ferguson et al in the TNP ACAID system. Both of these possibilities invite experimental verification. In fact, we have recently obtained evidence that aqueous humor contains a potent antiproliferative activity that is revealed when aqueous humor is added to culture fluid in a mixed lymphocyte response in vitro. Since it has been shown that aqueous humor is not cytotoxic to lymphocytes (personal communication, C. Kaiser), the potential effects of these growth-inhibited lymphocytes on systemic immune responses will be important to describe.

The physiologic relevance of ACAID remains conjectural. In the context that immune privilege in the anterior chamber of the eye is extended to solid tissue allografts and tumor cells, suppressed DH to the alloantigens on these grafts appears to be a critical factor in promoting graft survival. However, the physiologic relevance of ACAID with respect to soluble antigens is less obvious. Several soluble autologous molecules are uniquely present within the eye and have been used to induce autoimmune ocular disease in experimental animals: crystalline lens proteins, retinal S Ag, IRBP. The fact that autoimmunity can be induced in experimental animals indicates that lymphocytes capable of recognizing these antigens must exist in adult animals. Some have suggested that the reason normal animals do not develop autoimmunity is that unique intraocular antigens normally remain sequestered within the eye, hidden from the immune system. In the case of S Ag, evidence suggests that this molecule is neither present in the blood, nor in the aqueous humor. However, trauma to the eye, or inflammation of the uveal tract may release S Ag from its retinal sanctuary. This would permit the possibility of access of this autoantigen to the immune system by conventional pathways, that is, lymphatic drainage to regional lymph node, where a destructive, autoimmune process is likely to be...
generated. It could be reasoned that during trauma/inflammation S antigen may be released into the aqueous humor, in which case the potential for inducing ACAID exists. Thus, suppression of DH would be favored and the probability of autoimmune retinitis reduced. In apparent confirmation of this idea, we have recently prevented S Ag-induced EAU in susceptible adult Lewis rats by pretreating them with intracameral injections of soluble S Ag.\textsuperscript{25}

**Abbreviations Used**

AC (Anterior chamber)
ACAID (Anterior chamber-associated immune deviation)
DH (Delayed hypersensitivity)
S Ag (Retinal soluble antigen)
IRBP (Interphotoreceptor retinoid binding protein)
BSA (Bovine serum albumin)
IC (Intracameraly)
SC (Subcutaneously)
CFA (Complete Freund's adjuvant)
IFA (Incomplete Freund's adjuvant)
PSS (Physiological salt solution)
HBSS (Hanks' balanced salt solution)
IV (Intravenously)

**Key words:** soluble antigens, ACAID, retinal S antigen, immune deviation, anterior chamber

**Acknowledgments**

The authors thank Drs. J. P. Williamson, B. Ksander, L. A. Smith and K. R. Aoki for useful discussion and Ms. M. Mammolenti for her excellent technical assistance.

**References**