Injection of Soluble Antigen into the Anterior Chamber of the Eye Induces Expansion and Functional Unresponsiveness of Antigen-Specific CD8\(^+\) T Cells

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*J Immunol* 2002; 169:5630-5637; doi: 10.4049/jimmunol.169.10.5630

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Injection of Soluble Antigen into the Anterior Chamber of the Eye Induces Expansion and Functional Unresponsiveness of Antigen-Specific CD8+ T Cells

Kyle C. McKenna, Yijun Xu, and Judith A. Kapp

The injection of soluble Ag into the anterior chamber (a.c.) of the eye induces systemic tolerance, termed a.c.-associated immune deviation (ACAID), characterized by Ag-specific inhibition of delayed-type hypersensitivity responses and a reduction in complement-fixing Abs. Recently, we have shown that CD8+ CTL responses are also inhibited in ACAID. In this study, we have used an adoptive transfer approach to follow the fate of Ag-specific CD8+ TCR transgenic (OT-I) T cells in vivo during the induction and expression of ACAID. C57BL/6 (B6) recipients of OT-I splenocytes that were injected with chicken OVA in the a.c. displayed reduced OVA-specific delayed-type hypersensitivity and CTL responses, compared with those of mice given OVA in the subconjunctiva or an irrelevant Ag human IgG in the a.c. OT-I T cells increased 9-fold in the submandibular lymph nodes and 3-fold in the spleen following an a.c. injection with OVA, indicating that expansion rather than deletion of Ag-specific CD8+ T cells was induced by this treatment. OT-I T cells expanded equivalently upon administration of OVA in CFA to mice previously given OVA in the a.c. or subconjunctiva. However, the lytic activity attributed to OT-I T cells was reduced on a per-cell basis in mice previously given OVA in the a.c. We conclude that tolerance of CTL responses in mice given Ag via the a.c. results from unresponsiveness of Ag-specific CD8+ T cells. The Journal of Immunology, 2002, 169: 5630–5637.
ocular APC that have processed exogenous Ag may also activate CD8+ T cells.

The goal of the present study was to determine the mechanism of CTL inactivation in ACAID. Using an adoptive transfer approach, the fate of Ag-specific CD8+ T cells was monitored in vivo during the induction and expression of ACAID. We show that the injection of soluble Ag in the a.c. of the eye causes expansion of Ag-specific CD8+ T cells that have reduced lytic capacity. These data demonstrate that inhibited CTL responses in ACAID are due to functional unresponsiveness and not clonal deletion of Ag-specific CD8+ T cells.

Materials and Methods

Experimental animals

Female C57BL/6 (B6; H-2b) mice were purchased from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD). C57BL/6-TgN (TCK-1) (14) transgenic mice, also referred to as OT-I, were a generous gift of Dr. M. Bevan (University of Washington, Seattle, WA) and were bred and maintained in the animal facilities at Emory University. The transgenic mice have been backcrossed for 10 generations to B6 mice and are considered congenic with B6 mice. All mice were 6–8 wk old. Mice were bred and maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, DC).

Material and Methods

Ages and adjuvants

Grade VI chicken egg OVA and human IgG (HuIgG) were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in PBS at a concentration of 25 mg/ml. CFA containing heat-killed Mycobacterium tuberculosis strain H37Ra and IFA were purchased from Difco Laboratories (Detroit, MI). OVA (2 mg/ml) in CFA, and OVA (0.5 mg/ml) in IFA emulsions were prepared by mixing equal volumes of aqueous Ag and adjuvant.

Target cell lines

E.G7-OVA, an H-2b tumor cell line that expresses the chicken OVA gene (15) kindly provided by Dr. M. Bevan), was used as a CTL target in these studies. E.G7-OVA was cultured in standard growth medium (SGM) (RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, gentamicin, penicillin, and streptomycin) at 37°C in 5% CO2 atmosphere. E.G7-OVA was maintained free of mycoplasma and periodically cultured with 250 μg/ml of neomycin to maintain expression of the transfected OVA gene.

Ag administration

For all Ag injections, mice were anesthetized with 0.1 ml of a solution containing 10 mg/ml ketamine (Sigma-Aldrich) and 2 mg/ml xylazine (Bayer, Shawnee Mission, KS) delivered i.m. in the thigh. For subconjunctival (scon) and a.c. injections, the eye was also anesthetized by topical drops of proparacaine HCl from Alcon Laboratories (Humacao, Puerto Rico). For scon injections, 50 μg of OVA or HuIgG in 2 μl of PBS was injected into the subconjunctiva at the base of the globe by reflecting the lid with forceps. For a.c. injections, the cornea was punctured by inserting a 30-gauge needle directly above the pupil and parallel to the iris. Aqueous humor was allowed to flow out and was removed by an ophthalamic sponge (Microsponge, Alcon Laboratories). An air bubble was then introduced into the a.c. by injecting a 1.5 μl volume of air with a Hamilton microsyringe (Hamilton, Reno, NV) fitted with a 33-gauge beveled needle. Next, 50 μg of OVA or HuIgG in 2 μl of PBS was injected with a microsyringe fitted with a 33-gauge blunt needle. The air bubble seals the corneal puncture and prevents leakage of Ag. For s.c. injections, 200 μg of OVA in CFA was injected into both hind footpads (100 μg per foot in 50 μl). For intradermal injections, 200 μg of OVA in CFA was administered in two injections (100 μg per injection in 50 μl) in the back.

Adoptive transfer and enumeration of OT-I T cells by flow cytometric analysis

Spleens from naive OT-I mice were removed and single cell suspensions were prepared. RBC were removed by hypotonic lysis and then splenocytes were washed and resuspended at 2–4 × 107 cells/ml in Cellgro HBSS from Mediatech (Herndon, VA). More than 90% of CD8+ T cells in OT-I mice express the Vb5/Vα2 transgenic TCR and bind OVA577–264 H-2Kb tetramers (data not shown). Due to a normal complement of B cells, transgenic T cells accounted for 10 ± 3% (n = 5) of total splenocytes in naive OT-I mice. OT-I T cells from naive OT-I mice were CD44hi and forward scatter (FSC)high, which is indicative of a naive, unactivated phenotype (data not shown). OT-I splenocytes (1.0–2.0 × 108) in 0.5 ml HBBS were then injected into naive B6 mice via the lateral tail vein. In some experiments, OT-I splenocytes were labeled with Cell Tracker Green 5- (6-) CFDA SE from Molecular Probes (Eugene, OR), by incubating a splenocyte suspension (2.0 × 107 cells/ml) with Cell Tracker Green at a final concentration of 5 μM for 15 min at 37°C. Cells were then washed twice with HBBS before transfer. B6 recipients of OT-I splenocytes were euthanized by CO2 asphyxiation. Right and left axillary, brachial, inguinal submandibular, and popliteal lymph nodes and the spleen were then removed. These tissues were processed into single-cell suspensions, as described above, and then stained with FITC- or PerCP-labeled anti-CD8a, PE-labeled TCR Vb5/IV5/2,5 mAbs from BD PharMingen (San Diego, CA), and APC-labeled OVA577–264 H-2Kb tetramers (16). Tetramers were provided by Dr. J. Altman (Emory University, Atlanta, GA).

Flow cytometric analysis of stained cells was performed using a FACS-Calibur flow cytometer (BD Biosciences, San Jose, CA). From a live lymphocyte gate, determined by forward and side scatter, a CD8+ gate was drawn by plotting CD8+ cells against side scatter and 1.0–2.0 × 108 CD8+ events were collected. OT-I T cells were defined as CD8+ TCRβ5/2/S/OVA577–264 H-2Kb tetramer+ and the percentage of OT-I T cells in each tissue was determined by analysis using FlowJo version 3.3 data analysis software (TreeStar, San Carlos, CA). Absolute numbers of OT-I T cells were determined by multiplying the percentage of OT-I T cells by the total cell yield in each respective tissue. In some experiments, CFSE incorporation by OT-I T cells was also measured. CFSE is a fluorescent dye that, upon nuclear incorporation, is distributed equally to daughter cells upon cell division (17). In these experiments, 2.5–5.0 × 107 CD8+ TCRβ5/2+ cells were collected for analysis.

CTL assay

After injection of OVA in CFA, 1.0–3.0 × 107 draining lymph node cells were incubated with the indicated concentration of OVA in 10 ml of SGM for 3 days in an upright 25-cm2 tissue culture flask. Following incubation at 37°C in 5% CO2 atmosphere, the number of live cells was determined by trypan blue exclusion and a standard 4-h Ch1 release assay was performed at various E:T ratios using E.G7-OVA cells labeled with Na2Cr2O7 (DuPont, Boston, MA) as targets (15).

To determine the lytic activity attributed to OT-I T cells on a per-cell basis, the percentage of OT-I T cells in each effector cell culture was determined by flow cytometric analysis as described above. To distinguish viable from nonviable cells, Via-Probe (7AAD) from BD Pharmingen was added to stained cultures. Only Via-Probe-negative cells from a lymphocyte gate, determined by forward and side scatter, i.e., viable lymphocytes, were used to determine the percentage of the OT-I T cells. The percentage of OT-I T cells was then used to calculate the number of OT-I T cells added to the CTL assay. Data were corrected for differences in the number of OT-I T cells and are presented as lytic activity plotted against OT-I T cell number. In other experiments, OT-I T cells from OT-I-transferred mice receiving indicated treatments were added to draining lymph node cell cultures of similarly treated nontransferred mice.

DTH assay

Mice were given a s.c. injection with 100 μg OVA in CFA at the base of the tail. Seven days later, mice were injected s.c. in the right footpad with 12.5 μg OVA in IFA in 25 μl. An equivalent volume of PBS in IFA was injected in the left footpad. Twenty-four hours later, DTH was measured as footpad swelling using a micrometer (Mitutoyo 227-101; MTI, Paramus, NJ). The footpad swelling in response to OVA was calculated by the formula:

\[
\text{DTH} = \frac{\text{Swelling in response to OVA} - \text{Swelling in response to control}}{\text{Swelling in response to control}} \times 100
\]

Statistical analysis

Differences in OT-I T cell numbers, lytic activity, or DTH responses between indicated groups of mice or cell cultures were compared by Student’s t test. Values of p < 0.05 were considered significant.

Results

OVA-specific CTL and DTH responses are down-modulated in OT-I-reipient mice given soluble OVA in the a.c.

The injection of OVA in CFA primes for CTL responses that are mediated by CD8+ αβ T cells specific for OVA577–264 presented
by H-2Kb (12). However, the frequency of these Ag-specific T cells in naive, as well as immunized, mice is below the threshold of detection by flow cytometry (data not shown). As a result, the adoptive transfer approach of Kearny et al. (18) was used with OT-I TCR transgenic mice (14) to increase the frequency of these OVA-specific CD8+ T cells in vivo.

Priming for CTL and DTH responses, generated by the administration of OVA in CFA, is inhibited if normal B6 mice are first given OVA in the a.c. (4). To determine whether B6 recipients of OT-I splenocytes developed immune responses that were similar to those of normal B6 mice, both CTL and DTH responses were measured in OT-I recipients. This is an important control because increasing the number of Ag-specific T cells might alter the regulation of DTH and CTL responses resulting from injection of Ag in the a.c. Consistent with our previous observations (4), the administration of OVA in CFA primed for robust CTL responses in the draining lymph nodes of OT-I-transferred mice, and these CTL responses were markedly reduced if recipients were first given OVA in the a.c. (Fig. 1A). In addition, both nontransferred and OT-I recipients that were previously injected with OVA in the a.c. showed significantly reduced DTH responses compared with mice that were untreated in the eye (Fig. 1B). Although the magnitude of the DTH response in untreated OT-I recipients (1.25 ± 0.12 mm) was significantly greater ($p = 0.027$) than that of untreated nontransferred mice (1.10 ± 0.17 mm), the percentage of inhibition of DTH responses in mice given OVA in the a.c. was equivalent between OT-I recipients (25% inhibition) and nontransferred mice (22% inhibition), and both were statistically different from control mice (Fig. 1B). These data indicate that increasing the frequency of OVA-specific CD8+ T cells did not change the priming for CTL responses in vivo or abrogate inhibition of CTL and DTH responses resulting from the administration of OVA in the a.c. Thus, the adoptive transfer model is an appropriate model for AICAID.

Enumeration of Ag-specific CD8+ T cells following Ag administration

There are several potential mechanisms by which CTL responses might be inhibited by administration of Ag via the a.c. Inhibition might result from immune deviation of CD4+ T helper cells (6). Other potential mechanisms that could account for the inhibition of CTL activity include clonal deletion, functional unresponsiveness, immune deviation of CD8+ T cells, or activation of regulatory cells.

To determine the fate of CTL precursors after administration of OVA, OVA-specific CD8+ T cells were tracked in vivo by flow cytometric analysis. CD8+V85+OVA257-264-H-2Kb tetramer+ cells were easily visualized in the lymph nodes and spleens of B6 recipients of OT-I splenocytes where they consistently represented <1.0% of total cells in these tissues (Fig. 2: "No Ag"). Footpad administration of OVA in CFA induced OT-I T cell expansion in the popliteal lymph nodes and spleen, while OVA injected in the a.c. caused expansion in the submandibular lymph nodes and the spleen (Fig. 2).

In mice injected with OVA in CFA, the cell yield in the draining popliteal lymph node increased dramatically, 43-fold, compared with the cell yield in untreated mice (Table I, experiment 1). Combined with a 4-fold increase in the percentage of OT-I T cells, this treatment resulted in a 168-fold expansion of OT-I T cells. Although the cell yield in the spleen was unchanged, a 2-fold increase of OT-I T cells was also observed in this compartment due to an increased percentage of OT-I T cells. No significant increase in the cell yield or number of OT-I T cells was observed in submandibular or axillary lymph nodes following the OVA in CFA s.c. injection. The injection of OVA in the a.c. caused a 2-fold increase in cell yield in the submandibular lymph nodes. Combined with a 2.4-fold increase in the percentage of OT-I T cells, this resulted in a 6-fold increase of OT-I T cells. In addition, OT-I cells increased 4-fold in the spleen, while there was no significant increase of OT-I T cells in popliteal or axillary lymph nodes.

To determine whether the increase in OT-I T cells in the submandibular lymph nodes and the spleen was Ag specific, mice were injected in the a.c. with the irrelevant Ag HulG3. Three days later, ipsilateral and contralateral submandibular lymph nodes and

![FIGURE 1. OVA-specific CTL and DTH responses are down-modulated in adoptive recipients after administration of OVA in the a.c. Priming of CTL precursors was measured in the popliteal lymph nodes of B6 recipients of OT-I splenocytes. OT-I recipients (three mice per group) received either OVA or HulG in the a.c. on day 0 and then were given OVA in CFA s.c. in the rear footpads on day 7. Five days later, right and left popliteal lymph nodes were removed, pooled, and stimulated with 20 μg/ml OVA in vitro for 3 days. A standard C11 release assay was then performed with E.G7-OVA cells as targets (A). The displayed experiment is representative of two experiments with similar results. DTH responses were measured in groups of 11–12 B6 mice or B6 mice that received OT-I splenocytes that were injected with OVA in the a.c. or were left untreated. The data presented were combined from two experiments, and each symbol represents measurements from an individual mouse (B).](http://www.jimmunol.org/)

![FIGURE 2. Enumeration of OT-I T cells in recipient mice following exposure to Ag. OT-I T cells, demarcated by a box, were enumerated in OT-I-recipient B6 mice 3 days after injection of OVA in the a.c. or OVA in CFA in the rear footpads. Events displayed are from a CD8 gate. The percentage of OT-I T cells of total cells in each tissue is shown in the upper right corner of each plot. Right and left lymph nodes were combined for analysis. The plots are representative of one of four mice, all of which showed similar trends.](http://www.jimmunol.org/)

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spleens were removed and OT-I T cells were enumerated. The number of OT-I T cells in the lymph nodes and spleens of mice given HulG in the a.c. was comparable to that of recipient mice that were left untreated (Table I; compare “HulG a.c.” of experiment 2 to “Untreated” of experiment 1). Mice given OVA in the a.c. showed increased OT-I T cell numbers in the ipsilateral submandibular lymph nodes and spleen that were equivalent to or slightly greater than the increases observed in experiment 1. OT-I T cells did not increase in the contralateral lymph node. These data suggest that the submandibular lymph nodes and spleen drain the a.c. of the eye and that the increase of OT-I T cells was Ag specific.

Previous work of Perez et al. (19) showed that injection of OVA into the subconjunctiva resulted in the expansion of Ag-specific CD4+ T cells in the submandibular lymph nodes of DO11.10 recipients. This route of Ag administration does not induce systemic tolerance (20). Therefore, OT-I T cells in the submandibular lymph nodes were measured after scon administration of OVA to determine whether CD8+ OT-I T cells also expanded following this treatment. A significant 15.6-fold increase in OT-I T cells was observed in the ipsilateral submandibular lymph nodes 3 days after the administration of OVA in the subconjunctiva (Table I, experiment 3). Like the administration of OVA in the a.c., the expansion of OT-I T cells was Ag-specific and confined to the ipsilateral submandibular lymph node.

The kinetics of OT-I T cell accumulation in the submandibular lymph nodes were similar between mice receiving OVA in the a.c. and those receiving OVA scon, peaking 3 days after Ag exposure and decreasing by day 5 (Fig. 3). The number of OT-I T cells on day 3 was 2.6-fold greater \((p = 0.004)\) in mice given OVA scon \((156 \pm 16 \times 10^3\) cells) compared with that of mice given OVA in the a.c. \((60 \pm 0.7 \times 10^3\) cells). These data demonstrate that OT-I T cells expanded in the draining lymph nodes when OVA was given by a route that induces tolerance (a.c.) or immunity (scon).

### Table I. Recovery of transferred OT-I T cells in recipient B6 mice following Ag administration

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Lymphoid Tissue</th>
<th>Cell Yield ((\times 10^3))</th>
<th>Total OT-I T Cells ((\times 10^5))</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Submandibular lymph nodes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td>365.0 ± 145.0</td>
<td>13.5 ± 4.9</td>
<td>−1.1</td>
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<tr>
<td></td>
<td>OVA/CFA s.c.</td>
<td>325.0 ± 114.0</td>
<td>12.7 ± 6.3</td>
<td>−1.1</td>
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<tr>
<td></td>
<td>OVA a.c.</td>
<td>845.0 ± 313.0</td>
<td>77.1 ± 26.3</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>Popliteal lymph nodes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td>23.5 ± 10.2</td>
<td>1.2 ± 0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OVA/CFA s.c.</td>
<td>1,020.0 ± 709.0</td>
<td>202 ± 155</td>
<td>168.3</td>
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<tr>
<td></td>
<td>OVA a.c.</td>
<td>27.8 ± 15.7</td>
<td>1.3 ± 0.7</td>
<td>1.1</td>
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<td></td>
<td>Axillary lymph nodes</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td>105.0 ± 47.5</td>
<td>4.2 ± 1.6</td>
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<tr>
<td></td>
<td>OVA/CFA s.c.</td>
<td>75.0 ± 25.0</td>
<td>4.4 ± 2.7</td>
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<td>OVA a.c.</td>
<td>56.0 ± 23.0</td>
<td>3.0 ± 1.3</td>
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<tr>
<td></td>
<td>Spleen</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Untreated</td>
<td>5,380 ± 1,530</td>
<td>143 ± 19.2</td>
<td></td>
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<tr>
<td></td>
<td>OVA/CFA s.c.</td>
<td>5,800 ± 1,890</td>
<td>289 ± 215</td>
<td>2.0</td>
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<tr>
<td></td>
<td>OVA a.c.</td>
<td>6,730 ± 1,840</td>
<td>532 ± 189</td>
<td>3.7</td>
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<tr>
<td>2</td>
<td>Ipsilateral submandibular lymph nodes</td>
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<td></td>
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<td></td>
<td>HulG a.c.</td>
<td>275 ± 140</td>
<td>9.5 ± 5.4</td>
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<td>OVA a.c.</td>
<td>645 ± 193</td>
<td>87.9 ± 40.0</td>
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<td>Ipsilateral submandibular lymph nodes</td>
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<tr>
<td></td>
<td>HulG scon</td>
<td>298 ± 44.4</td>
<td>6.0 ± 2.3</td>
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<tr>
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<td>OVA scon</td>
<td>498 ± 93.0</td>
<td>93.8 ± 45.5</td>
<td>15.6</td>
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<td>Contralateral submandibular lymph nodes</td>
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<tr>
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<td>HulG a.c.</td>
<td>173 ± 49</td>
<td>5.5 ± 1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OVA a.c.</td>
<td>270 ± 179</td>
<td>6.1 ± 4.9</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>8,700 ± 1,450</td>
<td>118 ± 25.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OVA a.c.</td>
<td>13,000 ± 896</td>
<td>407 ± 85.6</td>
<td>3.5</td>
</tr>
</tbody>
</table>

* B6 mice were given \(1.0 \times 10^7\) OT-I splenocytes i.v. Two days later, 50 μg of HulG or OVA was administered to the eye by the indicated routes or 200 μg of OVA in CFA was injected in the rear footpads (four mice per group).Three days later, the spleen and right and left axillary, submandibular, and popliteal lymph nodes were harvested. In experiment 1 right and left lymph nodes were combined for analysis. OVA-treated mice were compared to untreated mice on the day of challenge is indicated (Fig. 3). The number of OT-I T cells on day 3 was 2.6-fold greater \((p = 0.004)\) in mice given OVA scon \((156 \pm 16 \times 10^3\) cells) compared with that of mice given OVA in the a.c. \((60 \pm 0.7 \times 10^3\) cells). These data demonstrate that OT-I T cells expanded in the draining lymph nodes when OVA was given by a route that induces tolerance (a.c.) or immunity (scon).
Measurement of OT-I T cell division following Ag exposure

To determine whether the increased numbers of OT-I T cells in the lymph nodes and spleens of mice receiving OVA in the a.c. or OVA in CFA s.c. were the result of OT-I T cell division, OT-I splenocytes were labeled with CFSE before adoptive transfer. Decreased CFSE fluorescence intensity is an indicator of cell division. On day 0, groups of three transferred mice were given HuIgG in the a.c., OVA in the a.c., OVA in CFA s.c., or were left untreated. Three days later, lymph nodes and spleens were removed, pooled, and CFSE fluorescence intensity of gated OT-I T cells was measured by flow cytometry. OT-I T cells in untreated mice expressed high levels of CFSE (CFSE\textsuperscript{high}) in all tissues (Fig. 4).

Using the fluorescence intensity of OT-I T cells in untreated mice as a baseline for CFSE incorporation, a marker of OT-I\textsuperscript{CFSE\textsuperscript{low}} cells was drawn. CFSE\textsuperscript{low} cells accounted for 4–5% of OT-I T cells in the submandibular and popliteal lymph nodes and spleens of untreated mice. Mice injected with HuIgG in the a.c. showed a similar percentage of CFSE\textsuperscript{low} cells to that of untreated mice in both the lymph nodes and spleens, indicating that OT-I T cells did not divide after the administration of an irrelevant Ag. This was consistent with the observation that OT-I T cells did not increase in mice given HuIgG a.c. (Table I; experiment 2). Mice injected with OVA in the a.c., however, showed a marked increase in CFSE\textsuperscript{low} cells in all lymph nodes tested, as well as in the spleen. However, the most pronounced increase in OT-I\textsuperscript{CFSE\textsuperscript{low}} cells occurred in the draining submandibular lymph node and in the spleen. Similarly, mice injected with OVA in CFA s.c. showed increases in OT-I\textsuperscript{CFSE\textsuperscript{low}} cells in the draining popliteal lymph node and spleen where OT-I T cells were also significantly increased (Table I; experiment 1). These data suggest that increases in OT-I T cells following the administration of OVA in the a.c. or OVA in CFA s.c. involved cell division. It is notable that injection of OVA in the a.c. induced proliferation of OT-I T cells in the nondraining (contralateral submandibular and popliteal) lymph nodes, yet this injection failed to cause a net increase in the number of OT-I T cells at these sites (Table I). The number of CFSE\textsuperscript{high} OT-I T cells decreased to similar levels in the draining and non-draining lymph nodes, but the nondraining lymph nodes did not accumulate as many CFSE\textsuperscript{low} cells as the draining nodes. This suggests that small amounts of OVA must reach all peripheral lymphoid tissues after injection in the eye but it reaches sufficient levels to stimulate a net expansion of OT-I T cells only in areas that directly drain the injection site. Whether cells that proliferated in nondraining lymph nodes failed to accumulate because they died or left the lymph node is not known.

**OT-I T cell expansion following secondary administration of OVA in CFA**

Data from the previous experiments clearly demonstrated that OT-I T cells expanded following administration of OVA in the a.c., indicating that clonal deletion could not explain decreased CTL activity in these mice. To determine whether tolerance was induced by injection of OVA in the a.c., it was necessary to challenge the mice subsequently with OVA in CFA. Therefore, we next determined the fate of OT-I T cells upon a subsequent Ag exposure with OVA in CFA of mice previously given OVA in the a.c., OVA s.c., or HuIgG in the a.c. Seven days after ocular administration of Ag, the number of OT-I T cells in axillary, brachial, and inguinal lymph nodes was equivalent between all treatment groups (Fig. 5). At this time point, the primary expansion of OT-I T cells in mice given OVA in the a.c. or s.c. had returned to baseline levels equivalent to those of mice that were given the irrelevant Ag HuIgG in the a.c. OT-I T cells increased significantly in all treatment groups 3 days after injection of OVA in CFA. The number of OT-I T cells was similar in mice previously given OVA in the a.c. (152.7 ± 8.3 × 10\textsuperscript{3}) or OVA s.c. (130.6 ± 43.4 × 10\textsuperscript{3}), but significantly less than mice given HuIgG in the a.c. (538.4 ± 156.6 × 10\textsuperscript{3}). These data are consistent with a previous report showing that, once T cells undergo clonal expansion and retraction, their expansion is not as great as the primary response upon secondary exposure with the same Ag (18).

**Functional comparison of OT-I T cells following a.c. and s.c. administration of OVA**

Differences between CTL activity after OVA in CFA injection of mice previously given HuIgG or OVA in the a.c. could be attributed to decreased numbers of OT-I T cells, decreased functional

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**FIGURE 4.** Ag-specific expansion of OT-I T cells. CFSE\textsuperscript{low} labeled OT-I splenocytes were transferred to naive B6 recipients 2 days before specific Ag exposure, and lymph nodes and spleens were removed for flow cytometric analysis 3 days later. Plots display CFSE fluorescence intensity of OT-I T cells from indicated tissues pooled from 3 mice receiving the same treatment. The percentage of CFSE\textsuperscript{low} cells of OT-I T cells is indicated by bars. This experiment was repeated three times with similar results.
Administration of OVA in the a.c. Seven days after the administration of OVA or HuIgG in the a.c., OT-I recipient mice were injected with OVA in CFA. A group of six OT-I recipient mice were injected with equivalent amounts of HuIgG or OVA in the a.c., or OVA in the subconjunctiva. Seven days later, OT-I T cells were enumerated in pooled axillary, brachial, and inguinal lymph nodes from three mice per group. The remaining mice were injected with OVA in CFA in the back, and OT-I T cells were enumerated in the same lymph nodes on day 10. This experiment was repeated twice with similar results.

activity, or both. Thus, the lytic activity of effector cell cultures was corrected to account for differences in OT-I T cell numbers between groups of mice given HuIgG or OVA in the a.c. Consistent with data shown in Fig. 5, a greater number of OT-I T cells was present in cultures from OVA in CFA-primed mice that were previously given HuIgG in the a.c. than in cultures from primed mice previously given OVA in the a.c. (data not shown). However, when the lytic activity was corrected for the differences in OT-I T cell numbers in the CTL assay, the lytic activity in cultures from primed mice previously given OVA in the a.c. was lower than that in cultures from primed mice given HuIgG in the a.c. (Fig. 6B). This suggests that OT-I T cells have decreased lytic capacity in mice given OVA in the a.c. However, there is a caveat to this experiment. The CTL precursors did not differentiate under identical conditions, because the number of OT-I T cells stimulated in vitro was not equal between the two treatment groups. Precursor frequency has been shown to affect the differentiation of OT-I T cells into lytic effectors (21). Therefore, a second approach was used in which an equivalent number of OT-I T cells from OVA in CFA-primed mice given OVA in the a.c. and from those given HuIgG in the a.c. were stimulated in vitro (Fig. 6B). In these experiments, draining lymph nodes were harvested 10 days after OVA-in-CFA administration. This time point was chosen because OT-I T cells expanded to peak levels and contracted in the draining lymph node. However, the percentage of OT-I T cells was slightly higher in primed mice given HuIgG a.c. Therefore, draining lymph node cells containing $2.0 \times 10^5$ OT-I T cells from OVA in CFA-primed OT-I transfused mice receiving indicated treatments were diluted with draining lymph node cells from nontransferred mice primed with OVA in CFA after receiving the same treatments. This process equalized both the number of OT-I T cells and number of primed lymph node cells in cultures from mice given OVA in the a.c. or HuIgG in the a.c. This experiment also showed that CTL activity was significantly decreased in cultures from mice given OVA in the a.c. (Fig. 6B). Thus, OT-I T cells from mice given OVA in the a.c. display decreased lytic capacity as well as decreased numbers of OT-I T cells compared with those from mice receiving HuIgG via the a.c. before OVA in CFA.

To determine whether the decreased lytic capacity of OT-I T cells from mice that were given OVA in the a.c. represented tolerance or merely decreased secondary responsiveness to Ag, CTL activity in these mice was compared with the CTL activity of mice given OVA scon, a route that induces immunity. The number of OT-I T cells from draining lymph nodes of mice given OVA in the a.c. or OVA scon, before priming with OVA in CFA, were equalized by diluting them with lymph node cells of nontransferred mice, as described above. CD8$^+$ V$\beta$8$^+$ OVA$\alpha$57–264 tetramer$^+$ cells were undetectable in primed nontransferred mice receiving either ocular treatment (data not shown). The lytic activity in cultures from OVA in CFA-primed nontransferred mice (Fig. 7, △ and ○) was significantly decreased in cultures from mice given OVA in the a.c. (74% inhibition) when compared with cultures from mice given OVA scon. Lytic activity in cultures of lymph node cells from recipients of OT-I T cells was higher than in nontransferred cultures, indicating that OT-I cells contributed to the total lytic activity (Fig. 7, △ and ○). Although both cultures contained the same number of input OT-I T cells, the lytic activity attributed to OT-I T cells was lower in cultures from mice given OVA in the a.c. (42% inhibition) compared with OVA-scon cultures. Therefore, OT-I T cell lytic activity was decreased on a per-cell basis in cultures from mice given OVA in the a.c. and was the result of tolerance rather than a reflection of previous exposure to the same Ag.

![FIGURE 5](image5.png)

**FIGURE 5.** OT-I T cell expansion following administration of OVA in CFA. Groups of six OT-I-recipient mice were injected with equivalent amounts of HuIgG or OVA in the a.c., or OVA in the subconjunctiva. Seven days later, OT-I T cells were enumerated in pooled axillary, brachial, and inguinal lymph nodes from three mice per group. The remaining mice were injected with OVA in CFA in the back, and OT-I T cells were enumerated in the same lymph nodes on day 10. This experiment was repeated twice with similar results.

![FIGURE 6](image6.png)

**FIGURE 6.** Decreased lytic capacity of Ag-specific CTL after administration of OVA in the a.c. Seven days after the administration of OVA or HuIgG in the a.c., OT-I-recipient mice were injected with OVA in CFA. A, Three days later, draining lymph nodes were harvested, and equal numbers of lymph node cells were stimulated in vitro with OVA for 3 days, and then a CTL assay was performed. The percentage of OT-I T cells was measured after in vitro stimulation and used to calculate the number of OT-I T cells added to the CTL assay. B, Ten days after administration of OVA in CFA, draining lymph nodes were harvested, equivalent numbers of OT-I T cells were stimulated in vitro for 3 days, and CTL activity was measured.

![FIGURE 7](image7.png)

**FIGURE 7.** Administration of OVA in the a.c. induces CD8$^+$ T cell tolerance. Groups of three OT-I-transferred or nontransferred mice injected with OVA in the a.c. (▲ and △) or scon (● and ○) 7 days earlier were then injected with OVA in CFA. Ten days later, draining lymph nodes were harvested and lymph node cells were stimulated with OVA for 3 days. CTL responses from lymph node cells of nontransferred mice (△ and ○) given indicated treatments were compared with lymph node cells from OT-I-transferred cultures containing $2 \times 10^4$ OT-I T cells (▲ and ●) that were given similar treatments. Each symbol represents the mean of triplicate measurements. Error bars represent the SD of the mean. E.G7-OVA cells were used as targets. This experiment was repeated twice with similar results.
Discussion

Previous studies from our laboratory have shown that the administration of soluble Ag via the a.c. of the eye results in a form of tolerance that reduces both CD8+ T cell-mediated cytotoxic and CD4+ T cell-mediated DTH responses (4). In this study, we confirm and extend those observations, showing that the inhibition of CTL responses is the result of functional unresponsiveness rather than clonal deletion of Ag-specific CD8+ T cells.

To determine whether the reduction in CTL responses was the result of functional unresponsiveness of Ag-specific T cells, we used an adoptive transfer approach, involving the transfer of transgenic T cells of known specificity into naive syngeneic recipients to follow the fate of these cells in vivo during the induction and expression of ACAID. This approach has previously been used by two laboratories (19, 22) to track Ag-specific cell division.

The increased number of OT-I T cells in the submandibular lymph nodes and spleen following the administration of OVA in the a.c. indicates that these cells were functionally unresponsive. We conclude that decreased CTL responses in ACAID are the result of functional unresponsiveness rather than clonal deletion of Ag-specific CD8+ T cells.

Acknowledgments

We thank Jing Wen for excellent technical assistance and Lily Wang for preparation of tetratomers. We also thank R. Lee Reinhardt and Peter Reichert of Marc Jenkins laboratory for excellent training in essential techniques of visualizing Ag-specific T cells by the method of adoptive transfer of transgenic T cells. James Zimring contributed invaluable discussions on experimental design.

References


