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

An immune response in the eye is required to eliminate pathogens that might damage the delicate microanatomy of the eye and cause blindness. However, this immune response poses a threat to the eye, because nonspecific inflammation can lead to tissue damage. As an evolutionary adaptation, a highly regulated immune response, which is capable of eliminating pathogens while minimizing nonspecific inflammation, occurs in the eye. This phenomenon is reflected by extended survival of foreign tissues placed in the anterior chamber (a.c.), the vitreous cavity, and the subretinal space of the eye, compared with that of tissues grafted in conventional sites such as the peritoneal cavity or the skin (1, 2). In addition, the injection of antigenic material into the a.c. of the eye of mice and rats induces a unique form of systemic tolerance, termed a.c.-associated immune deviation (ACAID) (3). For example, recipients of soluble Ag, such as OVA, in the a.c. display inhibited delayed-type hypersensitivity (DTH) and CTL responses when an immunogenic form of OVA is injected s.c. (4). Nevertheless, total IgG production is unaffected, while the isotypes of IgG Abs that fix complement, IgG2a, IgG2b, and IgG3, are inhibited in some strains of ACAID mice (5). The deficiency in DTH and IgG isotypes that fix complement suggests that Th1 functions of CD4+ T cells are inhibited in this form of tolerance to exogenous Ags.

In support of the theory that Th1 function is inhibited in ACAID, Takeuchi et al. (6) have shown that CD4+ T cells are deviated toward a Th2 phenotype when stimulated by TGF-β-treated peritoneal exudate cells (PEC). TGF-β is an immune suppressive cytokine present in the aqueous humor (7), the fluid filling the a.c., and PEC cultured in aqueous humor or TGF-β become ACAID inducing when transferred to naive mice (8). Qualitative changes to the APC, induced by TGF-β, correlate with quantitative changes in the expression of costimulatory molecules and the production of cytokines. For instance, PEC cultured in the presence of aqueous humor or TGF-β show decreased expression of CD40 and decreased production of IL-12 (6). CD4+ T cells stimulated with TGF-β-treated PEC secrete IL-4 but not IFN-γ, whereas CD4+ T cells stimulated with untreated PEC secrete IFN-γ exclusively (6). In addition, transgenic CD8+ T cells stimulated with Ag-pulsed TGF-β-pretreated PEC became efferent suppressors that inhibited DTH responses when transferred to naive mice (9). The deviation of CD4+ T cells from a Th1 to a Th2 phenotype combined with suppressor T cell generation explains, in part, the reduced DTH responses and the reduction in complement-fixing Abs observed in ACAID.

The mechanisms involved in the inhibition of CTL responses in ACAID are not yet understood. CD8+ αβ T cells primarily recognize endogenously synthesized peptides presented by MHC class I molecules on the cell surface of APC (10). However, phagocytic cells have been shown to process exogenous proteins, load peptides onto MHC class I molecules, and stimulate CD8+ T cells (11). Moreover, mice given OVA emulsified in CFA display robust Ag-specific CTL responses (12, 13). Induction of this CTL response requires adjuvant and phagocytic cells (13). Therefore,
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 procedures on animals were conducted according to the principles in the guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, DC).

Ages and adjuvants

Grade VI chicken egg OVA and human IgG (HulG) 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. RBC were removed by hypotonic lysis and then splenocytes were used to determine the percentage of the OT-I T cells.

Target cell lines

E.G7-OVA, an H-2 b tumor cell line that expresses the chicken gene encoding OVA (2 mg/ml) in CFA, and OVA (0.5 mg/ml) in IFA emulsions were prepared. RBC were removed by hypotonic lysis and then splenocytes were used to determine the percentage of the OT-I T cells. 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 fluorescence dye that distributes equally to daughter cells upon cell division (17). In these experiments, 2.5-5.0 × 106 CD8+ TCRβ5+ cells were collected for analysis.

C TL assay

After injection of OVA in CFA, 1.0-3.0 × 106 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. Flowing incubation at 37°C in 5% CO2 atmosphere, the number of live cells was determined by trypan blue exclusion and a standard 4-h Cr51 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, Viabix-Probe (7AAD) from BD PharMingen was added to stained cultures. Only Viabix-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; MTL, Paramus, NJ). The footpad swelling in response to OVA was calculated by the following formula: thickness of Ag-in-IFA footpad (mm) − thickness of PBS-in-IFA footpad (mm).

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 recipient 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 OVA.257-264 presented

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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 ACAID.

**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). However, CTL responses induced by OVA in CFA are independent of CD4+ T cells (12). 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+Vα85 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 HuIgG. Three days later, ipsilateral and contralateral submandibular lymph nodes and
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 subconunctiva 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 subconunctiva (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 ± 16 × 10^3 cells) compared with that of mice given OVA in the a.c. (60 ± 0.7 × 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 (× 10^4)</th>
<th>Total OT-I T Cells (× 10^3)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Submandibular lymph nodes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>365.0 ± 145.0</td>
<td>13.5 ± 4.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA/CFA s.c.</td>
<td>325.0 ± 114.0</td>
<td>12.7 ± 6.3</td>
<td>−1.1</td>
<td></td>
</tr>
<tr>
<td>OVA a.c.</td>
<td>845.0 ± 313.0</td>
<td>77.1 ± 26.3</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>Popliteal lymph nodes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>23.5 ± 10.2</td>
<td>1.2 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA/CFA s.c.</td>
<td>1,020.0 ± 709.0</td>
<td>202 ± 155</td>
<td>168.3</td>
<td></td>
</tr>
<tr>
<td>OVA a.c.</td>
<td>27.8 ± 15.7</td>
<td>1.3 ± 0.7</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Axillary lymph nodes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>105.0 ± 47.5</td>
<td>4.2 ± 1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA/CFA s.c.</td>
<td>75.0 ± 25.0</td>
<td>4.4 ± 2.7</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>OVA a.c.</td>
<td>56.0 ± 23.0</td>
<td>3.0 ± 1.3</td>
<td>−0.7</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>5,380 ± 1,530</td>
<td>143 ± 19.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA/CFA s.c.</td>
<td>5,800 ± 1,890</td>
<td>289 ± 215</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>OVA a.c.</td>
<td>6,730 ± 1,840</td>
<td>532 ± 189</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>2 Ipsilateral submandibular lymph nodes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HulG a.c.</td>
<td>275 ± 140</td>
<td>9.5 ± 5.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA a.c.</td>
<td>645 ± 193</td>
<td>87.9 ± 40.0</td>
<td>9.3</td>
<td></td>
</tr>
<tr>
<td>Contralateral submandibular lymph nodes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HulG a.c.</td>
<td>173 ± 49</td>
<td>5.5 ± 1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA a.c.</td>
<td>270 ± 179</td>
<td>6.1 ± 4.9</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HulG a.c.</td>
<td>8,700 ± 1,450</td>
<td>118 ± 25.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA a.c.</td>
<td>13,000 ± 896</td>
<td>407 ± 85.6</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>3 Ipsilateral submandibular lymph nodes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HulG scon</td>
<td>298 ± 44.4</td>
<td>6.0 ± 2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA scon</td>
<td>498 ± 93.0</td>
<td>93.8 ± 45.5</td>
<td>15.6</td>
<td></td>
</tr>
<tr>
<td>Contralateral submandibular lymph nodes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HulG scon</td>
<td>184 ± 96.1</td>
<td>4.3 ± 3.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA scon</td>
<td>255 ± 42.3</td>
<td>6.3 ± 3.2</td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>

* B6 mice were given 1.0 × 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 in experiment 1 to mice injected with HulG a.c. in experiment 2 or mice injected with HulG scon in experiment 3 by a two-tailed t test with unequal variance. Boldface indicates p < 0.05.

![FIGURE 3. Kinetics of expansion of OT-I T cells in the submandibular lymph nodes following the injection of OVA in the a.c. or OVA scon.](http://www.jimmunol.org/)
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 spleen cells 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 HulG 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 (CFSEhigh) 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 CFSElow cells was drawn. CFSElow 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 HulG in the a.c. showed a similar percentage of CFSElow 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 HulG a.c. (Table I; experiment 2). Mice injected with OVA in the a.c., however, showed a marked increase in CFSElow cells in all lymph nodes tested, as well as in the spleen. However, the most pronounced increase in OT-I CFSElow 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 CFSElow 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 CFSEhigh 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 CFSElow 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 scon, or HulG 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 scon had returned to baseline levels equivalent to those of mice that were given the irrelevant Ag HulG 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 × 10⁴) or OVA scon (130.6 ± 43.4 × 10⁴), but significantly less than mice given HulG in the a.c. (538.4 ± 156.6 × 10⁴). 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 scon administration of OVA

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

FIGURE 4. Ag-specific expansion of OT-I T cells. CFSE-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. The percentage of CFSElow cells of OT-I T cells is indicated by bars. This experiment was repeated three times with similar results.
HuIgG in the a.c., OT-I-recipient mice were injected with OVA in CFA. A
administration of OVA in the a.c. Seven days after the administration of OVA or
added to the CTL assay. After in vitro stimulation and used to calculate the number of OT-I T cells
a CTL assay was performed. The percentage of OT-I T cells was measured
slightly higher in primed mice given HuIgG a.c. Therefore, drain-
ning lymph node cells containing $2.0 \times 10^7$ OT-I T cells from OVA
in CFA-primed OT-I-transferred mice receiving indicated treat-
ments were diluted with draining lymph node cells from nontrans-
ferred 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 toler-
ance 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 equal-
ized by diluting them with lymph node cells of nontransferred
mice, as described above. CD8$^+$ V8$^+$ OVA$^{57-264}$ 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.

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. 6A).
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 iden-
tical 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 effect the differentiation of OT-I T
cells into lytic effectors (21). Therefore, a second approach was
used which an equivalent number of OT-I T cells from OVA in
CFA-primed mice were stimulated in vitro (Fig. 6B). In these ex-
periments, draining lymph nodes were harvested 10 days after
OVA-in-CFA administration. This time point was chosen because
OT-I T cells have 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, drain-

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, bra-
chial, 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. Decreased lytic capacity of Ag-specific CTL after admin-
istration 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. 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 CD4+ T cells (DO11.10) in BALB/c recipients after the injection of whole OVA or OVA peptides in the a.c. of the eye. We used the same model Ag, OVA, to track CD8+ TCR transgenic cells (OT-I) (14) in B6 recipients. We extended the previous studies by determining the fate of Ag-specific T cells after OVA in CFA administration of mice previously given OVA in the a.c. or OVA scon. The number of OT-I T cells increased in the submandibular lymph nodes and spleen following the administration of OVA in the a.c. Thus, expansion rather than deletion of Ag-specific CD8+ T cells is elicited by delivery of Ag to the a.c. The increase was Ag specific and involved cell division.

The expansion of OT-I T cells in both the submandibular lymph nodes and spleen suggests that Ags delivered in the a.c. may be drained by both the afferent lymphatics and the venous system. In support of this interpretation, aqueous humor has been shown to flow directly into the venous system through the trabecular meshwork via Schlemm’s canal and also into the extracellular spaces of the ciliary body (23). This process is aided by an incomplete endothelial cell layer of the anterior part of the ciliary body, which is believed to provide access to the afferent lymphatics via the episcleral spaces (23). Dendritic cells are present in both the ciliary body and the trabecular meshwork (24–26). Thus, Ags injected into the a.c. that are processed by ocular APC, theoretically could exit the eye via Schlemm’s canal into the venous system and hence to the spleen, or via the uveal-scleral route through the ciliary body to the suprachoroidal space to the episcleral spaces, which are drained by the conjunctival lymphatics (23).

The increased number of OT-I T cells in the submandibular lymph nodes and spleen following an a.c. injection with OVA indicate that clonal deletion does not explain the decreased CTL responses in ACAID. Therefore, alternative mechanisms of CTL inactivation were tested. First, the magnitude of OT-I T cell expansion was evaluated between ACAID and non-ACAID mice. Ag delivered to the subconjunctiva, a route that drains into the submandibular lymph nodes but does not induce tolerance (20), induced greater expansion of OT-I T cells in the submandibular lymph nodes than did the administration of OVA in the a.c. This difference in T cell expansion may be explained by availability of Ag. Because Ag injected into the a.c. escapes the eye by both the afferent lymphatics and venous system, the amount of Ag present in the submandibular lymph node may be lower in mice given OVA in the a.c. than those given OVA scon. Conversely, Ag injected into the subconjunctiva is likely to drain exclusively to the lymph nodes, resulting in more available Ag to stimulate naive T cells. In support of this interpretation, Kaech et al. (27) have recently shown that the amount of available Ag determines the number of T cells that are recruited from the naive T cell pool for activation and proliferation. Nevertheless, the injection of OVA in the a.c. or scon induced OT-I T cell expansion in the draining lymph nodes with similar kinetics of expansion and contraction. Upon secondary stimulation with OVA in CFA, OT-I T cells expanded equivalently in mice previously given OVA in the a.c. or OVA scon, indicating again that clonal deletion could not explain decreased CTL activity in mice given OVA in the a.c.

Expansion of CD4+ T cells with a subsequent loss of effector function has been reported for CD4+ TCR transgenic T cells in recipient mice after tolerance induction by intravenous (18, 28) and mucosal administration (29) of Ags. In addition, viral immune evasion due to unresponsive CD8+ T cells has been observed in chronic lymphocytic choriomeningitis virus infections (30) and recently in response to polyoma virus-induced tumors (31). To determine whether Ag-specific CD8+ T cells were functionally unresponsive after ACAID induction, we stimulated equivalent numbers of OT-I T cells harvested from mice given OVA in CFA that were previously injected with OVA in the a.c. or OVA scon to assure that CTL precursors in each treatment group differentiated under similar conditions. In these experiments, the lytic activity attributed to OT-I T cells was significantly less in mice given OVA in the a.c. indicating 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 tetramers. 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


