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Induction of Bystander Suppression by Feeding Antigen Occurs despite Normal Clonal Expansion of the Bystander T Cell Population

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The induction of bystander suppression, whereby the response against one Ag is suppressed when it is presented in the context of an Ag to which tolerance is already established, would be an important property of oral tolerance, because it would allow treatment of autoimmune and hypersensitivity responses where the initiating Ag is not known. Although bystander suppression has been described in oral tolerance, it is not known how its effects are mediated at the level of the bystander T cells. In addition, previous studies have not compared regimes in which Ag is fed in a tolerogenic or immunogenic manner, meaning that the possible effects of Ag competition have not been excluded. In this study we have used two populations of Ag-specific TCR transgenic CD4+ T cells to examine the cellular basis of bystander suppression associated with oral tolerance in mice in vitro and in vivo. Our results show that bystander responses can be inhibited by feeding Ag and that these effects are more pronounced in mice fed protein in tolerogenic form than after feeding Ag with mucosal adjuvant. However, the expansion of the bystander-specific CD4+ T cells is not influenced by the presence of oral tolerance. Thus, bystander suppression does not reflect clonal deletion or reduced clonal expansion of the bystander T cells, but may act by altering the functional differentiation of bystander T cells. The Journal of Immunology, 2004, 173: 6059–6064.

Oral administration of Ag has long been recognized to induce peripheral tolerance of a wide range of systemic immune responses, and this has been proposed as therapy for autoimmune disease (reviewed in Refs. 1 and 2). One of the most useful immunoregulatory mechanisms that may be induced by oral tolerance is bystander suppression, in which responses to a second, unrelated Ag can be inhibited when it is presented together with the Ag to which tolerance is already established. Functional bystander suppression has been described in a number of in vivo models of oral tolerance in autoimmune disease, where feeding Ag can prevent the induction of disease when animals are challenged with the tolerizing Ag and the autoantigen together or if the two Ags are colocalized in the same anatomical site (3, 4). There are similar reports using model Ags (5, 6), but the cellular basis of this phenomenon remains unclear, and the way in which naive T cells specific for the bystander Ag can be suppressed is not known.

A number of regulatory T cell populations have been reported to suppress the proliferation of bystander T cell populations in vitro, either via the production of cytokines such as IL-10 or TGF-β (7–11) or through cell-cell contact (12–14). However, few studies have examined bystander tolerance at the cellular level in vivo. In one report (15), two different populations of TCR transgenic CD4+ T cells were used to study bystander suppression after i.v. injection of soluble Ag, and there was no inhibition of the clonal expansion of the bystander T cell population. However, although similar protocols have been shown to induce bystander suppression in vivo (16, 17), this was not confirmed functionally after the cotransfer of transgenic T cells (15), and it remains possible that tolerance induced by feeding Ag has different effects on the expansion and differentiation of bystander T cells from those found after parental induction of tolerance.

A further issue that has not been addressed in previous studies of bystander tolerance is whether the phenomenon is induced only by feeding protocols in which the initial oral exposure to Ag produces tolerance. Thus, the possibility that Ag-exposed T cells may simply prevent activation of a bystander population by competing for access to Ag and/or growth factors due to their increased frequency or better ability to interact with APC (18) has not been excluded.

In this study we have investigated the cellular basis of bystander suppression in oral tolerance by making use of two populations of TCR transgenic CD4+ T cells of different Ag specificities whose clonal expansion can be monitored directly. We show that feeding OVA in a tolerogenic regimen is associated with suppression of in vivo responses to a second Ag (hemagglutinin (HA)) when administered together with OVA at the time of challenge and that similar, but lesser, effects may occur in the presence of productively primed T cells. However, the clonal expansion of the bystander HA-specific CD4+ T cell population was not affected by the induction of oral tolerance, suggesting that bystander suppression alters the functional differentiation of naive T cells, rather than causes a quantitative difference in their clonal expansion.

Materials and Methods

Animals

BALB/c mice were purchased from Harlan Olac (Bicester, U.K.). DO11.10 mice, with CD4+ T cells specific for OVA323–339 peptide in the context of

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I-A^d recognized by the KJ1-26 clonal Ab (19), were obtained originally from Dr. N. Lycke (University of Gothenburg, Gothenburg, Sweden), whereas 6.5 mice containing TCR transgenic CD4^+ T cells specific for the HA111–119 peptide of influenza HA A protein and I-E^d recognized by the 6.5 clonal Ab (20) were obtained from Prof. M. K. Jenkins (University of Minnesota Medical School, Minneapolis, MN). All mice were maintained on an OVA-free diet at the Central Research Facility, University of Glasgow, under specific pathogen-free conditions and were first used between 6 and 8 wk of age. Procedures were conducted according to United Kingdom Home Office guidelines.

Adaptive transfer of Ag-specific lymphocytes

Lymph nodes and spleens were homogenized by rubbing through Nitex mesh (Cadisch & Sons., London, U.K.) in RPMI 1640 medium (Life Technologies, Paisley, U.K.); the resulting cell suspensions were washed twice by centrifugation at 400 × g for 5 min and resuspended in RPMI 1640. The proportions of Ag-specific T cells were evaluated by flow cytometry, and syngeneic recipients received 3 × 10^6 Ag-specific T cells in a volume of 0.2 ml injected i.v. In some experiments cells were labeled with the fluorescent dye CFSE (Molecular Probes, Eugene, OR) immediately before use (21) by resuspending at 10^7 cells/ml in 10 μM CFSE in PBS/0.1% BSA (Sigma-Aldrich, Poole, U.K.) for 10 min at 37°C, and then were washed three times before cell culture.

Oral and systemic administration of Ags

Oral tolerance was induced by feeding OVA (Sigma-Aldrich) dissolved in 0.2 ml of saline using a rigid steel gavage tube or by inclusion of OVA in the drinking water at a dose of 5 mg/ml overnight. On the basis that mice consumed an average of 4 ml of water/mouse over the 18-h period, this gave a total dose of 20 mg of OVA. Control mice were fed OVA together with 20 μg of cholera toxin (CT; Sigma-Aldrich) or were fed saline alone.

Mice were challenged by s.c. injection in the hind footpad with 100 μg of HA111–119 peptide (Sigma-Genosys, Cambridge, U.K.), either alone or together with 100 μg of OVA emulsified in CFA (Sigma-Aldrich) in a total volume of 50 μl.

Flow cytometry

Aliquots of 10^6 cells in 12 × 75-mm polystyrene tubes (BD Falcon, Oxford, U.K.) were resuspended in 100 μl of FACS buffer (PBS, 2% FCS, and 0.05% NaN₃) containing biotinylated KJ1.26 or 6.5 anti-TCR Abs and used for access to Ag or growth factors.

Measurement of Ag-specific immune responses in vivo

Ag-specific delayed-type hypersensitivity (DTH) responses were assessed 3 days after systemic immunization, a described previously (22–24). Briefly, mice were anesthetized using halothane, and the thickness of the unimmunized rear footpad was measured using calipers (Kroeplin, Munich, Germany) before the s.c. injection of 100 μg of heat-aggregated OVA or 100 μg of HA111–119 peptide in 50 μl of saline. Footpad thickness was measured again 24 h later, and Ag-specific DTH responses were calculated by subtracting the footpad increments found after challenge of naive BALB/c mice.

Measurement of Ag-specific proliferation and cytokine production in vitro

Fourteen days after systemic immunization, single-cell suspensions of draining popliteal lymph nodes were prepared by rubbing through Nitex mesh as described above. After washing, cells were counted and resuspended at 10^6 cells/ml in complete medium alone or with 1 mg/ml OVA or 100 μg/ml HA111–119. The cells were then cultured in a total volume of 200 μl at 37°C in 5% CO₂ in air for 72–120 h in flat-bottom, 96-well tissue culture plates (Costar; Nuncopore, High Wycombe, U.K.), and proliferation was assessed by addition of 1 μCi/well [3H]Thym (West of Scotland Nucleotide Dispensary, Glasgow, U.K.) for the last 18 h of culture. DNA-bound radioactivity was harvested onto glass-fiber filter mats, and thymidine incorporation was measured on a 1205 Betaplate scintillation counter (all from Wallac Oy, Turku, Finland). To assess cytokine production, the supernatants of 1-ml cultures containing 4 × 10^6 cells stimulated as described above in 24-well plates (Costar) were sampled after 48 h. These were centrifuged at 13,000 × g and stored at −20°C until analysis by a standard sandwich ELISA protocol (IFN-γ capture R4-6A2; IFN-γ detection XMG1.2; BD Pharmingen). The plates were developed using tetramethylbenzidine substrate and read at 630 nm using a microplate reader, and the levels of cytokine in supernatants were calculated by comparison with recombinant cytokine standards (R&D Systems, Minneapolis, MN).

Purification of CD4^+ T cells

CD4^+ T cells were purified by magnetic separation using a CD4^+ T cell isolation kit (Miltenyi Biotec, Bisley, U.K.) according to the manufacturer’s instructions. After labeling cells with Abs and microbeads, the cells were washed in MACS buffer (PBS, 0.5% BSA, and 2 mM EDTA (both from Sigma-Aldrich)) and resuspended in 500 μl of MACS buffer before running through a mass spectrometry column (Miltenyi Biotech). The negatively selected fraction of cells that passed through the column was analyzed for purity by flow cytometry and was found to be routinely >95% CD4^+.

Statistical analysis

The results are represented either as the mean ± 1 SD or as readings from individual animals as appropriate. Normalized data were analyzed using Student’s t test, whereas other datasets were compared using a Mann-Whitney U test. Statistical analysis was performed using Minitab software (State College, PA).

Results

Induction of bystander tolerance by different feeding protocols

We first determined the ability of different regimens of Ag feeding to induce functional bystander suppression of responses to a second, unrelated Ag. BALB/c mice were fed OVA and challenged systemically with OVA mixed with HA peptide in adjuvant. Two protocols were used to induce tolerance: a single high dose of 20 mg of OVA, or the same dose given in the drinking water overnight; these protocols have been reported to involve different regulatory mechanisms (25). As a control, mice were also fed OVA in an immunogenic form, with CT as an adjuvant, to allow us to examine whether prior exposure to Ag in any form might influence the bystander population by competition from differentiated CD4^+ T cells for access to Ag or growth factors.

Two weeks after immunization with both Ags, draining lymph node cells from mice fed saline were responsive to both OVA and HA upon restimulation in vitro, as assessed by proliferation and IFN-γ production. In all cases, the response to HA was 1 order of magnitude lower than that of the OVA-specific response (Fig. 1). Mice fed a single dose of 20 mg of OVA or given OVA in their drinking water showed OVA-specific tolerance in vitro, as illustrated by a significantly suppressed proliferative response to OVA (Fig. 1a). Surprisingly, mice fed OVA/CT also had reduced proliferative responses compared with saline-fed controls, possibly reflecting an alteration in the kinetics of the recall response in these animals, although these were significantly higher than those found in the mice fed OVA alone (Fig. 1a). The HA-specific proliferative response showed a similar trend, with the mice fed OVA as a single dose or overnight having lower responses to HA peptide than cells from saline-fed mice (Fig. 1b). Again, mice fed OVA/CT had HA-specific responses lower than controls, indicating that some of the reduced responses may be due to competitive effects of Ag-experienced T cells. However, the responses of OVA/CT-fed mice were significantly higher than those of OVA-tolerant mice (Fig. 1b), suggesting the additional presence of active suppression of the bystander responses in tolerized animals.

Assessment of IFN-γ production in these cultures showed an identical pattern, with significantly lower levels of OVA- and HA-specific cytokine production in cultures from mice fed OVA in
FIGURE 1. Induction of functional bystander suppression by feeding Ag. BALB/c mice were fed a single dose of 20 mg of OVA (20 mg) with or without 20 μg of CT or were given OVA overnight (o/n) in their drinking water and immunized s.c. with 100 μg of OVA and 100 μg of HA emulsified together in CFA 10 days later. Controls were fed saline 10 days before immunization. Two weeks after immunization, cells from the draining lymph node were restimulated in vitro with either 1 mg/ml OVA protein or 100 μg/ml HA peptide. Data shown are the mean proliferative responses after 5 days of culture (cpm ± 1 SD) of three mice per group (a and b) or the mean concentration of IFN-γ (nanograms per milliliter) in cultures of cells pooled from three mice per group ± 1 SD (c and d). *, p < 0.05 vs saline-fed mice; #, p < 0.05 vs OVA/CT-fed mice.

tolerogenic protocols (Fig. 1, c and d). Together these results indicate that feeding soluble proteins can induce bystander suppression to an unrelated Ag. To examine this phenomenon at the cellular level, we used TCR transgenic CD4+ T cells to allow the regulatory processes to be monitored directly at the level of Ag-specific T cells.

Orally tolerized CD4+ T cells do not inhibit naive T cell expansion in vitro

First, we determined whether T cells from orally tolerized mice could suppress the clonal expansion of naive T cells specific for an unrelated Ag in vitro. CD4+ T cells were purified from mice fed 20 mg of OVA 10 days after feeding and were cultured with naive, CFSE-labeled, 6.5 TCR transgenic HA-specific T cells in vitro. The cultures were stimulated with either OVA, HA, or both Ags together, and the division of HA-specific cells was assessed by flow cytometry after 72 h. As shown in Fig. 2, HA-specific cells stimulated with HA peptide expanded efficiently, as measured by the loss of CFSE. The clonal expansion of these cells was identical in the presence of CD4+ cells from mice that had been fed OVA alone or together with CT regardless of whether the cultures were stimulated with HA only or with OVA and HA together (Fig. 2). Thus, CD4+ T cells from orally tolerized mice do not regulate the clonal expansion of a naive bystander population of CD4+ T cells in vitro.

Naive bystander CD4+ T cells expand normally in vivo despite induction of functional tolerance

We next explored whether the clonal expansion of bystander CD4+ T cells was inhibited in vivo under conditions where functional bystander tolerance could be induced. BALB/c mice received 3 × 10^6 OVA-specific DO11.10 cells 24 h before feeding them a single dose of OVA alone or with CT, and 7 days later (by which time the primary response of the DO11.10 cells had waned, and there was no residual OVA capable of naive T cell stimulation (data not shown) (26)) the recipients were transferred with HA-specific 6.5 T cells. One day after the transfer of HA-specific T cells, mice were challenged with HA, either alone or together with OVA, and the expansion of each TCR transgenic population was monitored by flow cytometry at various times after immunization. At the time of challenge, 8 days after feeding OVA, the proportion of DO11.10 cells had returned to approximately the same level as that in saline-fed controls, confirming that the primary expansion induced by feeding Ag had ended (Fig. 3, a and b). These T cells expanded efficiently in the draining lymph node in response to systemic challenge, with the peak of expansion occurring 3 days after immunization (Fig. 3, a and b). The proportion of OVA-specific T cells at the peak of the response was less in mice fed soluble OVA or OVA/CT than that in saline-fed mice (Fig. 3, a and b), confirming previous reports of impaired clonal expansion by Ag-experienced T cells (27, 28). However, the absolute number of OVA-specific T cells was not significantly altered. The clonal expansion of HA-specific T cells was assessed in the same lymph node suspensions. In mice fed saline and immunized with HA/CFA, there was good clonal expansion of the HA-specific 6.5 T cells, with the peak of the response again occurring 3 days after immunization (Fig. 3, c and d). These responses were identical in magnitude and kinetics in saline-fed mice that had been immunized with HA and OVA together (Fig. 3, c and d) as well as in mice that had been fed either OVA or OVA/CT before transfer and challenge.

The remaining adoptive transfer recipients were then assessed for a number of immune parameters to examine the induction of
bystander tolerance by these protocols. Thus, 2 wk after immunization with OVA and HA in adjuvant, draining lymph node cells were restimulated in vitro with each of the Ags. By this time the initial expansion of Ag-specific T cells had returned to baseline levels, which were not significantly different among the three groups (percentage of KJ1.26+ CD4+ cells: saline-fed, 0.343±0.021; OVA-fed, 0.356±0.046; OVA/CT-fed, 0.223±0.125). Lymph node cells from saline-fed mice transferred with DO11.10 and 6.5 cells proliferated in response to stimulation with either OVA or HA (Fig. 4, a and b) and also produced IFN-γ (Fig. 4, c and d). As anticipated, OVA-specific proliferation and IFN-γ production were decreased in lymph node cells taken from mice previously fed the tolerizing dose of soluble OVA and challenged with OVA and HA in vivo, demonstrating the presence of OVA-specific tolerance. This suppression was not evident in mice that had been fed OVA/CT (Fig. 4, a and c). HA-specific proliferation and IFN-γ production were also significantly suppressed in mice that had been fed soluble OVA to induce tolerance, but not in saline-fed mice or mice fed OVA/CT (Fig. 4, b and d).

Finally, HA-specific DTH responses were also suppressed in adoptive transfer recipients fed OVA when assessed 3 wk after challenge with OVA and HA compared with those found in saline- and OVA/CT-fed recipients (saline- vs OVA-fed, p = 0.016; OVA/CT- vs OVA-fed, p = 0.030; Fig. 5). No significant suppression was seen in mice fed OVA/CT (p = 0.735). These results further confirm the presence of functional bystander tolerance in vivo and in vitro despite the normal clonal expansion of the bystander T cell population.

Discussion

The ability of oral Ag to induce suppression of bystander responses has been demonstrated previously in a number of models (3–6), although to date the mechanisms involved have remained elusive. The prospect of bystander tolerance induced by oral Ag may be of enormous therapeutic potential depending on the fate of the bystander-specific CD4+ T cells. We therefore established a model of bystander suppression associated with oral tolerance, in which Ag-specific CD4+ T cells could be identified and monitored during the induction of bystander tolerance. Our results demonstrated that although different doses of soluble Ag without adjuvant were able to induce suppression of bystander responses, this bystander tolerance was not due to deletion or inhibition of expansion of the bystander population, suggesting that this phenomenon is due to a functional alteration of this population.

Initial experiments assessed the ability to induce suppression of a bystander response of two different doses of fed Ag. As expected, administration of a single bolus of OVA or prolonged exposure via drinking water induced OVA-specific tolerance, as assessed in vitro and in vivo. In addition, both regimens suppressed the bystander response to HA. This was unexpected, because it has been suggested that high and low doses of Ag induce different mechanisms of tolerance, with only multiple low doses of fed Ag inducing active suppression (25). However, additional experiments in which adoptive transfer recipients were fed a single bolus of OVA also suggested that functional bystander suppression could be induced effectively by this regimen of OVA feeding, suggesting that this higher dose of fed Ag may also induce active suppressive mechanisms. Previous studies of bystander suppression in vivo have also shown that a dose of 10 mg of OVA is capable of inducing bystander tolerance as efficiently as lower doses fed by gavage or in the drinking water (6). Thus, the ability of different doses of fed Ag to induce bystander suppression in oral tolerance warrants re-examination. Importantly, despite inducing an altered recall response compared with naive animals, the inclusion of CT as a priming control regimen was not able to induce bystander suppression, indicating that the phenomenon was not simply due to an alteration in the kinetics of the bystander response induced by prior exposure to Ag by the oral route.

In an attempt to examine the mechanism of bystander suppression further, we used two Ag-specific CD4+ T cell populations of different specificities to visualize the response of tolerized and naive T cells after challenge with both Ags. We found that the Ag-driven division of bystander, HA-specific T cells in vitro when cultures contained both OVA and HA was not altered by the presence of CD4+ T cells purified from orally tolerized mice compared with CD4+ T cells from naive mice. CD4+ T cells from mice fed OVA with CT also had no effect on the division of bystander T cells in vitro. Thus, orally tolerized CD4+ T cells could not suppress clonal expansion of naive, bystander CD4+ T cells in vitro. Because it was possible that our in vitro system did not reproduce...
duplicate samples of lymph node cells pooled from three mice per group (c and d). Cells that had previously been exposed to oral Ag was somewhat lower in tolerized mice compared with primed animals. These data seen in mice fed OVA and adjuvant, with Ag-specific proliferative responses after a 72-h culture for three mice per group (a and b) and IFN-γ production (nanograms per milliliter) at 48 h of culture of triplicate samples of lymph node cells pooled from three mice per group (c and d). * p < 0.05 vs saline-fed mice; #, p < 0.05 vs OVA/CT-fed mice.

FIGURE 4. Adoptive transfer recipients show evidence of functional bystander suppression in vitro after induction of oral tolerance. BALB/c mice were transferred with DO11.10 and 6.5 T cells, fed OVA, and immunized with OVA and HA as described in Fig. 3. Two weeks after immunization with OVA and HA in CFA, cells from the draining popliteal lymph node were restimulated in vitro with either 1 mg/ml OVA (a and c) or 100 μg/ml HA 

The situation in a tolerant animal in vivo, we then used an adoptive transfer model in which both T cell populations could be tracked during the induction of bystander tolerance. After parenteral challenge with OVA, the clonal expansion of OVA-specific CD4+ T cells that had previously been exposed to oral Ag was somewhat reduced compared with the response of naive CD4+ T cells under the same conditions, confirming previous reports (27, 28). However, the clonal expansion of naïve HA-specific T cells in response to OVA and HA was identical regardless of whether the host mice had been orally tolerized by feeding OVA and despite the induction of effective bystander tolerance, as assessed by cytokine production and proliferation in vitro and DTH responses in vivo. As a definitive marker of tolerance at the single-cell level still remains to be identified, we cannot conclude that these Ag-specific CD4+ T cells that expand are the same cells that eventually become tolerized in a bystander fashion. Nonetheless, our results show clearly that such Ag-specific T cells can undergo clonal expansion normally despite the presence of functional tolerance.

A novel finding from our study was that prior exposure to Ag in either tolerogenic or immunogenic form could modulate subsequent responses by bystander T cells in vivo, suggesting that competition for Ag or growth factors by Ag-experienced cells may contribute to this phenomenon. Nevertheless, we would emphasize that feeding soluble OVA had a tolerogenic effect greater than that seen in mice fed OVA and adjuvant, with Ag-specific proliferation, cytokine production, and DTH responses all significantly lower in tolerized mice compared with primed animals. These data would suggest that competition cannot explain all aspects of tolerance, and that active regulation is an additional, important mechanism for this.

Initial in vitro studies of T regulatory (Treg) or anergized CD4+ T cells have suggested that Treg cells may act by suppressing the proliferation of naive bystander T cells either directly (29, 30) or by preventing the ability of APC to prime naive T cells (31, 32). However, other recent reports have suggested that CD4+ T cells that become tolerized in vivo may not necessarily show a defect in clonal expansion, because CD4+ T cells tolerized in the periphery can polarize naive CD4+CD25+ T cells toward an IL-10-secreting phenotype without altering their clonal expansion (34), suggesting that cell division and functional differentiation are not necessarily linked in tolerized T cells or in T cells influenced by Treg.

Our results are also consistent with a previous study in which DO11.10 T cells tolerized by i.v. injection of OVA were unable to suppress the clonal expansion of bystander HA-specific T cells after rechallenge with both Ags together (15). However, these authors did not examine whether functional tolerance to the bystander Ag was induced in these mice. Others have suggested the ability of i.v. Ags to induce mechanisms of active suppression (16, 17); thus, it is possible that although bystander suppression is induced by this route, it occurs despite normal clonal expansion, similar to results in oral tolerance.

Together our findings suggest that the induction of tolerance to a bystander Ag may not necessarily affect the clonal expansion of Ag-specific T cells, and they support results from other forms of peripheral tolerance induction, in which clonal expansion of Ag-specific T cells is not suppressed despite the presence of functional tolerance (15, 28, 35). Thus, tolerized CD4+ T cells with bystander activity may act primarily by altering the functional capabilities of naive T cells rather than by preventing the initial activation of these cells by APC or by inhibiting their clonal expansion in a manner similar to that seen in other forms of peripheral tolerance (15, 28, 35) with normal early events, but later failure to acquire effector function.
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