

Oral Tolerance

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The intestinal immune system discriminates between potentially harmful and harmless foreign proteins. The basis for this differential response may be related to the conditions of antigen presentation by antigen-presenting cells, as determined by their phenotype or activation state. How these conditions affect specific immunologic unresponsiveness to later challenge with an antigen is not known. Two possible mechanisms are the induction of anergy or deletion of responsive cells and the activation of regulatory cells or mediators, and the mechanism may very depending on the tolerizing regimen used. Should regulatory cells be involved, they are speculated to induce tolerance through their production of inhibitory cytokines, such as IL-4, IL-10, and TGF- β . Studies using specific antibodies and selective genetic knockout (KO) strains of mice, however, have provided conflicting data. A final intriguing possibility is that tolerance results from cognate interactions between T cells and APCs, so that tolerant T cells or APCs prime T cells they contact to deliver a tolerogenic signal to the next T cell they encounter, possibly through a function dependent on interactions between Notch family receptors and their ligands. As with many questions in mucosal immunology, definition of the mechanisms of oral tolerance (OT) has proved difficult to address experimentally, but promising approaches include study of the distribution of fed antigen, of targeted genetic KOs, and of transgenic strains.

An essential feature of the intestinal immune system is its ability to discriminate between potentially harmful and harmless foreign proteins. Under normal physiological conditions the response to soluble food antigens is tolerance, while pathogenic stimuli such as invasive organisms or antigens administered in the presence of inflammatory agents elicit active immunity. The outcome of exposure to orally administered antigen (Ag) has important consequences as oral tolerance (OT) has been proposed as a therapy for inflammatory disorders, yet it represents a barrier to oral vaccination with subunit vaccines. Meanwhile pathological conditions such as inflammatory bowel disease (IBD) may be associated with a breakdown in oral tolerance leading to active immunity to food antigens or commensal bacteria. The basis of this dichotomy remains uncertain but may reflect differences in the interactions between antigen-presenting cells (APCs) and T cells resulting from variation in the anatomical location, phenotype, and activation state of the APC population. The mechanism of oral tolerance is also unclear and may be the result of deletion, anergy, or some form of active regulation depending on the dose and frequency of feeding. Thus, some of the major questions facing mucosal immunologists are where, why, and how does fed Ag induce tolerance or immunity?

WHERE AND WHY? ROLE OF THE ANTIGEN PRESENTING CELL

As noted above, the basis for the differential response of the intestinal immune system to harmless or pathogenic forms of

Ag is uncertain but may be related to the circumstances and location of Ag presentation. Presentation of Ag to T cells in the context of appropriate costimulation provided by APCs results in T cell priming and the development of a productive immune response (1, 2). This is characterized by the differentiation of T cells into proliferating, cytokine-producing effector cells that can also provide cognate help for B cells. In contrast, presentation of Ag to T cells in the absence of costimulation is believed to result in the induction of tolerance with a concomitant failure of T cell effector and helper functions (1, 2). It has been proposed that the provision of costimulation, and therefore the induction of tolerance or immunity, may depend on the phenotype and activation state of APCs. Whereas "professional" APCs that express adequate levels of costimulatory molecules (activated dendritic cells [DCs], activated macrophages, and activated B cells) prime T cells efficiently, "nonprofessional" APCs (naive/resting B cells, resting DCs, and class II MHC-expressing tissue cells) have reduced costimulatory potential and tolerate T cells (1–3). This probably reflects the influence that the physical form and route of administration of the Ag plays in dictating the location, phenotype, and activation state of the APC. As many professional APCs express low levels of costimulatory molecules in their resting state, anergy or deletion of T cells may result from an absence of inflammatory mediators to upregulate costimulatory molecules on these APCs. Indeed, while Ag presentation by DCs is usually associated with immunological priming, a number of studies of peripheral and oral tolerance have suggested that these cells may be tolerogenic if presenting Ag in the absence of inflammation (4, 5). Thus, administration of Ag associated with an adjuvant results in T cell priming, whereas the same Ag induces T cell tolerance in the absence of adjuvant (3). Such presentation may also lead to polarization or immune deviation to immunoregulatory helper T type 2 (Th2), helper T type 3 (Th3), or regulatory T type 1 (Tr-1) cells, all of which have been implicated as mediators of oral tolerance (6–10). Immune deviation of this kind may be particularly useful in regulation of intestinal immune responses to harmless Ags, as a bias toward Th2/Th3-like responses, and away from helper T type 1 (Th1) would favor harmless IgA production and avoid immunopathology. This may reflect B cell presentation of Ag, as this has been associated with tolerance and preferential Th2 induction (2, 6–8, 11). However, this remains controversial, for while the local manifestations of oral tolerance may be dependent on distinct Th cell subsets, this does not appear to be the case for the systemic consequences of feeding Ag (12, 13). It is also important to note when considering oral vaccination or the therapeutic use of oral tolerance that orally administered Ags can induce tolerance or priming at both local and systemic sites (6–8). It therefore seems likely that both local and peripheral APC–lymphocyte interactions are important in these situations. However, it is unclear exactly where and when these interactions take place, which cells are involved, and whether the interactions differ between oral tolerance and priming. Studies using adoptive transfer systems or MHC–peptide tetramers to track Ag-specific T cells *in vivo* have yielded conflicting results, with some studies suggesting that responses to orally administered Ags are initiated locally in

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the gut and then disseminate (14, 15) while another proposes simultaneous activation of T cells throughout the animal after feeding (16). These studies concentrated on the behavior of T cells in response to fed Ag and did not investigate the underlying cellular interactions. Furthermore, there was no attempt to address differences between responses to immunogenic or tolerogenic forms of fed Ag and further detailed studies of this type are required. In addition to determining the kinetics and gross anatomical locations of the interactions underlying the induction of oral tolerance and priming in the studies described above, it will be important to examine the localization of immunologically relevant Ag (i.e., that associated with class II MHC), using peptide-MHC antibodies (17, 18). Studies using these reagents have shown localization of Ag on resting splenic B cells after intravenous administration (17, 18). Ag administered in this way is known to induce peripheral tolerance and these studies have indicated that this may be the result of Ag presentation by resting B cells. Interestingly, oral tolerance has been reported to be normal in B cell KO mice (19).

While the activation state, phenotype, and location of the APCs involved in oral tolerance and priming remain elusive, so do the mechanisms of tolerance. Furthermore, how these two areas relate to each other will also be important to determine, that is, does the mechanism of oral tolerance vary with and/or as a result of the APC type.

HOW? POTENTIAL MECHANISMS OF ORAL TOLERANCE

Oral tolerance (OT) is defined as the specific immunological unresponsiveness to challenge with an antigen induced by its prior feeding (6, 8, 20–22). Unresponsiveness of T cells in the periphery may be achieved in a number of ways: the T cell may encounter the antigen under circumstances that result in the subsequent functional or actual elimination of the cell (anergy or deletion, respectively). Alternatively, regulatory cells or mediators may be induced that can modify the immune response. As noted above, which of these mechanisms operates in OT is controversial and has been suggested to depend on the tolerizing regimen employed. Thus, it has been proposed that clonal deletion may result from feeding high doses of antigen while some form of active regulation may be a feature of low-dose/repeated feeding regimens (6, 8, 20–22).

DELETION

If clonal deletion of antigen-specific T cells is induced by feeding a high dose of antigen, this would be expected to result in stable, long-lasting tolerance, with the caveat of repopulation by naive, Ag-specific cells that could be activated at a later stage. Clonal deletion of CD4⁺ T cells via apoptosis *in vivo* has been demonstrated after oral administration of soluble chicken ovalbumin (OVA) to T cell receptor (TCR) transgenic mice (23), although this was not observed after feeding myelin basic protein (MBP) to appropriate transgenic mice (24). Furthermore, the physiological relevance of studying immune responses in fully transgenic animals is questionable. The more physiologically relevant system, in which small numbers of identifiable TCR transgenic T cells are adoptively transferred into normal syngeneic recipients (25), has yielded conflicting results (15), with one study finding T cell deletion after feeding large doses of antigenic peptide while another reported no evidence of deletion in mice fed more conventional tolerogenic doses of OVA protein. The fact that oral tolerance can be induced normally in *lpr* mice (26) argues against a role for Fas-dependent apoptosis in the intact animal. However,

studies indicate a possible role for p55 tumor necrosis factor receptor (TNFR)-mediated apoptosis in oral tolerance (26), consistent with the role of the TNF-TNFR interaction in mediating cell death and peripheral tolerance in other systems (27). Despite these observations, it remains unclear whether TNF has its effects via deletion, anergy, and/or immune deviation and whether these effects are on T cells directly or via the induction/activation of other cells and molecules. Furthermore, definitive exploration of OT in KO mice is limited by the fact that the p55 TNFR is also critically important for the organogenesis of lymphoid tissues, in particular the Peyer's patches. Thus, the apparent defect in oral tolerance in these animals (A. M. Mowat, J. L. Viney, and P. Garside, unpublished observations) could reflect gross defects in intestinal antigen handling and priming of mucosal T cells. In addition, it is difficult to assess oral tolerance in these mice, as systemic immune responses in these animals are impaired (26). Thus, the role of TNF and the TNFR (and other family members implicated in apoptosis) during clonal deletion in oral tolerance requires further definitive investigation.

REGULATORY T CELLS

An alternative to clonal deletion of individual specific T cells is the possibility of induction of regulatory cells. The induction of active regulatory cells could maintain tolerance via suppression of new, naive emigrants. However, there are inherent dangers in this strategy. For example, inappropriate activation of regulatory cells as a result of infection or inflammation could result in immunopathology. On the other hand, nonspecific bystander suppression may dominate. While this would be an important and useful phenomenon to exploit therapeutically in the context of autoimmune diseases where the target antigen is unknown, it may prove problematic if responses to oral vaccines or pathogens are suppressed. Regulatory cells may mediate bystander suppression via the production of inhibitory cytokines such as transforming growth factor β (TGF- β), which can exert nonspecific suppressive effects on other antigen-reactive cells in the vicinity, irrespective of their specificity (6, 8, 20–22). While a number of studies have now cast considerable doubt on earlier suggestions of a role for CD8⁺ T cells as suppressor cells in oral tolerance there is a general consensus that CD4⁺ T cells may be able to fulfill this role and that these cells can transfer oral tolerance *in vivo* (6, 8, 20–22). The original theory to explain these results was that oral tolerance reflected the downregulation of Th1 CD4⁺ cells by Th2 CD4⁺ cells. However, oral tolerance to OVA can suppress both Th1 and Th2 responses quite adequately (13) and normal oral tolerance can be induced in the absence of Th2 cells in interleukin 4 (IL-4) and STAT6 (signal transducer and activator of transcription 6) KO mice (12, 13). More recently the role of CD4⁺ T cells as active mediators of oral tolerance has been explained by the presence of populations of regulatory T cells such as Th3, Tr-1, or CD38⁺-CD45RB^{low}CD4⁺ T cells, which produce suppressive cytokines (*see below*) and that can inhibit Th1-mediated immunopathologies such as experimental autoimmune encephalomyelitis and colitis (10, 28, 29). It will be important to understand the mechanisms and interactions underlying the induction and effects of such cells if they are to be exploited therapeutically.

REGULATORY CYTOKINES

Although distinct subpopulations of T cells have been implicated in oral tolerance, it is also possible that it may be caused by the preferential production of individual inhibitory cytokines in response to fed protein. Those that have received most interest are IL-4, IL-10, and TGF- β .

As we have noted, IL-4 does not appear to be essential, despite some evidence of its preferential upregulation. As IL-10 suppresses Th1 activity via downregulation of the expression of costimulatory molecules and IL-12 production by APCs (30), it was an attractive candidate as a mediator of oral tolerance, particularly where it appeared to be mediated by Th2 cells. Initial reports suggested that the production of IL-10 was enhanced in oral tolerance (6, 8, 20–22), and IL-10-producing “Th3” clones can be isolated from animals tolerized by feeding MBP (6, 8, 20–22). IL-10 dependent, OVA-specific TCR transgenic T lymphocytes have been shown to mediate non-specific bystander suppression of experimental colitis when adoptively transferred *in vivo* and activated by feeding OVA (10). Similarly, the ability of CD45RB^{low} T cells to ameliorate colitis in this model has been demonstrated to depend on IL-10 secretion by these cells (31). However, we have found that marked suppression of IL-10 occurs in mice fed OVA (13), and normal oral tolerance occurs in mice depleted of IL-10 (32). Studies of oral tolerance in IL-10 KO animals have been difficult as they spontaneously develop severe IBD under conventional animal house conditions. However, studies using “healthy” IL-10 KO animals have indicated that prevention of experimental autoimmune uveitis (EAU) via the induction of oral tolerance was normal in these animals (33). More recently, the source of the IL-10 in tolerance has come under scrutiny, with some studies indicating that IL-10 production by lamina propria APCs might be important in local immunoregulation in the gut (34, 35). Thus, the role and source of this cytokine in oral tolerance remains unclear.

TGF- β is the mediator currently receiving most attention. Abundant in the normal intestine (6, 8, 20–22), it is produced by cells of both hemopoietic and epithelial origin and is important in regulating epithelial homeostasis and IgA switching (6, 8, 20–22). TGF- β also has well-documented suppressive effects on many aspects of the immune response (6, 8, 20–22). Increased production of this cytokine has been reported in a number of models of oral tolerance (6, 8, 20–22) and TGF- β -secreting T cell clones can be produced from animals tolerized in this way (6, 8, 20–22). Furthermore, the bystander suppressive effects exerted by these cells *in vitro* and *in vivo* can be prevented with anti-TGF- β (6, 8, 20–22). Local TGF- β production has also been associated with the prevention of experimental colitis in a number of models (6, 8, 10, 20–22, 28) and regulatory Th3 and Tr-1 cells (*see above*) are a potent source of this cytokine (10, 28). Studies suggest that TGF- β and the IL-12/IFN- γ pathway play opposing roles in intestinal immune regulation and that modulation of these cytokines may be the key to induction of mucosal immunity and/or tolerance (36). However, enhanced TGF- β production is not a universal finding in all models of mucosal tolerance (6, 8, 20–22), and we have recently demonstrated normal oral tolerance in IL-12 KO mice (37). Furthermore, it has been reported that anti-TGF- β had no effect on oral tolerance in a model of EAU. As with IL-10 KO animals, studies of conventional TGF- β KO animals have been difficult and restricted. Homozygous TGF- β KO pups die soon after weaning (no longer acquiring TGF- β from mother's milk) as a result of severe and widespread inflammation. Attempts to study oral tolerance in these animals supported a role for TGF- β in oral tolerance but were difficult to interpret as they used neonates and anti-LFA-1 treatment to prevent inflammation (38). Thus, the role of TGF- β in oral tolerance requires further investigation.

COGNATE INTERACTION

A final intriguing possibility is that tolerance may require cognate interactions between T cells and/or APCs. It has been

proposed that regulatory and/or anergic cells may mediate their suppressive effects directly via the production of inhibitory cytokines or indirectly by competing for growth factors, MHC-peptide complexes, or costimulatory molecules on APCs (39). However, evidence indicates that the effects of regulatory cells may be mediated via a cognate interaction. In this context, it is significant that CD4⁺ T cells have been shown to activate APCs in a contact-dependent manner to subsequently deliver help to CD8⁺ T cells of different specificity (40). Similarly, tolerized T cells have been shown to modify APCs such that they can induce tolerance in subsequently encountered naive T cells (41). In such a system, a cognate interaction with a tolerant T cell or APC “primes” the cell they have contacted to deliver a tolerogenic signal to the next T cell they encounter. The mechanism by which this cognate interaction mediates tolerance remains unclear. While a variety of soluble (cytokines, chemokines) or membrane-bound molecules (CTLA-4, TNF- α , etc.) (42) could fulfill this cognate function, a novel study has revealed a possible role for the receptor-ligand pair Notch-Serrate in such a mechanism of tolerance (43). These molecules are important in cell fate decisions in a number of situations, but of particular interest is their role in the cell fate decisions of thymocytes. The induction of tolerance or immunity may be based on similar cell fate decisions by CD4⁺ T cells in the periphery, and therefore, a study hypothesized that Notch family members and their ligands may be important in immunoregulation. This work demonstrated that overexpression of the Notch ligand Serrate by APCs results in the induction *in vivo* of a regulatory population of CD4⁺ T cells that can transfer antigen-specific tolerance. The importance of Notch receptor/ligand family members in oral tolerance remains to be investigated.

The mechanisms and cellular interactions underlying OT are important to determine because, as we have noted above, these may influence the anatomical and functional extent of its effects. For example, immunomodulation via direct cognate interactions might be expected to be relatively more discriminating and localized than that mediated via the production of soluble mediators. Furthermore, identifying the source and/or target (e.g., APC and CD4⁺ T cell) of any immunomodulatory molecules and their effects may allow the development of targeted intervention. Which of the mechanisms described above operates in oral tolerance, to what extent this is influenced by the tolerizing regimen, and whether they are mutually exclusive remains unclear. As with many questions in mucosal immunology it has been extremely difficult to address these points in a defined experimental manner. However, advances in techniques to determine the distribution of immunologically relevant fed Ag and monitor the behavior of antigen-specific lymphocytes *in vivo* combined with increasingly defined and targeted KO and transgenic technology mean that answers to the questions posed above may become accessible in the near future.

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