Intracameral Injection of Antigen Potentiates the Production of Antigen-Specific T Cell Proteins in Serum After the Induction of Delayed-type Hypersensitivity

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**Purpose.** To determine whether the introduction of antigen into the anterior chamber induces the production of extracellular antigen-specific T cell proteins (T cell antigen-binding molecules [TABM]) specific for the antigen.

**Methods.** Balb/c mice received an intracameral or subconjunctival injection of trinitrophenylated spleen cells (TNP spleen cells) before skin sensitization and challenge with picryl chloride. The production of TNP-specific TABM in serum was quantified by enzyme-linked immunosorbent assay-based antigen binding and immunoblotting using a rabbit antiserum raised against a monoclonal antigen-specific T cell protein that induces suppressor T cells.

**Results.** Intracameral, but not subconjunctival, injection of TNP spleen cells before contact sensitization increased the level of TNP-specific TABM in serum. At 2 days, TABM levels began to rise and peaked at 5 days after contact sensitization. Anterior chamber injection of TNP spleen cells alone into nonimmunized mice did not induce a detectable increase of TNP-specific TABM in serum. Mice that did not receive an intracameral TNP spleen cell injection but were made contact sensitive to TNP showed a diminished TNP-specific TABM response. Specificity of the TABM induced by intracameral-injection and sensitization for the TNP hapten was documented by showing that the induced TABM binds TNP-bovine serum antigen (BSA) but does not bind either BSA alone or azobenzencarsonate-ovalbumin and that immune sera raised against ovalbumin did not contain an increase in TNP-specific TABM. TNP-specific TABM in the sera of mice receiving an intracameral injection of TNP spleen cells, followed by contact sensitization with picric chloride (PCI), were purified by affinity for TNP and were resolved by polyacrylamide gel electrophoresis and immunoblotting as Mr 110,000 polypeptides.

**Conclusions.** Serum levels of TNP-specific TABM induced by contact sensitization alone are enhanced fourfold to fivefold when sensitization is preceded by the intracameral, but not the subconjunctival, injection of TNP spleen cells. The authors propose that besides suppression of systemic delayed type hypersensitivity, the intracameral injection of antigen into nonimmunized mice may prime an increased production of TABM, which may indicate systemic activation of the immunoregulatory T cell circuit.

The anterior chamber (AC) injection of antigen results in a prolonged inhibition or reduction of the systemic delayed type hypersensitivity (DTH) response to the antigen and the production of IgG2a antibodies, whereas the production of both IgM and IgG1 antibodies is unaffected. The mechanisms, signals, or both that affect this anterior chamber-associated immune deviation (ACAID) are complex and appear to involve cellular and humoral mechanisms at either the splenic or the eye level. For example, when a soluble antigen is injected into the anterior chamber, intraocular bone marrow-derived dendritic cells have been shown to enter the circulation and to migrate to the spleen. Cell interactions in the spleen are thought to result in the activation of antigen-specific
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MATERIALS AND METHODS

Animals
Female 6- to 8-week-old Balb/c mice were obtained from River Run Breeders of Harlan Laboratories (Indianapolis, IN) or Jackson Laboratories (Bar Harbor, ME) and maintained by the Center for Laboratory Animal Care at the University of Connecticut Health Center in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Antigens
2,4,6-trinitrobenzene sulfuric acid (TNBS) was obtained from Sigma Chemical (St. Louis, MO). 2,4,6-trinitro-1-chlorobenzene (TNCB or picryl chloride, PCI) was obtained from Chemtronix (Swannanoa, NC). TNP-BSA (10 mg/ml) was prepared by mixing 500 mg BSA and 500 mg TNBS in 50 ml of 0.1 M Na2CO3. The solution was stirred overnight at room temperature and then dialyzed against 1 L of 0.01 M NaHCO3.

Preparation of Hapten-Modified Lymphoid Cells
Spleens were obtained from mice killed by cervical dislocation and were gently teased apart in phosphate-buffered saline (PBS, pH 7.2). Spleen cells were centrifuged at 500g for 5 minutes and were treated with distilled water for 5 seconds to lyse the erythrocytes. After lysis, the cells were washed two times with PBS, suspended in PBS and 10 mM TNBS, and incubated for 10 minutes at room temperature. The cells were then washed three times with PBS, and the TNF spleen cells were resuspended in PBS at 1 x 10^6/ml.

Antisera
TABM secreted into ascitic fluid by a mouse T cell hybrid specific for azobenzene arsonate (Hyb51H7D) were purified by precipitation of ascitic fluid with 43% (NH4)2SO4, ion exchange chromatography of the precipitated proteins, and affinity for azobenzene arsonate (ABA). The Mr 110,000 ABA-specific polypeptides were used to immunize rabbits. The antiserum is specific for TABM secreted by T cell hybrids and T cell clones and does not bind immunoglobulins, albumin, globin, or T cell membranes.

Anterior Chamber Inoculations of TNP-Modified Spleen Cells
Injections into the AC were performed using a 30-gauge needle cannula inserted in plastic tubing and attached to a microinjection on a mounted Hamilton (Reno, NV) syringe, as described. For each planned injection, 2 µl of air followed by 5 µl of TNF spleen cells (1 x 10^6/ml) were drawn into the tubing. Each mouse was anesthetized with ketamine-xylazine (0.2 ml of a 1:10 in 0.9% NaCl saline), and, for AC inoculations, an oblique transcorneal paracentesis was performed with microscopic control using the 30-gauge needle cannula. Intracameral injections were performed under a dissecting microscope, and 5 µl TNF spleen cells (5 x 10^6 TNF spleen cells) were inoculated, followed by 2 µl of air. Anterior chambers that failed to maintain the air bubble were re-injected. Twenty-four hours after AC injection, animals were immunized for contact sensitivity. Subconjunctival injections were made with a 30-gauge needle and a Hamilton syringe.

Immunization for Contact Sensitivity
Animals were immunized for contact sensitivity by topical (belly skin) application of 0.1 ml of 7% PCI in 5:1 acetone—olive oil base according to the method of Ferguson et al. Mice were challenged 5 days later by
FIGURE 1. Titration of TNP-binding TABM in the sera of mice injected with TNP spleen cells and sensitized and challenged for DTH. Five Balb/c mice received an AC or subconjunctival injection of TNP spleen cells, and, 1 day later, 100 μl 7% PCI was applied to the abdomen. Some mice received no AC injection but were sensitized and challenged. Five days later, 15 μl 1% PCI was applied to the left ear, and the mice were bled 2 days after that. The sera were pooled, and 100 μl of diluted immune or nonimmune pooled serum was added to microtiter trays coated with 1 μg/well TNP-BSA. Bound TABM were detected with rabbit anti-TABM and alkaline-phosphatase-conjugated goat anti-rabbit immunoglobulin. Titer is taken as the highest reciprocal dilution giving an optical density of 0.2 (NRS = 0.1). AC denotes mice that received an AC injection of TNP spleen cells before sensitization; S.con denotes mice that received a subconjunctival injection of TNP spleen cells before sensitization; DTH denotes mice that were sensitized and challenged for DTH but received no AC or subconjunctival injection; NMS denotes normal (nonimmune) mouse serum. Data are representative of three to five experiments, with three to five mice per group in each experiment. TNP = trinitrophenol; TABM = T cell antigen binding molecule; AC = anterior chamber; BSA = bovine serum antigen; NRS = normal rabbit serum; DTH = delayed type hypersensitivity.

the application of 0.015 ml of 1% PCI in acetone-olive oil (3:1) to one ear. Twenty-four and 48 hours later, the DTH response was determined by measuring ear swelling with an engineer's micrometer (Mitutoyo, Tokyo, Japan).

Ag Binding Enzyme-Linked Immunoabsorbent Assay

Microtiter trays (Dynatech, Torrance, CA) were coated with 1 μg/well of TNP-BSA in 0.1 M NaHCO₃, pH 9.25, by 16 hours incubation at room temperature. The trays were blocked by incubation for 1 hour at 37°C with 200 μl 0.1% gelatin per well. Dilutions of immune or nonimmune mouse serum were added at 100 μl per well, and the trays were incubated for 1.5 hours at 37°C. After washing with 1 M K₂HPO₄, 0.1% Triton X-100, 0.85% NaCl, pH 7.4, 100 μl per well of rabbit anti-TABM, or normal rabbit serum at a dilution of 1:500 was added and incubated and washed as above. A 1:1000 alkaline phosphatase-conjugated goat anti-rabbit IgG (TAGO, Burlingame, CA) 100 μl per well was then added and incubated for 1.5 hours at 37°C, after which the trays were washed. Finally, 100 μl per well of 1 mg/ml p-nitrophenylphosphate in substrate buffer (105 mg/ml diethanolamine and 100 μg/ml MgCl₂, pH 9.8) was added to each well. The trays were incubated for 3 to 10 minutes at room temperature, and absorbance optical density was read.

Immunoblot Analysis

Balb/c mice received an AC injection of TNP spleen cells and were immunized for contact sensitivity as described above. Two days after challenge, sera were obtained and pooled. Three hundred microliters of immune or nonimmune serum was diluted 1:10 with 0.05% Tween/PBS, and 1 ml of each was added to 300 μl of TNP-BSA Sepharose beads (10 mg TNP-BSA/1 ml beads). After the beads were rotated for 1 hour at 4°C, they were washed 3× with 0.05% Tween-PBS and eluted with 0.5 ml sodium dodecyl sulfate (SDS) sample buffer. One hundred-microliter aliquots of eluate were reduced by the addition of β-mercaptoethanol to a final concentration of 5% and boiled for 5 minutes. Four microliters of the eluate was resolved by 10% SDS-polyacrylamide (Phastsystem; Pharmacia, Piscataway, NJ) gel and electrophoretically transferred to an immobilon poly(vinylidene difluoride) membrane. After the membrane was blocked with 3% dry milk for 1.5 hours at 37°C, it was incubated overnight with 1:500 rabbit anti-TABM antiserum at 4°C. After 1 hour of washing, peroxidase-conjugated goat anti-rabbit IgG (1:1000) was added, and the membrane was again incubated for 4 hours at 4°C. Finally, the membrane was suspended in Tris, 0.03% H₂O₂ buffer, and diaminobenzidine (Chemicon, Temecula, CA) was added. Color developed in 15 to 30 minutes, and the membrane was washed with wash buffer and dried at 37°C.

RESULTS

Intracamerall Injection of TNP Spleen Cells Followed by Contact Sensitization Enhances a Rise in TNP-Specific Serum TABM

To determine the effect of AC injection of TNP spleen cells on the production of TNP-specific serum TABM, mice received an AC or subconjunctival injection of TNP spleen cells before sensitization with PCI. Sera were obtained 2 days after topical sensitization and challenge with PCI (9 days after ocular injection) and
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FIGURE 2. Specificity of serum TABM. Five Balb/c mice received an intracameral injection of $5 \times 10^5$ Balb/c TNP spleen cells, and DTH was induced as described in Figure 1. Two days after PCI was applied to the ear, the mice were bled. (A) One hundred microliters of a 1:20 dilution of immune serum was added to microtiter tray wells coated with 1 µg/well TNP-bovine serum albumin (TNP-BSA), bovine serum albumin, or azobenzenezaronate (ABA) ovalbumin. TABM binding was detected with anti-TABM. The optical density obtained with normal rabbit serum (0.15 ± .03) was subtracted from the optical density obtained with anti-TABM. (B) Five Balb/c mice received $5 \times 10^5$ Balb/c TNP spleen cells AC, and DTH was induced as described in Figure 1. Two days after PCI was applied to the ears, the mice were bled. Pooled sera obtained from mice receiving AC, TNP spleen cells (•), and pooled sera obtained from ovalbumin-sensitized mice 2 days after receiving an intracameral injection of ovalbumin (●) were titrated against 1 µg/well TNP-BSA. Data represent the mean ± SE optical density of three replicates. TABM = T cell antigen-binding molecule; DTH = delayed type hypersensitivity; PCI = picryl chloride; TNP-BSA = trinitrophenol-bovine serum antigen; AC = anterior chamber.

were titrated in TNP-BSA-coated wells in microtiter trays. TABM bound to TNP were detected by the addition of rabbit anti-TABM and alkaline phosphatase conjugated goat anti-rabbit immunoglobulin. As shown in Figure 1, although the titer of nonimmune serum was 1:40, there was an increase in TNP-specific TABM in the sera of mice sensitized without an earlier AC injection (1:640).

However, the titer of TNP-specific TABM in the sera of AC-injected, sensitized mice was fourfold to eightfold higher than the titer in the sera of mice that did not receive AC injections of TNP spleen cells. In some experiments, AC-injected sensitized mice did not have specific TNP-serum TABM titers greater than those found in nonimmune serum (data not shown). Moreover, the titer of TNP-specific TABM in the sera of mice receiving an subconjunctival injection was no greater than that for noninjected sensitized mice.

Mice that received subconjunctival injections had a mean swelling increment of $19.5 ± 3.7 \times 10^{-3}$ cm, and mice that received no ocular injection of TNP spleen cells had a mean swelling increment of $14.4 ± 2.24 \times 10^{-3}$ cm, whereas the increment in unsensitized mice challenged with the antigen or unchallenged sensitized mice was 1 to $1.3 ± 0.05 \times 10^{-3}$ cm. Mice that were AC injected had a mean swelling increment of $5.85 ± 2.25 \times 10^{-3}$ cm.

Characteristics of AC-Amplified Serum TABM

To examine the antigenic specificity of elevated serum TABM levels found after AC injection and sensitization, immune serum was added to microtiter tray wells coated with 1 µg/well TNP-BSA, BSA, or ABA-ovalbumin (OVA). As shown in Figure 2A, serum TABM
amplified by AC immunization and sensitization bound to TNP–BSA but did not bind significantly to BSA or ABA–OVA. Further, an antisera containing OVA-specific murine TABM, obtained from OVA-sensitized mice receiving an AC injection of OVA, did not show an increase in TNP-specific TABM (Fig. 2B).

To determine the molecular size of TNP-specific TABM, pooled sera from AC-injected sensitized mice or nonimmune serum was mixed with TNP–BSA-Sepharose beads. The bound protein eluted from beads was reduced and resolved by SDS–PAGE and electroblotted onto an immobilon membrane. When anti-TABM serum was then added to blotted proteins (Fig. 3), sera obtained from AC-injected sensitized mice contained a Mr 110,000 reduced TNP-specific TABM, which was not detected in the sera of nonimmune mice.

**Kinetics of Appearance of Serum TABM After Intracameral Injection and Sensitization**

The results presented above indicate that AC injection of TNP spleen cells, followed by sensitization and challenge with picryl chloride, potentiated a rise in TNP-specific TABM that occurred after sensitization. To examine the kinetics of TABM production, groups of mice received AC injections of TNP spleen cells, with or without subsequent sensitization and challenge with PCI, and were bled at various times thereafter to quantitate TNP-specific serum TABM. All sera were titrated, and, as shown in Figure 4, there was no increase in TNP-specific TABM in mice injected intracameral only with TNP spleen cells. Ten days after injection, TNP-specific serum TABM did not increase (data not shown). Two days after sensitization, an increase in serum of TNP-specific TABM was observed in AC-injected mice, whereas sensitized mice that did not receive an AC injection did not have an increase in serum of TNP-specific TABM. At day 6, TNP-specific TABM in the sera of AC-injected mice were at least twice that of sensitized mice that did not receive AC injections. In fact, TNP-specific TABM in the latter group on day 6 was approximately equal to AC-injected mice on day 5. On day 6, the ears of the mice were challenged by topical application of PCI. In the sera of AC-injected mice, TNP-specific TABM increased further, and twofold to threefold higher optical density readings were obtained with sera from AC-injected mice than non-AC-injected mice.

**DISCUSSION**

Serum TABM have been shown to be T cell-derived proteins by the demonstration that serum TABM detected by ELISA-based antigen binding share epitopes with TABM produced by T cell hybrids and TcR. In addition, immunized SCID mice (which do not produce TABM), when reconstituted with thymocytes, produce TABM but do not produce immunoglobulins. Thus, the induction of a serum TABM response reflects the activation of T lymphocytes. We found that some TNP-specific TABM were produced in response to Co. In addition, immunized SCID mice (which do not produce TABM), when reconstituted with thymocytes, produce TABM but do not produce immunoglobulins. Thus, the induction of a serum TABM response reflects the activation of T lymphocytes. We found that some TNP-specific TABM were produced in response to...
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FIGURE 4. Kinetics of appearance serum TABM after intracameral injection. Twenty Balb/c mice received AC injections of TNP spleen cells on day 0 and were sensitized and challenged with PCI. Twenty mice received no AC injection. Five mice were bled at each time, and pooled sera were assayed for TNP-binding TABM. Data represent the mean ± SE of five replicates of the optical density obtained with 100 μl of a 1:33 dilution of serum. The optical density of nonimmune serum (0.15 to 0.3) has been subtracted. Hatched bars = AC injection, sensitization, and challenge. Closed bars = sensitization and challenge only. TABM = T cell antigen-binding molecule; AC = anterior chamber; PCI = picryl chloride; TNP = trinitrophenol.

contact sensitization and challenge with PCI. Preliminary results (Streilein et al, unpublished observation) suggest that doses of PCI optimal for DTH stimulation do not stimulate TABM, whereas supraoptimal doses of PCI stimulate TABM. Thus, some TABM production may be an element common to most immune responses. We found that the production of TABM in response to sensitization was accelerated and enhanced at least fourfold by the intracameral injection of antigen before sensitization. The TABM potentiated by intracameral injection of TNP spleen cells in the present study were antigen specific, as indicated by the observation that the TABM would bind TNP but not ABA or BSA, and TNP-specific TABM were not increased by AC injection of ovalbumin. In addition, serum TABM potentiated by AC injection is similar in molecular size to TcR Ca positive BSA-specific serum TABM induced by systemic immunization with BSA and adjuvants and to monoclonal TABM, which show some amino acid sequence homology to pre-TcR.

Because the AC injection of TNP spleen cells alone without sensitization did not induce a rise in the titer of TNP-specific TABM, it appears that AC injection into nonimmune mice may prime the mice to amplify the TABM response to a subsequent systemic antigenic stimulus. TABM levels specific for injected antigen do rise in serum after the AC injection of a soluble protein antigen into sensitized, splenectomized mice or into nonsensitized asplenic mice that have received AC injections of BSA and are then challenged with BSA. These observations support our contention that the intracameral route provides a "signal" that primes T cells that produce TABM and/or activates TABM-producing T cells primed from a previous sensitization. Perhaps this "signal" is a blood-borne dendritic cell derived from the iris or ciliary body that has been shown to transfer ACAID to naive mice. The increase in antigen-specific TABM in serum after the intracameral injection of TNP spleen cells and sensitization shown in this article is also consistent with previous studies of the appearance in serum of an antigen-specific "factor" that can transfer immunosuppression after asplenic mice received an AC injection of TNP spleen cells and epicutaneous sensitization with PCI. Serum TABM detected by an antiseraum raised against purified TsIF, and the serum factor detected by monoclonal anti-TsIF, both bear TcR Ca determinants and may thus be related. The observation here that antigen-specific TABM reached nearly maximal levels coincident with the time at which mice were challenged for DTH, and the serologic similarity of TABM...
to TdF induced by AC injection of TNP spleen cells, also suggests that TdBM may serve an immunoregulatory role. When sufficient immunoregulatory T cells are present (as in immune animals), TdBM could activate these cells. According to this view, the demonstration of an immunosuppressive role for TdBM would require the presence of adequate numbers of immunoregulatory T cells. If TdBM, in concert with antigen, induced the proliferation of or activation of these immunoregulatory T cells, the steady rise in serum TdBM 3 to 9 days after ear challenge would suggest that the molecules we detect may serve a role that becomes more evident several days or weeks after challenge, i.e., indicating that the TdBM may serve to perpetuate the suppressed state. In either case, the production of TdBM is probably not obligated to the induction of ACAID but is, rather, one manifestation of the amplification effect of ACAID on immune T cells. That is, when the proliferation of immunoregulatory T cells has already been induced by a previous sensitization, TdBM may serve to activate these cells. Experiments are now in progress to investigate this point.

Key Words

ACAID, immunoregulation, serum, T cells, TdBM

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