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In Vivo Behavior of Peptide-Specific T Cells During Mucosal Tolerance Induction: Antigen Introduced Through the Mucosa of the Conjunctiva Elicits Prolonged Antigen-Specific T Cell Priming Followed by Anergy¹

Rita M. Egan,* Chris Yorkey,[†] Richard Black,[†] Wai Khan Loh,[†] Julia L. Stevens,[‡] Eugene Storzynsky,[§] Edith M. Lord,[§] John G. Frelinger,[§] and Jerold G. Woodward^{2†}

The mucosa of the conjunctiva is an important site of entry for environmental Ags as well as Ags emanating from the eye itself. However, very little is known about T cell recognition of Ag introduced through this important mucosal site. We have characterized the in vivo process of CD4 T cell recognition of Ag delivered via the conjunctival mucosa. Application of soluble OVA to the conjunctiva of BALB/c mice induced potent T cell tolerance. APC-presenting OVA peptide in vivo was only found in the submandibular lymph node and not in other lymph nodes, spleen, or nasal-associated lymphoid tissue. Similarly, in TCR transgenic DO11.10 adoptive transfer mice, OVA-specific CD4⁺ T cell clonal expansion was only observed in the submandibular lymph node following conjunctival application of peptide. These experiments thus define a highly specific lymphatic drainage pathway from the conjunctiva. OVA-specific T cell clonal expansion peaked at day 3 following initiation of daily OVA administration and gradually declined during the 10-day treatment period, but remained elevated compared with nontreated adoptive transfer mice. During this period, the T cells expressed activation markers, and proliferated and secreted IL-2 in vitro in response to OVA stimulation. In contrast, these cells were unable to clonally expand in vivo, or proliferate in vitro following a subsequent OVA/CFA immunization. These results suggest that Ag applied to a mucosal site can be efficiently presented in a local draining lymph node, resulting in initial T cell priming and clonal expansion, followed by T cell anergy. *The Journal of Immunology*, 2000, 164: 4543–4550.

The conjunctival tissue surrounding the eye is a mucosal surface in continual contact with tear fluid and is an important point of entry for environmental Ags and infectious agents (1). The conjunctiva, along with the secretory lacrimal gland, share many features in common with other mucosal-associated lymphoid tissues (1, 2). Like gut-associated lymphoid tissue, conjunctival-associated lymphoid tissue (CALT)³ contains lymphocytes interspersed in the epithelial layer and in the substantia propria. In the human, the intraepithelial lymphocytes are predominately CD8⁺ T cells, as in the intestine (3). In the substantia propria, there are equal proportions of CD8⁺ and CD4⁺ T cells that can aggregate into follicles, and a small number of B cells and

mast cells. The conjunctival epithelium also contains dendritic cells and Langerhans cells capable of functioning as APC (4). Human mucosal lymphocyte-1 Ag (β_7 integrin) is present on conjunctival lymphocytes as in other mucosal tissues (5), suggesting that CALT is part of a common mucosal system that directs the selective homing of $\alpha_4\beta_7$ integrin-expressing T cells (6, 7). The mechanism of Ag transport across the epithelial cells into the conjunctival mucosa is unknown. Although some investigators have discussed the presence of M cells in the conjunctival epithelium (5), convincing data demonstrating their existence in CALT have not yet been forthcoming (8). Nevertheless, the conjunctiva contain lymphoid elements common to other mucosal tissues and appear to be a part of a common mucosal immune system.

Infectious agents entering through mucosal sites generally elicit immune responses with a Th2 characteristic and dominated by IgA Ab production (6, 9). On the other hand, the predominant response to inert, T-dependent Ags is one of tolerance (10, 11). The phenomenon of oral tolerance has been extensively studied, and multiple mechanisms appear to be involved, including active suppression and anergy (12). Ag delivered to the nasal-associated lymphoid tissue (NALT) (13) or the bronchial-associated lymphoid tissue (14, 15) has also been shown to induce a state of tolerance. This has led to the suggestion that the common mucosal-associated lymphoid tissues have evolved specialized mechanisms to generate T cell tolerance toward mucosally delivered Ags. Although extensive analysis has been done on T cells tolerized as a result of oral tolerance, studies are hampered by the difficulty in controlling the site and dosage of Ag coming in contact with the intestinal mucosa. Nevertheless, two classes of tolerized T cells have been described depending on the dose of Ag used to feed the

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³ Abbreviations used in this paper: CALT, conjunctival-associated lymphoid tissue; CFSE, 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester; DTH, delayed-type hypersensitivity; IFA, immunofluorescence assay; LN, lymph node; NALT, nasal-associated lymphoid tissue; PPD, purified protein derivative; X-Gal, 5-bromo-4-chloro-3-indolyl α -D-galactoside.

mice. Low dose feeding induces regulatory cells capable of suppression mediated by Th2 cytokines and TGF- α , while high dose feeding induces a state of anergy (12). One study using TCR transgenic T cells in an adoptive transfer model provided more direct evidence of clonal anergy among the Ag-specific T cells, but failed to demonstrate active regulatory cells (16). Despite these studies, major questions still remain concerning the sequence of events resulting in Ag-specific T cell tolerance in mucosal tissues. Furthermore, it is not clear whether the mechanisms of tolerance induction are the same for all mucosal sites. Clearly, the intestinal mucosa differs from other mucosal sites in terms of the shear load of antigenic stimulus, and therefore, it is important to evaluate other mucosal sites as well.

In this study, we demonstrate that application of Ag to the conjunctival mucosa induces potent immunological tolerance at doses considerably less than required for oral tolerance. Using a T cell hybridoma assay for APC and a TCR adoptive transfer system, we localized the major anatomical location of APC presentation and T cell recognition of conjunctival administered Ag to the submandibular lymph node (LN). Finally, we demonstrate that the continuous toleragenic application of Ag to the conjunctiva induces initial T cell priming and clonal expansion in the submandibular LN over a 10-day period, but ultimately results in a population of T cells that are anergic *in vivo* and *in vitro*. These findings suggest that tolerance in the form of T cell anergy can result from the continuous presentation of Ag in a specific LN draining the mucosal site of Ag contact.

Materials and Methods

Animals

DO11.10 transgenic mice were originally produced by Murphy et al. (17) and kindly provided by Dr. Marc Jenkins (University of Minnesota, Minneapolis, MN) with permission from Dr. Dennis Loh (Hoffman-LaRoche, Nutley, NJ). These mice have been backcrossed over 15 generations to BALB/c, and are currently being bred in our specific pathogen-free facility according to National Institutes of Health and American Association for the Accreditation of Laboratory Animal Care (AAALAC) guidelines. BALB/c mice were purchased from Charles River (Raleigh, NC) under a contract with the National Cancer Institute. The *s.c.* Ag injections described below were conducted under general anesthesia using Metophane (Pittman-Moore, Mundelein, IL).

Conjunctival administration of Ag and proliferative responses in BALB/c mice

BALB/c mice received various doses of OVA or PBS administered topically in a volume of 2.5 μ l to the conjunctiva of both eyes twice per day for 10 days, or a single application, as indicated in the figure legends. Four days following the last Ag administration, or 14 days following a single injection, OVA (100 μ g) in 0.1 ml of 1:1 CFA was injected *s.c.* in the back evenly distributed at the nape of the neck and both flanks. Seven days post-OVA/CFA immunization, cells from axillary, brachial, and inguinal LN were combined and incubated at 5×10^5 cells/well in 96-well plates containing OVA (25–100 μ g/ml) in RPMI media supplemented with 10% FCS, 10 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 5×10^{-5} M 2-ME in a humidified incubator with 7% CO₂ at 37°C. Cultures were incubated for 5 days with the addition of 1 μ Ci/well [³H]thymidine for the final 24 h. Cells were harvested onto filters, and the incorporated cpm determined on a Packard Matrix 96 direct beta counter (Downers Grove, IL). Results are expressed as the mean cpm \pm SEM of triplicate cultures.

Measurement of delayed-type hypersensitivity (DTH) responses by ear swelling

OVA or PBS was administered to the conjunctiva of groups of five mice, as described in the figure legends. On day 14, OVA (100 μ g) in 0.1 ml of 1:1 CFA was injected *s.c.* in the back and flanks. A group of unmanipulated (naive) BALB/c mice served as a negative control. On day 28 (14 days after the CFA injection), OVA (100 μ g in PBS) or PBS alone was injected in a volume of 10 μ l into the pinnae of the right or left ear, respectively.

Ear swelling was measured 24 and 48 h later and expressed as the mean (\pm SD) difference between right and left ears for groups of five mice.

Measurement of APC-presenting OVA *in vivo*

A T cell hybridoma specific for OVA_{323–339}/I-A^d and harboring an IL-2-*lacZ* fusion gene was produced by fusion of the BWZ.36 T cell hybridoma line with CD4⁺ T cells derived from the DO11.10 TCR transgenic mouse. BWZ.36 is a variant of BW5147 transfected with the IL-2 promoter-*lacZ* fusion construct (18). A hybridoma subclone, BDZ 26.2, was selected and was confirmed to express *lacZ* only in the presence of OVA_{323–339} and I-A^d-bearing APC (19). To assay for APC-presenting OVA, 5×10^5 BDZ cells/well were plated in triplicate in 96-well plates along with 5×10^5 LN or spleen cells/well or dissociated tissue from mice injected with OVA_{323–339}. As a positive control, OVA_{323–339} (10 μ g/ml) was added to wells containing BDZ cells only, or wells containing BDZ cells plus LN, spleen, or tissue. The cells were cultured for 18 h at 37°C and then fixed and stained with 5-bromo-4 chloro-3-indolyl α -D-galactoside (X-Gal), as previously described (19, 20). Activated cells were identified as blue staining cells, indicating expression of β -galactosidase, and quantitated by counting under an inverted microscope. The number of blue staining cells/well varies linearly with the number of added spleen cells over a three-log range in the presence of OVA_{323–339}, indicating that this method is semiquantitative (data not shown).

Adoptive transfer of transgenic T cells

Adoptive transfer of CD4⁺, KJ1-26⁺ T lymphocytes from transgenic mice was performed as previously described (21, 22). Briefly, LN and spleen cells from DO11.10 transgenic mice were treated with anti-CD8 mAb (hybridoma supernatant from clone 83.12.5) and rabbit complement, and the percentage of KJ1-26⁺, CD4⁺ T cells was determined by flow cytometry. A cell suspension containing 2.5×10^6 KJ1-26⁺, CD4⁺ T cells was injected *i.v.* by tail vein in a volume of 0.2 ml of HBSS into unirradiated BALB/c mice. Adoptive transfer recipients were used within 10 days of transfer. In some experiments, DO11.10 cells were stained immediately before transfer with the fluorescein-based dye 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE), as previously described (23).

Inoculation of Ag into adoptive transfer recipients

For *s.c.* injections, anesthetized mice (three in each group) were injected in the nape of the neck and in both flanks with a total of 100 μ g of OVA_{323–339} (synthesized by the University of Southern California Microchemical Facility, Los Angeles, CA) in 0.1 ml 1:1 CFA or IFA on day 0. For *i.v.* administration, 100 μ g OVA_{323–339} in 0.2 ml dH₂O was administered by tail vein on day 0. For conjunctival application, PBS or 25 μ g OVA_{323–339} in 2.5 μ l dH₂O was applied to the conjunctiva once per day. This was accomplished by restraining an unanesthetized mouse and applying 2.5 μ l of the OVA solution directly to the open eye using a microliter pipettor. In some cases, 100 μ g of OVA_{323–339} in 0.1 ml CFA was administered *s.c.* in the back on day 14.

On the days indicated in the text, mice were euthanized, and the submandibular, cervical, axillary, inguinal, and mesenteric LN or spleen removed separately for analysis. The individual LN and spleen of each of the three mice were combined for flow cytometry and proliferation assays.

Flow cytometry

KJ1-26 mAb (clonotypic, anti-TCR) (24) was purified from KJ1-26 hybridoma supernatant using a protein A-Sepharose column. KJ1-26 mAb was either biotinylated using a protein biotinylation kit (Amersham, Arlington Heights, IL), or FITC conjugated (25). All other Abs were obtained from PharMingen (San Diego, CA). For two-color flow cytometry, LN or spleen cells pooled from three mice were incubated with anti-CD4 PE and KJ1-26 FITC, washed twice, and analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) collecting 50,000 events. For three-color flow cytometry, LN or spleen cells were incubated with KJ1-26 FITC, anti-CD4 PE, and anti-CD44 biotin, followed by incubation with quantum red-streptavidin (Sigma, St. Louis, MO). A gate was drawn around the CD4⁺, KJ1-26⁺ cells or the CD4⁺, KJ1-26⁻ cells, and the fluorescence intensity of the CD44 staining was then determined on these populations. For CFSE experiments, cells were stained with CD4 PE and biotinylated KJ1-26, followed by avidin-quantum red.

Proliferation and IL-2 assays

For adoptive transfer recipients, proliferation assays were performed as described for BALB/c mice alone, except that 2×10^5 cells/well were used. For the measurement of IL-2, cells were incubated as above, except that Neutridoma (Boehringer Mannheim, Indianapolis, IN) was substituted

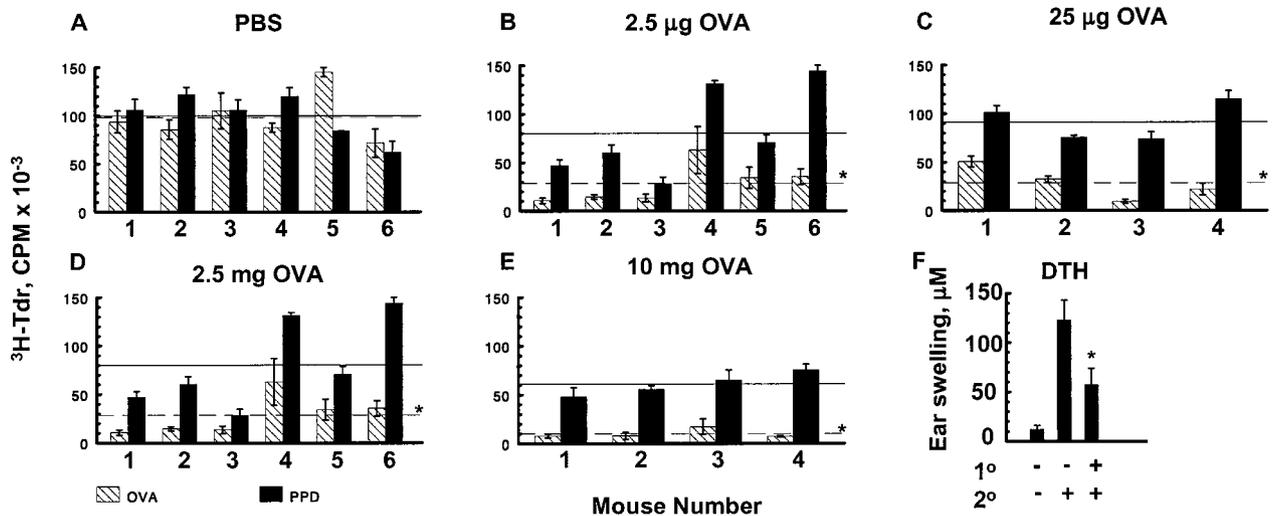


FIGURE 1. Conjunctival OVA induces tolerance. Groups of mice received conjunctival applications of PBS (A), 2.5 µg OVA in PBS (B), or 25 µg OVA (C) twice per day for 10 days. Mice were also given a single application of 2.5 mg (D) or 10 mg (E) OVA in both eyes. On day 14, all mice received OVA/CFA s.c. On day 21, axillary, brachial, and inguinal LN cells isolated from individual mice were cultured with 25 µg/ml OVA (▨) or PPD (■) for 5 days, and proliferation was determined by [³H]thymidine incorporation during the last 18 h. Results represent mean cpm ± SEM of triplicate cultures. Each pair of bars represents the response of an individual mouse. All samples had proliferative responses of less than 2500 cpm in the absence of Ag (not shown). LN cells from mice immunized with CFA alone did not show proliferation to OVA (not shown). The average response of all mice within a group is indicated by solid (PPD) and dotted (OVA) lines. The average OVA response was significantly ($p < 0.001$) reduced compared with the PPD response in all groups except A (denoted by *). F, Groups of five mice received either primary (1°) conjunctival applications of OVA (+) or PBS (-) as in group B above, followed by a secondary (2°) immunization with OVA/CFA (+) or nothing (-) on day 14. Mice were then challenged in the ear with OVA on day 28, and ear swelling was measured 24 h later. *, Significant ($p < 0.001$) decrease in ear swelling compared with the control.

for FCS. At 24 and 48 h, supernatant was analyzed for IL-2 production by ELISA (22).

Results

Conjunctivally administered OVA induces tolerance in BALB/c mice

To determine whether conjunctival application of Ag could induce tolerance, we applied different doses of OVA directly to the conjunctiva and measured OVA-specific T cell responsiveness. Low doses of OVA or PBS were applied to the conjunctiva twice per day for 10 days, or a single large dose was applied once. All mice were then immunized with OVA/CFA s.c. on day 14, and T cell proliferation to either OVA or PPD in the draining LN was assessed on day 21. The data in Fig. 1 show the proliferative responses of individual mice to optimal concentrations of either OVA or PPD. Because the degree of proliferation in response to CFA immunization can vary significantly in individual mice, the PPD response serves as an internal control for the response to OVA/CFA immunization in each mouse. The average proliferative response to OVA was significantly reduced compared with the average PPD response for all groups of mice that received conjunctival OVA, but not in mice receiving PBS. Surprisingly, we found that quite low doses (2.5 or 25 µg) of OVA induced potent tolerance if applied twice daily for 10 days (Fig. 1, A–C). To determine whether a single dose of OVA could induce tolerance, larger doses of OVA were applied to both eyes one time, and the mice were immunized 14 days later. A single application of 2.5 mg induced partial tolerance, while 10 mg induced almost complete tolerance (Fig. 1, D and E). The DTH response to OVA was also investigated. Application of 25 µg of OVA to the conjunctiva daily for 10 days resulted in a significantly depressed DTH response following immunization and challenge (Fig. 1F). These data demonstrate that application of Ag to the conjunctival mucosa induces a state of tolerance to a subsequent immunizing dose of Ag.

Ag applied to the conjunctiva drains specifically to the submandibular LN

Given that Ag introduction through the conjunctival mucosa induces tolerance, we wanted to determine the anatomic location of Ag presentation to T cells following conjunctival OVA application. To do this, we utilized a T cell hybridoma, BDZ 26.2, that expresses β-galactosidase only upon specific recognition of OVA/I-A^d complexes on APC (19). OVA_{323–339} peptide was applied to the conjunctiva of both eyes, and the LN and spleen were then tested for the presence of OVA_{323–339}-bearing APC 24 h later using the BDZ 26.2 cell line. In addition, because there is drainage from the conjunctiva to the nasal sinuses via the naso-lacrimal duct, we evaluated the presence of OVA_{323–339}-bearing APC in the NALT (26), and the conjunctiva itself. The only tissue showing significant numbers of APC-presenting OVA was the submandibular LN (Fig. 2). We also investigated other time points ranging from 2 to 96 h after administration of OVA_{323–339} and, while 24 h was optimum for OVA-bearing APC in the submandibular LN, we did not observe APC-presenting OVA_{323–339} in the other tissues at any of these time points (data not shown). Thus, these data establish the submandibular LN as the major site of Ag presentation following Ag application to the conjunctiva.

Conjunctival administration of Ag induces clonal expansion of Ag-specific, CD4⁺ T cells specifically in the submandibular LN

To determine the effect of conjunctival Ag administration on the Ag-specific T cell, we utilized an adoptive transfer system in which OVA_{323–339}-specific T cells derived from the DO11.10 transgenic mouse can be physically tracked using the clonotypic KJ1-26 mAb (21, 22). BALB/c mice received 2.5×10^6 KJ1-26⁺, CD4⁺ T cells from DO11.10 TCR transgenic mice i.v. and were used for experiments within 10 days. Adoptive transfer recipients received either daily OVA_{323–339} (25 µg) applied conjunctivally,

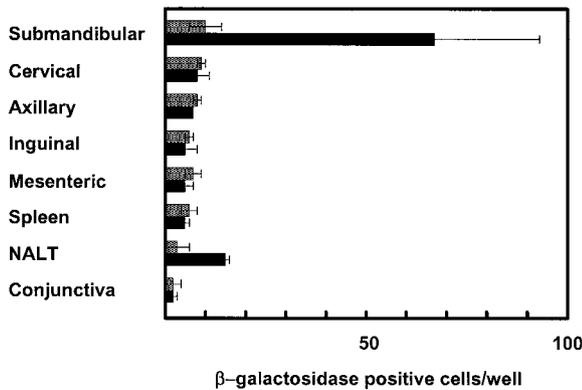


FIGURE 2. Localization of OVA/I-A^d-bearing APC in vivo following conjunctival OVA administration. Two mice were either given PBS (□) or a single dose of 100 μg OVA_{323–339} applied to the conjunctiva of both eyes (■), and then sacrificed after 24 h. Individual LN, spleen, NALT, and conjunctiva were removed, dissociated into single cell suspensions, and added to BDZ 26.2 cells in 96-well plates. As a positive control, OVA_{323–339} was also added to some of the wells from each group. After 18 h, the cells were fixed and stained with X-Gal. The total number of β-galactosidase-positive cells/well was determined by counting on an inverted microscope. Shown are the results in the absence of peptide added to the culture. In the presence of 1 μM OVA_{323–339}, the number of β-galactosidase-positive cells was as follows: LN and spleen, ~1 × 10⁴; NALT, 2128; conjunctiva, 130. The number of β-galactosidase-positive cells/well was found to vary linearly with increasing numbers of spleen cells added over a range of 10¹ to 5 × 10⁵ (data not shown).

OVA_{323–339}/CFA s.c. in the back, or no Ag. LN and spleen cells were obtained on days 3 and 6 following each route of OVA_{323–339} administration, and the presence of CD4⁺, KJ1-26⁺ T cells analyzed by flow cytometry (Figs. 3 and 4). KJ1-26⁺, CD4⁺ T cells were not detected in normal BALB/c mice and represented ~0.3% of LN or spleen cells of naive adoptive transfer recipients up to 10 days posttransfer (Fig. 3, A and B). As we (22) and others (21) have previously shown, s.c. administration of OVA_{323–339}/CFA in the back resulted in a large increase in KJ1-26⁺ T cells in the draining axillary and inguinal LN at day 6 (Fig. 3C). Following OVA_{323–339} applied conjunctivally, we observed Ag-specific T cell accumulation in the submandibular LN at day 3, with a persistence of the accumulation at day 6. However, no increase in KJ1-26⁺ cells was observed in cervical LN (Fig. 3, D–F). A similar analysis was performed on other distant LN or spleen and the results presented as the percentage of KJ1-26⁺ cells in those tissues at days 3 and 6 (Fig. 4). No increase in KJ1-26⁺ cells was found in other lymphoid tissue other than the submandibular LN.

FIGURE 3. Conjunctival OVA induces Ag-specific T cell expansion in draining LN. KJ1-26⁺, CD4⁺ T cells were quantitated in BALB/c mice (A), naive adoptive transfer mice (B), adoptive transfer mice immunized with OVA/CFA s.c. 6 days before assay (C), and adoptive transfer mice given conjunctival OVA_{323–339} (25 μg/day) for 3 days (E) or 6 days (D) before assay. Pooled axillary/inguinal LN cells (A, B, and C), cervical LN cells (D), or submandibular LN cells (E and F) from three mice were stained with anti-CD4 and KJ1-26 mAb and analyzed by flow cytometry. The percentage of KJ1-26⁺, CD4⁺ cells in C, E, and F represents significant increases ($p < 0.001$) as compared with B.

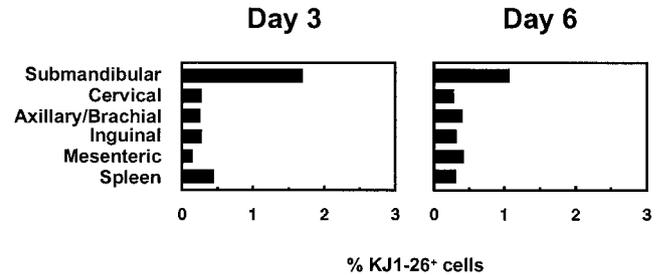
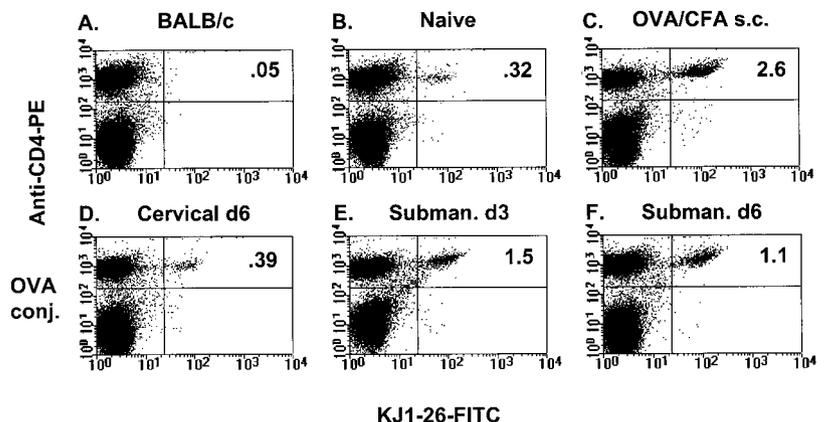


FIGURE 4. Conjunctival application of OVA elicits T cell clonal expansion primarily in the submandibular LN. Adoptive transfer recipients were treated as described for Fig. 3, D–F, and the percentage of CD4⁺, KJ1-26⁺ cells in different LN and spleen was determined by flow cytometry.

The observed increase in KJ1-26⁺ cells in the submandibular LN could be due to either a redistribution of KJ1-26⁺ cells in response to Ag, or to clonal expansion of the DO11.10 cells within the submandibular LN. To address this, DO11.10 cells were labeled with the fluorescein-based dye CFSE before transfer. CFSE stains intracellular proteins and remains stably associated with cells for long periods of time. Upon cell division, the level of staining is reduced by one-half in each daughter cell, thereby allowing the assessment of cell division using flow cytometry (23). As shown in Fig. 5, the CFSE staining in the naive CD4⁺, KJ1-26⁺ cells found within the submandibular LN was extremely bright and consisted of a single major peak. Following conjunctival OVA application, the CD4⁺, KJ1-26⁺ cells within the submandibular LN increased in number ~6.5-fold in 3 days, and the CFSE staining showed a pattern indicative of multiple cell divisions. By counting the peaks, it is possible to resolve discreet cell populations that range in the number of cell divisions from 1 to 7, with an average of 5 to 6 cell divisions. Thus, application of OVA to the conjunctival mucosa resulted in OVA-specific T cell clonal expansion within the submandibular LN, rather than redistribution of cells to that site. Furthermore, the location of KJ1-26⁺, CD4⁺ T cell clonal expansion coincides exactly with the location of Ag-bearing APC identified by the T cell hybridoma assay, further defining the submandibular LN as the primary draining LN from the conjunctival mucosa.

Following the injection of soluble peptide i.v., previous studies using this adoptive transfer system have shown that Ag-specific T cells undergo early clonal expansion with a peak at about day 3, followed by a rapid decline in numbers by day 6. The fact that continuous conjunctival application of OVA resulted in a higher than expected level of clonal expansion at day 6 suggested that the availability of Ag might be an important factor in maintaining T

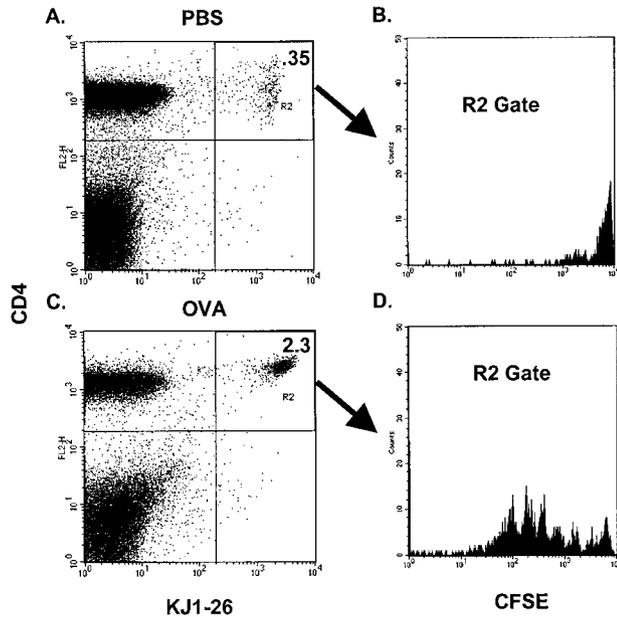


FIGURE 5. Conjunctival application of OVA₃₂₃₋₃₃₉ induces a high level of proliferation of CD4⁺, KJ1-26⁺ cells within the submandibular LN. PBS (A and B) or OVA₃₂₃₋₃₃₉ (C and D) was applied to the conjunctiva (25 μg/eye, once per day for 3 days) of adoptive transfer mice that had received CFSE-labeled DO11.10 cells. Submandibular LN cells were stained with anti-CD4 and KJ1-26 and analyzed by three-color flow cytometry. The two-color dot plot displaying CD4 vs KJ1-26 staining is displayed in A and C. The numbers in the upper right quadrant of A and C indicate the percentage of CD4⁺, KJ1-26⁺ cells of the total population. These double-positive cells in the upper right quadrant were gated (R2 gate) and further analyzed for CFSE (fluorescein) fluorescence in B and D.

cell clonal expansion. To address this issue, adoptive transfer mice were given OVA₃₂₃₋₃₃₉ via the conjunctiva continuously for 10 days, as a single dose i.v., or as a single dose s.c. emulsified in CFA, and the degree of clonal expansion was assessed over a 14-day period (Fig. 6). As previously shown, a single i.v. injection

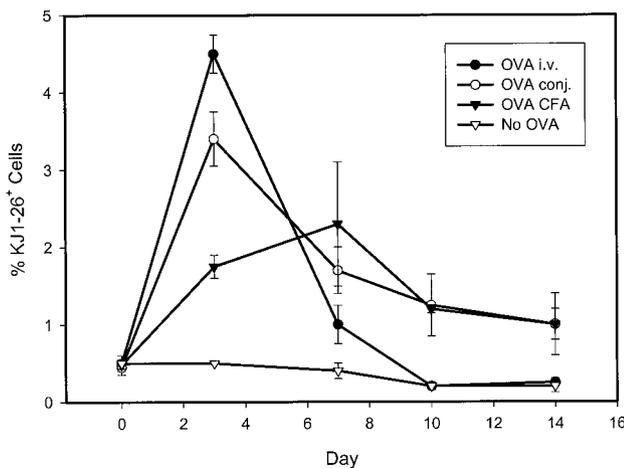


FIGURE 6. Conjunctival application of OVA₃₂₃₋₃₃₉ results in sustained clonal expansion of KJ1-26⁺, CD4⁺ cells in the draining LN. Adoptive transfer mice were given OVA₃₂₃₋₃₃₉ as a single i.v. injection of 100 μg (●), emulsified in CFA s.c. (▼), conjunctivally 25 μg/eye twice per day (○), or no Ag (▽). LN cells were prepared from pooled axillary and inguinal LN (●, ▼, ▽), or submandibular LN (○), and the percentage of KJ1-26⁺, CD4⁺ cells was determined by two-color flow-cytometric analysis on the day indicated. The data represent the mean ± SEM of the percentage of KJ1-26⁺, CD4⁺ cells determined from three individual mice analyzed separately.

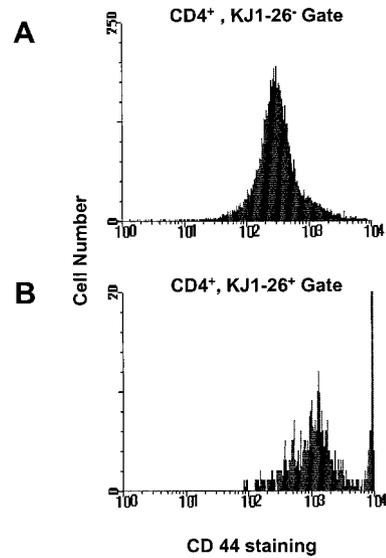


FIGURE 7. CD44 expression on conjunctivally primed submandibular LN cells. Submandibular LN cells from adoptive transfer mice given OVA₃₂₃₋₃₃₉ (25 μg/eye/day) conjunctivally for 3 days were stained with anti-CD4, KJ1-26, and anti-CD44, and analyzed by three-color flow cytometry. Shown is the histogram for CD44 intensity of cells gated in the CD4⁺, KJ1-26⁻ population (A), or cells gated in the CD4⁺, KJ1-26⁺ population (B).

of OVA induced a rapid increase in the number of KJ1-26⁺ cells, which peaked on day 3, followed by a rapid decline and reaching the level of the uninjected adoptive transfer mice by day 10. Also confirming previous results, injection of OVA₃₂₃₋₃₃₉ in CFA s.c. induced clonal expansion that peaked at a later time than the i.v. group, but was maintained at a significantly elevated level through day 14. Conjunctival application of OVA induced a rapid increase in KJ1-26⁺ cells like the i.v. group, but the expanded numbers of KJ1-26⁺ cells were maintained through day 14, like the CFA group. Thus, even though no adjuvant was present, the continuous daily application of OVA₃₂₃₋₃₃₉ resulted in a sustained clonal expansion of the KJ1-26⁺ cells. In agreement with this, these cells were found to show an activated phenotype, as assessed by an increase in CD44 staining (Fig. 7).

The KJ1-26⁺ cells in the submandibular LN are capable of proliferating and secreting IL-2

To assess the functional capacity of the expanded KJ1-26⁺ cells following conjunctival administration of OVA₃₂₃₋₃₃₉, LN or spleen cells were tested for their ability to proliferate and secrete IL-2 in response to OVA₃₂₃₋₃₃₉ stimulation in vitro. Various LN and spleen cells from adoptive transfer mice were analyzed 6 days after initiation of daily conjunctival application of OVA₃₂₃₋₃₃₉. The results in Fig. 8 show strong proliferation and IL-2 secretion from submandibular and cervical LN cells in response to OVA₃₂₃₋₃₃₉. At the cell density used (2 × 10⁵ per well), proliferation in this system is due to the transferred KJ1-26⁺ cells and not to other host-derived OVA-specific T cells present in the adoptive transfer recipient (21, 22). The fact that the cervical and, to a lesser extent, inguinal LN cells showed enhanced proliferation in this assay but no clonal expansion by day 6 may indicate that some of the conjunctivally applied OVA₃₂₃₋₃₃₉ is reaching more distant LN at levels sufficient to prime the DO11.10 cells, but not induce perceptible clonal expansion at the time points analyzed. Alternatively, KJ1-26⁺ cells initially activated in the submandibular LN

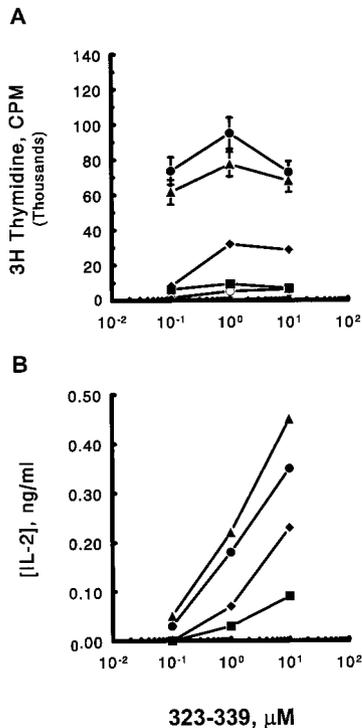


FIGURE 8. Conjunctival OVA₃₂₃₋₃₃₉ results in a primary proliferative response and IL-2 secretion of Ag-specific T cells to OVA₃₂₃₋₃₃₉. Three adoptive transfer mice received conjunctival OVA₃₂₃₋₃₃₉ (●, ▲, ◆, ■) or PBS (○) daily, and pooled submandibular LN (●), cervical LN (▲), inguinal LN (◆), or spleen cells (■) were assayed for proliferation (A) or IL-2 secretion in 48-h supernatants (B) in response to OVA₃₂₃₋₃₃₉ in vitro. Data represent the mean ± SEM [³H]thymidine incorporation (A) or IL-2 concentration (B) of triplicate cultures.

may have recirculated to other LN following initial clonal expansion. These results demonstrate that primary exposure of Ag applied to the conjunctiva in a manner that induces tolerance nevertheless results in the stimulation of Ag-specific T cells in the draining LN that are functional in vitro, at least at this early time point.

OVA-specific T cells primed as a result of initial conjunctival OVA administration are refractory to secondary stimulation in vivo

Our results in Fig. 1 showed that proliferative responses to s.c. OVA/CFA were diminished in BALB/c mice that had previously received soluble OVA₃₂₃₋₃₃₉ conjunctivally. To determine the in vivo behavior of the Ag-specific T cells under similar conditions, adoptive transfer mice were given OVA₃₂₃₋₃₃₉ conjunctivally for

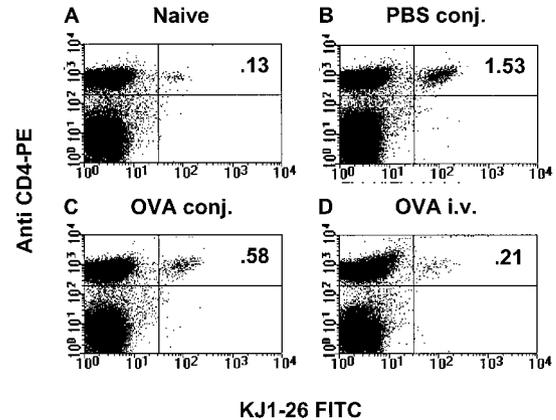


FIGURE 9. Prior treatment of adoptive transfer mice with conjunctival OVA₃₂₃₋₃₃₉ reduces the expansion of Ag-specific T cells to immunizing OVA₃₂₃₋₃₃₉/CFA. Adoptive transfer mice received either no manipulation (A), PBS conjunctivally (B), OVA₃₂₃₋₃₃₉ conjunctivally (C), or OVA₃₂₃₋₃₃₉ i.v. (D) on day 0. On day 14, OVA₃₂₃₋₃₃₉/CFA was injected s.c. (B–D), and pooled axillary, brachial, and inguinal LN cells from three mice were analyzed by flow cytometry 7 days later. The percentage of CD4⁺, KJ1-26⁺ cells is indicated in the upper right quadrant of the dot plot.

10 days, or as a single i.v. injection, and then immunized with OVA/CFA₃₂₃₋₃₃₉ 14 days later. The accumulation of KJ1-26⁺ cells in the LN draining the site of OVA/CFA injection was then determined 7 days later (Fig. 9). In adoptive transfer mice that received PBS conjunctivally, normal clonal expansion of the KJ1-26⁺ cells was observed in the DLN following OVA/CFA s.c. However, clonal expansion of KJ1-26⁺ cells in the LN draining the site of OVA/CFA injection was dramatically reduced in adoptive transfer mice that received prior OVA₃₂₃₋₃₃₉ conjunctivally. In agreement with previous studies, clonal expansion of KJ1-26⁺ cells was also reduced in adoptive transfer mice that received a prior i.v. injection of OVA₃₂₃₋₃₃₉, a well-characterized tolerogenic route of injection (21). To determine whether the remaining Ag-specific T cells in the LN draining the site of OVA/CFA immunization were functionally competent, their ability to proliferate to OVA₃₂₃₋₃₃₉ in vitro was determined and the results calculated per KJ1-26⁺ cell added to the culture (Table I). In mice tolerized by administration of OVA₃₂₃₋₃₃₉ conjunctivally or by i.v. injection, the KJ1-26⁺ T cells in the LN draining the site of OVA/CFA immunization proliferated poorly to OVA₃₂₃₋₃₃₉ compared with mice that received PBS conjunctivally. In contrast, LN cells from mice that received a primary injection of OVA/IFA s.c. showed a heightened ability to proliferate, indicating priming in this case. The fact that KJ1-26⁺ cells were present, but unable to proliferate, suggests that those cells were functionally impaired, i.e., anergic.

Table I. *In vitro* proliferation of OVA-specific T cells following secondary OVA/CFA immunization^a

Primary Ag Administration	% KJ1-26 ⁺ T Cells ^b	[³ H]Thymidine (cpm) ^c	cpm/KJ1-26 ⁺ Cell ^d
Conjunctival PBS	1.49	135,000	23
Conjunctival OVA	0.56	22,029	10
OVA i.v.	0.20	10,404	13
OVA/IFA	0.69	106,000	38

^a Adoptive transfer mice received a “primary” administration of either PBS or OVA₃₂₃₋₃₃₉ conjunctivally (25 μg/eye/day) for 10 days, a single i.v. injection of 100 μg of OVA₃₂₃₋₃₃₉ i.v., or a single s.c. injection of 100 μg of OVA₃₂₃₋₃₃₉ in IFA. All mice received a secondary injection of OVA/CFA 14 days later.

^b Axillary and inguinal LN were removed 7 days after the secondary immunization and analyzed for the percent CD4⁺, KJ1-26⁺ cells by flow cytometry.

^c LN cells were cultured in the presence of 2 μg/ml OVA₃₂₃₋₃₃₉ for 5 days, and [³H]thymidine incorporation was determined over the final 18 h of culture.

^d cpm/KJ1-26⁺ cell was calculated by dividing the cpm by the total number of KJ1-26⁺ cells added to each well (obtained by multiplying the % KJ1-26⁺ cells times 4 × 10⁵ cells/well).

Discussion

Our results demonstrate that potent immunologic tolerance can be achieved by exposure of Ag through the conjunctival mucosa. We have also identified the submandibular LN as the principal LN in which Ag-bearing APC are located and in which Ag-specific T cell clonal expansion occurs following conjunctival application of Ag. Clonal expansion was maintained at an elevated level and the T cells were responsive *in vitro* during the 10-day period of daily OVA_{323–339} application to the conjunctiva. However, in spite of the continuous Ag application, the number of Ag-specific T cells steadily declined over the 10-day period and, by day 14, the remaining OVA-specific T cells were refractory to secondary challenge with OVA/CFA. The fact that significant numbers of OVA-specific T cells were physically present, but unresponsive, indicates that they had become anergic *in vivo*.

The fact that APC-presenting OVA/I-A^d *in vivo* were only found in the submandibular LN and not NALT or mesenteric LN rules out that tolerance in our system was due to drainage of Ag through the naso-lacrimal duct and association with NALT or GALT. Rather, our results suggest that conjunctivally applied Ag drains directly to the submandibular LN and, to a lesser extent, the cervical LN. Recently, Wolvers et al. (27) have identified the same two LN as the principal LN draining the nasal cavity. (Although we have given them different names, our designation of submandibular LN corresponds to their designation of superficial cervical, and our designation of cervical corresponds to their designation of internal jugular.) Furthermore, they have shown that these two LN possess intrinsic properties promoting nasal tolerance not shared with other peripheral LN. Thus, it is possible that conjunctival tolerance and nasal tolerance operate via the same mechanism, by priming T cells within the same draining LN.

Although it ultimately results in tolerance, conjunctival administration of OVA peptide induces initial priming and clonal expansion of the KJ1-26⁺ cells within the draining LN of adoptive transfer mice. The CFSE experiments clearly showed that these cells underwent multiple rounds of cell division *in vivo*. They were also functional *in vitro*, as assessed by proliferation and IL-2 secretion. Their activated phenotype was also confirmed by CD44 staining. These results are also in agreement with our previous study showing priming of the KJ1-26⁺ cells following intraocular injection of OVA_{323–339} (22). Why then does tolerance develop after repeated conjunctival application of Ag? Our results demonstrate that the KJ1-26⁺ T cells remain partially responsive to repeated Ag stimulation via the conjunctiva for a short period of time (Fig. 6). However, the fact that the number of KJ1-26⁺ cells in the submandibular LN steadily declined over the 10-day period, in spite of continuous peptide application, indicates that these cells were progressively becoming refractory to Ag stimulation. By day 14, these cells were largely unresponsive to Ag challenge, whether in soluble form *i.v.* (data not shown) or in CFA *s.c.* (Table I). This unresponsiveness to secondary challenge in a distant *s.c.* site indicates that the conjunctival Ag treatment was effective in tolerizing the majority of KJ1-26⁺ cells in the adoptive transfer mouse, presumably due to recirculation of the T cells from the submandibular LN to other sites, and the likely systemic dispersion of some of the peptide during the 10-day treatment.

In their initial description of this adoptive transfer model, Kearney et al. (21) demonstrated that *i.v.* administration of peptide induced early clonal expansion of the KJ1-26⁺ cells, but that these cells became refractory to secondary Ag stimulation *in vivo* and *in vitro*. Similar results were also demonstrated following peptide administration in IFA *i.p.* (28). Also using the same adoptive transfer model, Van Houten and Blake (16) showed that feeding of

large quantities of OVA resulted in the systemic clonal expansion of KJ1-26⁺ cells, but that these cells subsequently became refractory to secondary stimulation. Similarly, several studies in other systems have also provided evidence that CD4⁺ T cell tolerance requires initial Ag-specific T cell priming (21, 22, 29, 30). Our results extend these studies to the conjunctival mucosa and collectively suggest that tolerance can result from systemic exposure and priming of Ag-specific T cells to soluble Ag, regardless of the route of Ag administration. In light of recent data showing that tolerance can be induced in B cell knockout mice (31), this initial priming most likely results from Ag presentation by dendritic cells in the LN and spleen.

In contrast to the initial priming and clonal expansion of the KJ1-26⁺ T cells in response to conjunctival OVA application, the expansion of KJ1-26⁺ T cells in response to a secondary immunogenic OVA/CFA immunization was suppressed and the remaining KJ1-26⁺ T cells were refractory to stimulation *in vitro*. This phenomenon has been noted previously for *i.v.* and *i.p.* tolerance (28, 32). The suppression of secondary clonal expansion following initial priming could be due to several, not mutually exclusive mechanisms. First, the initial primed cell population could induce regulatory T cells capable of down-regulating the secondary response to Ag in CFA. These cells could either be KJ1-26⁺ cells polarized to a Th2 phenotype (30, 31, 33–37), a TGF- β -secreting cell type (11), or a separate suppressor cell derived from the host that directly suppresses the KJ1-26⁺ cell. However, using a variety of *in vitro* and *in vivo* approaches, we have not detected any regulatory cells, suppressive cytokines, or bystander suppression during conjunctival tolerance (data not shown). Similarly, bystander suppression was not observed in the DO11.10 adoptive transfer system after *i.v.* tolerance (32, 38). Second, the initial priming of the Ag-specific cells could alter the traffic pattern of the cells due to alterations in the levels of adhesion molecules such as L-selectin. This could result in the increased migration of Ag-specific T cells from the LN draining the site of secondary immunization to the peripheral tissues in tolerized mice. However, this scenario would not explain the observed inhibition of the DTH response, nor the defect in the proliferative ability of cells from tolerized mice. Another possibility is that the repeated exposure of the KJ1-26⁺ T cells to Ag results in continuous apoptosis of the KJ1-26⁺ cells, which has been shown to be important in tolerance induction (39). Although it is likely that apoptosis of the KJ1-26⁺ T cells occurs as a normal consequence of activation-induced cell death, we have found no evidence that apoptosis contributes to tolerance in this system. Thus, we have been unable to induce tolerance by the injection of large numbers of irradiated (apoptotic) or unirradiated KJ1-26⁺ cells (R. Egan and J. Woodward, unpublished observations). The trivial explanation that continuous apoptosis depletes available KJ1-26⁺ cells is ruled out by our demonstration that KJ1-26⁺ cells are present at an elevated level in the tolerized mice before immunization with OVA/CFA (Fig. 6). Finally, repeated priming of the Ag-specific T cells with soluble Ag in the absence of adjuvant could have the effect of anergizing the T cells *in vivo*. Although T cell clonal anergy has been clearly demonstrated to occur *in vitro* as a consequence of TCR signaling in the absence of costimulation (40), it is much more difficult to demonstrate *in vivo*. Recently, using the DO11.10 adoptive transfer system, the Jenkins group has provided compelling evidence that the cells that are unresponsive following *i.v.* tolerance have in fact undergone prior clonal expansion and persist *in vivo* (32, 38). These results provide the strongest evidence to date that the Ag-specific, CD4⁺ T cell can be anergized *in vivo* as a consequence of soluble peptide exposure. A similar conclusion was reached by Van Houten et al. in their model of oral tolerance (16). Our results

showing that the KJ1-26⁺ cells were indeed present but hyporesponsive in vivo and in vitro following secondary peptide exposure extend this model to a system in which the in vivo clonal expansion and subsequent anergy are largely restricted to a single pair of LN draining the conjunctiva.

Collectively, our results and those of others suggest that repeated, systemic exposure of T cells to their cognate Ag in the absence of adjuvant or "danger" signals (41) induces initial T cell priming and clonal expansion, but eventually results in an anergic phenotype. Our results support a model whereby Ag applied to the conjunctiva is rapidly absorbed and drains to the submandibular LN. We propose that the subsequent mechanism of tolerance induction is similar if not identical to that which occurs following other routes of systemic Ag delivery, i.e., i.v. (21), i.p. (28), oral (42), and intraocular (43) routes. Thus, we support a model whereby these different anatomical sites of Ag delivery are viewed as facilitating systemic Ag exposure to T cells, primarily within LN and spleen. In this model, the mechanism of T cell tolerance would be the same, and only depends on the dose, timing, and affinity of Ag. It will therefore be critical to understand the signals in vivo that render T cells unresponsive after initial priming with Ag.

Dua et al. demonstrated that conjunctival instillation of retinal Ags in rats reduced the severity of experimental autoimmune uveitis and suggested that this was due to Ag-specific tolerance (44). The present study demonstrates that CD4⁺ T cell tolerance does indeed occur through a mechanism involving clonal anergy. Our results show that the Ag dosage required to induce conjunctival tolerance is considerably less than required for oral tolerance (12). Furthermore, the dosage can be controlled much more precisely and Ag application is much easier. Therefore, it is possible that conjunctival tolerance may have some clinical usefulness in inducing Ag-specific tolerance in autoimmune disease.

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