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Using Tolerance Induced Via the Anterior Chamber of the Eye to Inhibit Th2-Dependent Pulmonary Pathology

Kazumoto Katagiri, Jie Zhang-Hoover, Jun Song Mo, Joan Stein-Streilein, and J. Wayne Streilein

Anterior chamber-associated immune deviation (ACAID), a manifestation of ocular immune privilege, prevents Th1-dependent delayed hypersensitivity from developing in response to eye-derived Ags, thereby preserving vision. Since Th2-type cells have recently been shown to mediate destructive inflammation of the cornea, we wondered whether pre-emptive induction of ACAID could inhibit Th2 responses. Using a murine model of OVA-specific, Th2-dependent pulmonary inflammation, we pretreated susceptible mice by injecting OVA alone into the anterior chamber, or by injecting OVA-pulsed, TGF-β2-treated peritoneal exudate cells i.v. These mice were then immunized with OVA plus alum strategy that generates Th2-mediated OVA-specific pulmonary pathology. When pretreated mice were challenged intratracheally with OVA, their bronchoalveolar lavage fluids contained far fewer eosinophils and significantly less IL-4, IL-5, and IL-13 compared with that of positive, nonpretreated controls. Similarly, lung-draining lymph node cells of pretreated mice secreted significantly less IL-4, IL-5, and IL-13 when challenged in vitro with OVA. Moreover, sera from pretreated mice contained much lower titers of OVA-specific IgE Abs. We conclude that Ags injected into the anterior chamber of the eye impair both Th1 and Th2 responses. These results reduce the likelihood that ACAID regulates Th1 responses via a Th2-like mechanism. Thus, immune privilege of the eye regulates inflammation secondary to both Th1- and Th2-type immune responses. The Journal of Immunology, 2002, 169: 84–89.

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4 Abbreviations used in this paper: ACAID, anterior chamber-associated immune deviation; AC, anterior chamber; BAL, bronchoalveolar lavage; KLH, keyhole limpet hemocyanin; LN, lymph node; PEC, peritoneal exudate cell; i.t., intratracheal.
Materials and Methods

Mice
BALB/c mice were bred in our animal facility or were purchased from Taconic Farms (Germantown, NY) and used at 8–12 wk of age. All animals were treated according to the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research. All protocols were preapproved by the Animal Care and Use Committee of the Scheippers Eye Research Institute in accordance with National Institutes of Health guidelines.

Administration of Ag by various routes and immunization regimens
For induction of experimental allergic lung disease, the procedure of sensitization and challenge was modified from the method of Kung et al. (16). Briefly, mice were immunized i.p. with 10 μg of OVA (Sigma-Aldrich, St. Louis, MO) mixed with 2.25 mg of aluminum hydroxide (Imject Alum; Pierce, South Iselin, NJ) (alum) in 100 μl of PBS. The animals received a booster i.p. injection of this alum-OVA mixture 7 days later. Five days after the second i.p. injection, mice were intubated and challenged intratracheally (i.t.) with 50 μg of OVA dissolved in 50 μl of PBS.

For intraocular injection, each mouse received a 3-μl inoculation of OVA (50 μg) dissolved in HBSS into the anterior chamber (AC) of the right eye 7 days before sensitization. In some experiments, 50 μg of keyhole limpet hemocyanin (KLH; Calbiochem, Darmstadt, Germany) dissolved in PBS was inoculated into the AC.

For conventional sensitization, mice received a s.c. injection of 100 μg of OVA emulsified in CFA (Life Technologies, Grand Island, NY).

Generation of ACAID-inducing APCs
Serum-free medium was used for generation of ACAID-inducing APCs. This medium was composed of RPMI 1640 medium, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (all from BioWhittaker, Walkersville, MD), and 1 × 10^−3 M 2-ME (Sigma-Aldrich) supplemented with 0.1% BSA (Sigma-Aldrich), ITS−culture supplement (1 μg/ml iron-free transferrin, 10 ng/ml linoleic acid, 0.3 ng/ml Na2Se, and 0.2 μg/ml Fe(NO3)3) (Collaborative Biomedical Products, Bedford, MA). Peritoneal exudate cells (PEC) were obtained from normal BALB/c mice that received 3 ml of thioglycollate (Sigma-Aldrich) i.p. 3 days earlier. As described previously, PEC were washed, resuspended, placed in 24-well culture plates (1 × 10^6/well), and treated with 5 ng/ml porcine TGF-β1 (R&D Systems, Minneapolis, MN) and 5 mg/ml OVA in serum-free medium at 37°C in an atmosphere of 5% CO2. After overnight culture, plates were washed three times with culture medium to remove TGF-β2, Ag, and nonadherent cells. Adherent cells were harvested by vigorous pipetting and administered to naive mice i.v. 7 days before subsequent sensitization.

Bronchoalveolar lavage (BAL)
Mice were euthanized by an i.p. injection of sodium pentobarbital 1 or 3 days after i.t. challenge with OVA. The trachea was dissected free from the underlying soft tissues and an 0.58-mm diameter tube was inserted through a small incision in the trachea. BAL was performed 10 times through the tracheal cannula with 1-ml aliquots of pyrogen-free PBS warmed to 37°C. BAL fluid harvested in the first 1 ml was centrifuged and the supernatant was collected and kept at −70°C until use for cytokine assay. A hemocytometer was used to count the total number of BAL cells under phase-contrast microscopy. For differential cell count, cytocentrifugation preparations were made and stained with Diff-Quik (Green Cross, Osaka, Japan). A total of 1000 cells was counted to calculate the differential populations of alveolar macrophages, neutrophils, eosinophils, lymphocytes, and monocytes that were identified by standard morphology.

Cytokine production of lung-draining lymph node (LN) cells
Lung-draining LN cells were harvested 1 or 3 days after i.t. challenge with OVA. The LN cells (2 × 10^7/well) were cultured for 120 h in the absence or presence of OVA (1 ng/ml) in 96-well plates, as described by Janssen et al. (21). The LN cells were also cultured in a plate coated with anti-CD3 Ab (2C11, 10 μg/ml in PBS). Cells were cultured in medium consisting of RPMI 1640 (BioWhittaker), 10% heat-inactivated FCS (HyClone, Logan, UT), 2 mM L-glutamine, 10 mM HEPES buffer, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 1 × 10^−3 M 2-ME (Sigma-Aldrich). Supernatants were harvested and stored at −70°C until use for cytokine assays.

Cytokine assays
Content of cytokines in BAL and culture supernatants was analyzed by quantitative capture ELISA, according to the manufacturer’s instructions for IL-4, IL-5, IFN-γ (BD Pharmingen, San Diego, CA), and IL-13 (R&D Systems). The detection limits of the ELISAs were 16 pg/ml for IL-4 and IL-5, 62.5 pg/ml for IL-13, and 290 pg/ml for IFN-γ. In some experiments, samples were diluted with PBS containing 2% BSA to an appropriate concentration.

Measurement of OVA-specific IgG1, IgG2a/2b, IgG3, and IgE in sera
Serum samples were taken 10 days after the first sensitization with alum-OVA mixture. The concentration of OVA-specific IgG1, IgG2a/2b, IgG3, or IgE in the serum was determined by sandwich ELISA using alkaline phosphatase-conjugated ExtrAvidin (1/10,000 dilution; Sigma-Aldrich) and substrate p-nitrophenyl phosphate (Sigma-Aldrich) for color development. To determine serum levels of OVA-specific IgE, anti-IgE mAb (R35-72; BD Pharmingen) was used as capture Ab and biotin-conjugated OVA was used as detecting reagent. Microtiter plates (96 well) were coated overnight at 4°C with 2 μg/ml anti-IgE mAb diluted in PBS. After blocking with 2% BSA in PBS, diluted serum samples were added to the plate and incubated overnight at 4°C. After washing, biotin-conjugated OVA (2 μg/ml) was added into each well and incubated for 1 h at room temperature before color development. For OVA-specific IgG1, IgG2a/2b, and IgG3, OVA (10 μg/ml in carbonate buffer, pH 9.4) was used as capture reagent and biotin-conjugated anti-IgG1 (A85-1), IgG2a/2b (R2-40), or IgG3 (R40-82) (2 μg/ml; BD Pharmingen) was used as detecting Ab. Sera were diluted 100 times for optimal detection of IgG1, IgG2a/2b, and IgG3 and 1/5 for detection of IgE.

Delayed hypersensitivity assay
Mice were challenged by intradermal injection of Ag (200 μg of OVA/10 μl of HBSS) into the ear pinnae 7 days after final exposure to OVA. Ear swelling was measured 24 and 48 h later with an engineer’s micrometer (Mitutoyo; MTI, Paramus, NJ). Mice sensitized with OVA emulsified with CFA 7 days before challenge were used as positive control.

Statistical analyses
In experiments with groups of mice, each group contained at least five animals, and all experiments (both in vitro and in vivo) were repeated at least twice with similar results. The results displayed in the figures are representative of three or more experiments; SEM were calculated from a single experiment. Data were subjected to analysis by Student’s t test as appropriate. A p < 0.05 was considered to be significantly different.

Results
Effects of OVA injected intracameral or of OVA-pulsed PEC pretreated with TGF-β2 on OVA-specific humoral immune responses of mice with Th2 immunity
The first experiments were designed to determine whether mice initially exposed to OVA via an intracameral injection were able to develop the typical Th2-dependent spectrum of Abs when immunized subsequently with OVA and alum. Panels of adult BALB/c mice received OVA (50 μg/3 μl) into the AC of one eye. Seven days later these mice received an i.p. injection of OVA (10 μg) in aluminum hydroxide (alum), followed 7 days later by a second i.p. injection of OVA plus alum. Three days later tail vein blood was collected, and sera were separated and assayed quantitatively by ELISA for levels of OVA-specific IgG1, IgG2a, IgG2b, IgG3, and IgE Abs. Positive control mice received two i.p. immunizations with OVA plus alum, but no intracameral injection of OVA. An additional panel of BALB/c mice received a s.c. immunization with OVA (50 μg) in CFA; their sera were collected 10 days later. The results of this experiment are presented in Fig. 1A. Mice that received only i.p. injections of OVA plus alum (positive controls) generated high titers of OVA-specific IgE and IgG1, but low levels of IgG2a, 2b, and 3 Abs. By contrast, mice that similarly received i.p. injections of OVA plus alum following an AC injection of OVA (ACAID group) had barely detectable levels of OVA-specific IgE, but otherwise their levels of serum OVA-specific Abs
were similar to the positive controls. As anticipated, mice immunized with OVA plus CFA produced high levels of IgG2a, IgG2b, and IgG3 Abs, but low levels of IgE and IgG1 (data not shown).

Similar experiments were performed in which a panel of BALB/c mice was pretreated with an i.v. injection of BALB/c PEC that were incubated overnight with TGF-β2 and OVA. As before, these mice were immunized subsequently with two i.p. injections of OVA plus alum. Positive controls received only two i.p. injections of OVA plus alum, while an OVA-CFA control panel received a single s.c. injection of OVA plus CFA. The sera obtained from these mice were assayed for OVA-specific Abs. As revealed in Fig. 1B, mice that first received OVA-pulsed PEC treated with TGF-β2 displayed low levels of OVA-specific IgE, but levels of IgG1 and IgG3 that were higher than the positive controls. Together these findings indicate that mice first exposed to OVA via the AC or via OVA-pulsed, TGF-β2-treated PEC displayed an impaired capacity to produce IgE Abs when subjected subse-

quently to an immunizing regimen that, in normal mice, generated high titer IgE responses. Thus, the humoral immune response of ACAID is distinctly different from a typical Th2-type humoral response, and ACAID suppresses IgE Abs to the Ag first encountered through the AC.

Effects of OVA injected intracamerally or of OVA-pulsed PEC pretreated with TGF-β2 on airway inflammation of mice with a Th2 OVA pulmonary response

As before, positive control panels of BALB/c mice received an immunizing regimen of OVA plus alum designed to elicit an experimental model of allergic asthma. Five days after the second i.p. injection of OVA plus alum, the mice were challenged i.t. with OVA (50 μg). Three days later, the mice were euthanized, their bronchoalveolar spaces lavaged, and the fluid analyzed for its content of cells and cytokines. Experimental panels were pretreated as before with either an AC injection of OVA or an i.v. injection of OVA-pulsed, TGF-β2-treated PEC. Negative control mice received only an i.t. challenge with OVA. The results of these experiments are summarized in Fig. 2. As anticipated, the BAL of positive control mice contained a significant increase in total cells, compared with negative controls. By contrast, the BAL obtained from mice pretreated with OVA in the AC contained no more cells than the BAL of negative controls. The increased cellular content of positive control BAL was ascribable to eosinophils, lymphocyte, neutrophils, and monocytes. Similar results were observed with BAL from mice pretreated with an i.v. injection of OVA-pulsed, TGF-β2-treated PEC (data not shown). The accumulation of eosinophils in BAL is a characteristic feature of airway inflammation in experimental allergic lung disease of this type. Therefore, the virtual absence of eosinophils in the BAL of mice pretreated with OVA in the AC or by OVA-pulsed, TGF-β2-treated PEC indicates that these pretreatments mitigated this aspect of Th2-mediated pathology.

To determine whether the changes in cell content of BAL of mice pretreated with OVA in the AC before i.p. immunization with OVA plus alum were Ag specific, panels of BALB/c mice received an AC injection of OVA or KLH. Seven days later, both panels

FIGURE 1. Production of Ag-specific serum Abs. Mice were sensitized i.p. with OVA and alum twice after injection of OVA into AC (ACAID, a) or i.v. transfer of OVA-pulsed, TGF-β2-treated PEC (tolerogenic PEC, b). Mice sensitized with OVA/alum twice without any pretreatment were used as positive control and naive mice were used as negative control. Serum was harvested 10 days after first sensitization for estimations of OVA-specific Abs by ELISA. Mean OD readings for each group of sera are presented ± SEM. OVA-specific IgE values of mice with ACAID and of mice that received OVA-pulsed, TGF-β2-treated PEC were less than those of positive control (*, p < 0.000001; ***, p < 0.0001, respectively). OVA-specific IgG1 and IgG3 values of mice that received tolerogenic PEC were greater than those of positive controls and the latter was less than that of mice sensitized with OVA and CFA (***, p < 0.05).

FIGURE 2. Analysis of cells found in BAL fluid after i.t. OVA challenge. Mice pretreated with OVA in the AC followed by i.p. immunization with OVA plus alum (ACAID), mice that were only immunized i.p. with OVA plus alum (Positive control), and naive mice (Negative control) received an i.t. challenge with OVA. Three days later, BAL fluid was obtained and analyzed for content and types of cells. Mean values of cell numbers are presented ± SEM. Asterisks indicate mean values significantly less than those of positive control (**, p < 0.0001; *, p < 0.001).
received i.p. immunizing regimens of OVA plus alum. Three days after i.t. challenge with OVA, BAL was collected and the cellular content was assessed. As the results presented in Fig. 3 reveal, only the BAL from mice pretreated with OVA in the AC lacked high numbers of eosinophils. By contrast, the number of total cells and eosinophils were comparable to positive controls in the BAL of mice pretreated in the AC with KLH, but immunized and challenged i.t. with OVA. Thus, the changes observed in pulmonary inflammation of ocularly pretreated mice were Ag dependent and specific.

To determine the content of relevant cytokines in airway inflammation of the Th2 type, BAL was harvested 1 day after i.t. challenge of experimental and positive control mice described above. These BAL fluids were analyzed by ELISA for quantitative levels of IL-4, IL-5, and IL-13. As the results in Fig. 4 reveal, the BAL fluids from positive control mice contained large amounts of all three cytokines, whereas the BAL fluids obtained from mice pretreated with OVA in the AC contained much lower levels of IL-4, IL-5, and IL-13. Similar results were obtained with BAL from mice pretreated with OVA-pulsed, TGF-β2-treated PEC (data not shown). Together, the fluids obtained from lavaging the bronchoalveolar spaces of mice pretreated with OVA in the AC or with OVA-pulsed, TGF-β2-treated PEC were deficient in the cells (eosinophils) and cytokines (IL-4, IL-5, and IL-13) that are believed to play a prominent role in the pathogenesis of Th2-mediated allergic asthma.

Effects of OVA injected intracamerally or of OVA-pulsed PEC pretreated with TGF-β2 on phenotype of OVA-specific lymphoid cells in lung-draining LN

In experimentally induced allergic lung disease in mice, the LN that drain the airways of the lung (hilar and mediastinal) are documented to contain T cells of the Th2 phenotype that are responsible for causing the disease (21, 22). Therefore, we postulated that intracameral OVA pretreatment of mice destined to develop experimental allergic lung disease would impact the phenotype of OVA-specific cells in the draining LN. As before, panels of BALB/c mice were immunized i.p. with two injections of OVA plus alum; some panels were pretreated with AC injection of OVA, while other panels were pretreated with i.v. injections of OVA-pulsed, TGF-β2-treated APCs. All mice were then challenged i.t. with OVA. After 1 or 3 days, their lung-draining LN were harvested and cultured in vitro with anti-CD3 Abs or with OVA, respectively, for 120 h. The supernatants were then harvested and assayed by ELISA for IL-4, IL-5, IL-13, and IFN-γ. The results are displayed in Figs. 5 and 6. When

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Discussion

The systemic unresponsiveness that is elicited by introducing Ag first through the AC of the eye was first reported by Kaplan et al. in 1975 (23). Since then, a wide array of chemically and biologically diverse antigenic materials placed in the AC have been reported to elicit ACAID (5, 24, 25). Because the first reports of ACAID arose from tissue transplantation experiments and because delayed hypersensitivity and Th1-type immune responses have come to be considered dominant effectors of graft rejection, virtually all studies of ACAID have emphasized suppression of Th1-type immunity as a cardinal feature of the phenomenon. Since Th1 responses were first described as the reciprocal of Th2 responses and since Th2 cells were shown to suppress Th1-type responses (26), various investigators consider ACAID to be merely a Th2 response to Ags introduced into the AC of the eye. Fragmentary support for this possibility was generated by the experiments of Kosiewicz et al. (9) and of Niederkorn and D’Orazio (8), although other results cast doubt on the notion that ACAID is similar to Th2 responses. First, Kosiewicz et al. (9) also reported that unless mice that receive an AC injection of OVA are subsequently immunized systemically with OVA plus CFA, no evidence of OVA-responding Th2 cells can be found in spleen and LN. Instead, the spleens of these mice contain cells that secrete only TGF-β when stimulated with OVA in vitro. Second, mice deficient in IL-4 (because the relevant gene has been disrupted) readily acquire ACAID when Ag is injected into the AC. Because these genetically manipulated mice are incapable of developing Th2 responses, it seems unlikely that the ACAID they acquire can be Th2 mediated.

The results of the studies reported here diminish the possibility that the regulation of ACAID and Th2 are identical, at least for the heterologous protein Ag, OVA. Mice pretreated with either an AC injection of OVA or an i.v. injection of OVA-pulsed, TGF-β2-treated APCs failed to generate OVA-specific IgE responses when immunized i.p. with OVA plus alum. Moreover, when these mice were challenged i.t. with OVA, their BAL fluids contained few if any traces of a Th2-dependent inflammatory response: sparse eosinophils and lymphocytes were present, and only trivial amounts of IL-4, IL-5, and IL-13 were detected. By contrast, the positive control mice immunized with OVA plus alum generated robust OVA-specific IgE responses, and their BAL following intracheal challenge with OVA was rich in cells, especially eosinophils, and in IL-4, IL-5, and IL-13.

The failure of mice pretreated with OVA in the AC or with the surrogate ACAID-inducing APCs generated in vitro to generate intrapulmonary Th2 responses to OVA challenge was also evident when lymphoid cells were evaluated in the lung-draining LN. Whether stimulated in vitro with anti-CD3 Abs or with OVA, draining LN cells of OVA-pretreated mice failed to secrete significant amounts of IL-5 or IL-13. RT-PCR analysis of LN cells and BAL cells showed that the genes for IL-5, IL-13, and IFN-γ were either silenced or greatly repressed by OVA-AC treatment. These findings lead us to conclude that pretreatment of mice with AC injection of OVA or with an i.v. injection of OVA-pulsed, TGF-β2-treated APCs suppressed OVA-specific Th1 and Th2 responses comparably.

Our finding that induction of ACAID inhibits Th2-dependent pathology extends our knowledge of the range of immune effector systems altered in ocular immune privilege. Suppression of Th2-dependent pathology joins suppression of delayed hypersensitivity and suppression of complement-dependent inflammation. Yet even with the addition of suppressed Th2 responses to the immune privilege repertoire, there are immune effector systems that remain intact in ACAID. Mice with ACAID produce large amounts of IgG1 Abs. In that regard, it was of interest to learn in our present studies that, even though IgG1 is usually included among the Abs promoted by Th2 cells, IgG1 production persisted at high levels in mice that were pretreated with OVA in the AC followed by an i.p. immunization with OVA plus alum. Only IgE production, another Ab thought to be facilitated by Th2 cells, was diminished in these mice. Previous reports have demonstrated that mice with ACAID induced by minor histocompatibility Ag-bearing tumor cells acquire large numbers of activated CD8+ cytotoxic T cell precursors in their secondary lymphoid organs (27). Thus, on the cell-mediated arm of immune responses, mice with ACAID can still mount
cytotoxic T cell responses and, on the humoral immune side, mice with ACAID can still mount robust IgG1 responses to eye-derived Ags (3). In these animals, promotion of CD8+ T cell and IgG1 responses at the expense of Th1, Th2, and complement-fixing Ab responses serves to emphasize that the systemic immune response to eye-derived Ags is “deviant,” ergo the term ACAID.

Mice with Th2-biased immune responses produce large amounts of noncomplement-fixing IgG1 Abs as well as IgE. Our evidence indicates that induction of ACAID in mice that are subsequently exposed to a Th2-biasing immunization regimen prevents their production of IgE, while the production of IgG1 is preserved. At the very least, this outcome suggests that the regulations by T cells of IgG1 and IgE are distinct and that the regulatory T cells of ACAID suppress both complement-fixing Abs (IgG2a, IgG2b, IgG3) and IgE, whereas Th2 cells suppress complement-fixing IgG Abs, but not IgE.

It is worth pointing out that induction of ACAID offers for consideration a novel strategy with which to suppress an important Th2-dependent immunopathologic condition, allergic asthma. Previously, ACAID has been used experimentally to suppress or prevent rejection of orthotopic corneal allografts in mice (28, 29) as well as experimental autoimmune uveitis (30, 31), both of which are Th1-dependent pathologic conditions. Alternatively, attempts to use ACAID experimentally to prevent rejection of solid tissue allografts such as skin and heart have met with no success (J. W. Streilein, unpublished observations). We believe that the explanation for this conundrum resides in the fact that ACAID is immune deviation, i.e., a selective deficiency of one or more immune effector modalities, but not of all effector modalities. Whereas experimental allergic lung disease appears to be mediated solely by Th2 responses, and experimental autoimmune uveitis and acute rejection of orthotopic corneal allografts appear to be mediated solely by Th1 responses, rejection of skin or heart allografts can also be achieved by CD8+ T cells and perhaps by Ab-dependent cell-mediated cytotoxicity that uses noncomplement-fixing Abs. One prediction from these considerations is that induction of ACAID may be a useful therapeutic strategy, but only if the pathologic condition in question is mediated purely by the effector modalities that ACAID suppresses.

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