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Commentary

Oral tolerance

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Oral tolerance refers to the observation that if one feeds a protein and then immunizes with the fed protein, a state of systemic hyporesponsiveness to the fed protein exists. It was first described in 1911 when Wells (1) fed hen's egg proteins to guinea pigs and found them resistant to anaphylaxis when challenged. In 1946, Chase (2) fed guinea pigs the contact sensitizing agent dichloronitrobenzene (DCNB) and observed that animals had decreased skin reactivity to DCNB (2). Subsequently, numerous investigators have found that animals fed proteins such as ovalbumin or sheep red blood cells do not respond as well to these antigens when subsequently immunized but do respond normally to other antigens (3). The phenomenon of oral tolerance has also been observed in humans fed and immunized with keyhole limpet hemocyanin (4).

Immunologic tolerance is a basic property of the immune system that provides for self/non-self discrimination so that the immune system can protect the host from external pathogens without reacting against self. When the immune system reacts against itself, autoimmune disease results. For a time it was thought that self/non-self discrimination was a simple matter of deleting autoreactive cells in the thymus, but it is now clear that the maintenance of immunologic tolerance is a much more complicated process. Autoreactive cells, such as those reacting with brain, are not deleted and can be found in normal individuals (5, 6). Why these cells become activated and cause disease in some individuals whereas in others they remain harmless is a major question in basic immunology. How to control the autoimmune process once it has been initiated is a major problem in clinical medicine.

These two areas have come together in recent years as oral tolerance has been successfully used to treat autoimmune diseases in animal models (reviewed in ref. 7) and is now being applied for the treatment of human disease states (8, 9). Furthermore, an understanding of the basic mechanisms by which orally administered antigens induce immune tolerance is beginning to emerge. As with immunologic tolerance in general, oral tolerance involves multiple mechanisms (10–12). Thus the term oral tolerance is in some ways misleading, as it implies that there is one unique mechanism of tolerance induction when antigens are administered orally. This is not the case. Although the gut clearly has unique properties that favor tolerance induction, the type of tolerance induced must now be defined when factors that influence oral tolerance are investigated.

Orally administered antigen encounters the gut-associated lymphoid tissue (GALT), a very well-developed immune network that not only evolved to protect the host from ingested pathogens but also developed the inherent property of preventing the host from reacting to ingested proteins. The GALT consists of villi which contain epithelial cells capable of antigen presentation, intraepithelial lymphocytes, and lamina propria lymphocytes (13). In addition, there are Peyer's patches, lymphoid nodules interspersed below the villi, which are one of the primary areas in the GALT where specific immune responses are generated. Investigators have attempted to use the GALT to immunize for vaccines but have been hampered by the systemic hyporesponsiveness, or oral tolerance, that is naturally generated. Nonetheless, as described below, active induction of selected immune responses in the GALT is one of the primary mechanisms by which oral antigen suppresses systemic immunity.

In addition to stimulating the GALT, some oral antigen is absorbed. Although dietary antigens are degraded by the time they reach the small intestine, studies in humans and rodents have indicated that the degradation is partial and that some intact antigen is absorbed (14, 15). Absorbed antigen, either undegraded or partially degraded, appears to have an important role in the generation of one type of oral tolerance.

Mechanisms of Oral Tolerance (Fig. 1)

It is now known that two of the primary mechanisms by which oral tolerance is mediated occur via the generation of active cellular suppression or clonal anergy and that the determining factor is the dose of antigen fed (10–12). Low doses favor active suppression whereas high doses favor anergy. Active suppression is mediated by the induction of regulatory T cells in the GALT such as Peyer's patches. These cells then migrate to the systemic immune system. When higher doses of antigen are fed, clonal anergy results and can be demonstrated by reversal of systemic hyporesponsiveness by culturing with recombinant interleukin 2 (IL-2) (12). Anergy appears to be favored by the passage of antigen into the systemic circulation. Recently, we have found that clonal deletion may occur when large doses of antigen are fed (Y. Chen and H.L.W., unpublished work). Thus, oral tolerance is not a single immunologic event.

Active cellular suppression of immune responses has been studied extensively over the years and has remained ill-defined due to difficulties in cloning suppressor cells and defining their mechanism of action. More recently, it appears that one of the primary mechanisms of active cellular suppression is via the secretion of suppressive cytokines such as transforming growth factor β (TGF-β), IL-4, and IL-10 after antigen-specific triggering (16). In this sense the GALT is unique, as it favors the induction of cells which secrete these cytokines, Th2 as opposed to Th1 helper T cells, and T cells which secrete TGF-β, a potent immunosuppressive cytokine. T cells in lymphoid organs drained by mucosal sites secrete IL-4 as a primary T-cell growth factor, whereas those drained by nonmucosal sites secrete IL-2 (17). Oral tolerance has often been demonstrated by a decreased delayed-type hypersensitivity (DTH) response to the fed antigen (2, 3, 7) and it is known that DTH is a Th1 response inhibited by IL-4-producing Th2 cells. TGF-β plays an important role in local function of the gut, as it serves as a switch factor for IgA production in the mucosa (18) and may also be involved in the homing mechanism of the cells to high endothelial venules (19). TGF-β is produced by both $CD4^+$ and $CD8^+$ GALT-derived T cells (16, 20) and is an important mediator of the active component of oral tolerance. We have recently cloned TGF-β-secreting myelin basic protein (MBP)-specific $CD4^+$ cells from the mesenteric lymph nodes of SJL mice (16). These clones were structurally identical to Th1 disease-inducing clones in T-cell receptor usage, major histocompatibility complex (MHC) restriction, and epitope

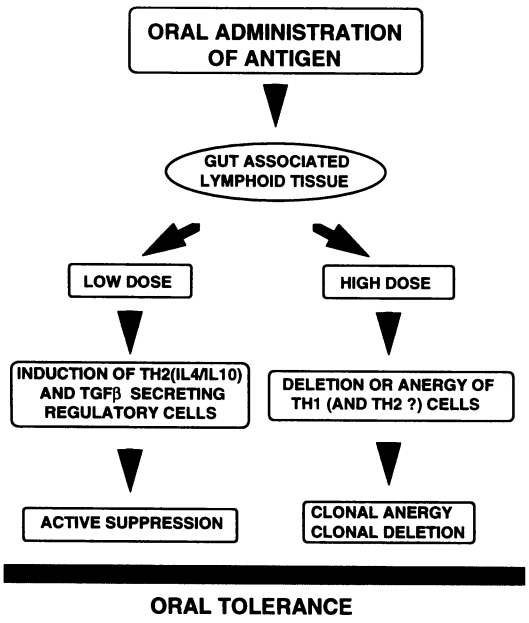

FIG. 1. Mechanisms of oral tolerance.

recognition but suppressed rather than induced disease. Thus, mucosally derived $CD4^+$ cells which primarily produce TGF-β may be a unique T-cell subset (Th3?) with both mucosal T helper function and downregulatory properties for Th1 and other immune cells.

Mucosal Immunity and Cholera Toxin

Mucosal immunity is responsible for protecting the mucosal surface of the host from pathogens and toxins, primarily through the production of secretory IgA. Particulate antigens, including live and killed microorganisms, can be effective oral immunogens, although most soluble protein antigens are poor mucosal immunogens and a variety of substances have been used as adjuvants for oral immunization for secretory IgA. The most potent mucosal adjuvant identified thus far is cholera toxin (CT).

CT consists of a central A subunit surrounded by a pentameric ring of B subunits. The B subunits bind to GM1 gangliosides on the surface of all eukaryotic cells and facilitate the entry of the central toxic A subunit. CT has been used as a mucosal adjuvant to enhance mucosal immunization as measured by IgA responses. In addition, it has been reported that feeding CT abrogates orally induced suppression of systemic immunity when fed with an unrelated protein antigen (21). Sun *et al.* (22) now report that when a protein is coupled to recombinant CT B subunit (CTB) and given orally, there is marked enhancement of peripheral immune tolerance to the protein but that CT itself abrogates CTB-induced suppression of systemic immunity (22).

The observation by Sun *et al.* raises the possibility of a more biologically potent way to induce peripheral tolerance by oral antigens and thus enhance the ability to suppress unwanted systemic autoimmune responses. The central immunologic question raised by the study is twofold: how does CTB enhance oral tolerance and how does CT itself abrogate it? It appears that CTB enhances oral tolerance by the more efficient induction of regulatory cells. Presumably, the special binding properties of CTB targets antigen through M cells in Peyer's patches, which is a primary area where regulatory cells are generated. In addition, it may target antigen onto the epithelium, another site where regulatory cell generation might occur (45). Indeed the authors were able to adoptively transfer protection from animals fed CTB coupled to antigen and very small doses of antigen were fed. Thus, it presumably increases the frequency of Th2 and TGF-β-secreting regulatory cells capable of mediating peripheral suppression. The abrogation of oral tolerance by CT may also be the result of an active immune process in the gut, as it has been shown that CT given orally with keyhole limpet hemocyanin primes both Th1 and Th2 responses (23), even though Th2 responses may be preferentially generated (24, 25). The priming of Th1 responses via the gut by CT in contrast to CTB may make it impossible to suppress systemic Th1 responses following peripheral immunization.

If oral tolerance is broadly defined as the inhibition of Th1 responses in the periphery either by large doses of antigen that leak into the circulation and lead to anergy or by gut-induced Th2-type regulatory cells, anything that favors Th1 over Th2 responses would abrogate oral tolerance. In this regard, the administration of large doses of interferon γ abrogates oral tolerance and we have found that orally administered IL-4 may enhance low-dose oral tolerance (Y. Chen and H.L.W., unpublished work). Lipopolysaccharide enhances oral tolerance to MBP and is associated with increased expression of IL-4 in the brain (26). Given that orally administered antigen preferentially stimulates Th2-type responses, it has been difficult to tolerize for systemic Th2 responses, though this may be achieved by the continuous feeding of large doses of antigen (D. Melamed, J. Fishman-Lobell, Z. Uni, H.L.W., and A. Friedman, unpublished work). Some have suggested that CT breaks oral tolerance by depleting $CD8^+$ cells (27), but this seems unlikely given that both $CD4^+$ and $CD8^+$ cells can mediate the active component of oral tolerance (16). Of note is that in T-cell receptor-transgenic animals, large doses of oral antigen can also lead to deletion of antigen-reactive cells in the GALT (Y. Chen and H.L.W., unpublished work).

Other investigations of the effect of CT on oral tolerance have produced a variety of results depending on the immune parameters measured and dosages of antigen fed. Investigators could not induce systemic hyporesponsiveness to CT itself as measured by antibody responses (21), whereas hyporesponsiveness as measured by DTH responses was observed (28). Pierre *et al.* (29) attempted to modulate oral tolerance to ovalbumin by CT and its B subunit and found that both compounds primed immune responses. Kikuta *et al.* (46) reported enhanced DTH responses by CTB given intranasally with influenza hemagglutinin vaccine. Based on the paper of Sun *et al.* (22), it now appears that these results were related to minor contamination of the CTB preparations with CT itself. Given what is now known about the mechanisms of oral tolerance and the availability of transgenic animals, investigation of CT and CTB can now be performed over wide dose ranges with the direct measure of cytokine-secreting regulatory cells and Th1 responses as measured by anergy and deletion. The question raised by the study of Sun *et al.* is whether CTB has unique mucosal stimulating properties that preferentially induce regulatory cells independently of Th1 responses and whether this effect is qualitatively different than multiple low dose feeding of uncoupled antigen. Furthermore, to what degree are local IgA responses linked to the induction of regulatory cells that mediate the active component of oral tolerance?

Bystander Suppression

Bystander suppression was described during the investigation of regulatory

cells induced by oral administration of low doses of MBP (30). It solves a major conceptual problem related to designing antigen- or T-cell-specific therapy of inflammatory autoimmune diseases such as multiple sclerosis, type I diabetes, and rheumatoid arthritis, in which the autoantigen is unknown or where there are reactivities to multiple autoantigens in the target tissue. In animal models of autoimmunity, during the course of the chronic inflammatory autoimmune process there is intra- and interantigenic spread of autoreactivity at the target organ (31–35). Similar findings have been observed in human autoimmune disease in which there are reactivities to multiple autoantigens from the target tissue. For example, in multiple sclerosis there is immune reactivity to three myelin antigens, MBP, proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) (5, 6). In type I diabetes, there are multiple islet-cell antigens that could be the target of autoreactivity, including glutamate decarboxylase, insulin, and heat shock proteins (36). Because regulatory cells induced by oral antigen secrete antigen-nonspecific cytokines after being triggered by the fed antigen, they suppress inflammation in the microenvironment where the fed antigen is localized. Thus, for an organ-specific inflammatory disease, one need not know the specific antigen that is the target of an autoimmune response but only feed an antigen capable of inducing regulatory cells which then migrate to the target tissue and suppress inflammation. Bystander suppression was demonstrated *in vitro* when it was shown that cells from MBP-fed animals suppressed proliferation of an ovalbumin-reactive cell line across a transwell, but only when triggered by the fed antigen (30). The soluble factor was identified as TGF-β. Bystander suppression has also been demonstrated in autoimmune disease models. One can suppress PLP peptide-induced experimental allergic encephalomyelitis (EAE) by feeding MBP (37), and MBP-specific T-cell clones from orally tolerized animals which secrete TGF-β also suppress PLP-induced disease. Other examples include the suppression of adjuvant- and antigen-induced arthritis by feeding type II collagen and the suppression of insulitis in the NOD mouse by feeding glucagon (7, 38).

Treatment of Autoimmune Diseases in Animals

A large series of studies have demonstrated that orally administered autoantigens can suppress several experimental models of autoimmunity and transplantation (Table 1; reviewed in ref. 7). The mechanism of suppression in these models depends on dosage administered; in some instances active suppression has been shown and in other instances, clonal anergy. Immunohistochemical studies have demonstrated the upregulation of antiinflammatory cytokines such as TGF-β and IL-4 in the target organ of animals fed low doses of autoantigens (26, 39). Importantly, for human trials, feeding after immunization and feeding in chronic disease models such as chronic EAE have been successful (38, 40). Thus, it does not appear that feeding an autoantigen to an already sensitized animal necessarily results in further priming. Suppression of disease, however, may be most effective when homologous protein is administered (41), a finding which has important implications for treatment of human autoimmune diseases for which recombinant human proteins might then be required. Although one can suppress the generation of antibodies by oral feeding, much larger doses are required, and since the gut preferentially induces Th2 responses, the degree to which oral tolerance will be successful in suppressing antibody-mediated diseases is unclear.

Table 1. Suppression of autoimmunity by oral tolerance

Condition	Protein fed
Animal models	
EAE	MBP, PLP
Arthritis	Type II collagen
Uveitis	S-antigen, IRBP
Diabetes (NOD mouse)	Insulin, glutamate decarboxylase
Myasthenia gravis	Acetylcholine receptor
Thyroiditis	Thyroglobulin
Transplantation	Alloantigen, MHC peptide
Human disease trials	
Multiple sclerosis	Bovine myelin
Rheumatoid arthritis	Chicken type II collagen
Uveoretinitis	Bovine S-antigen
Type I diabetes (planned)	Human insulin

Treatment of Autoimmune Diseases in Humans

The first attempts of oral tolerization may have been utilized by Native Americans, who were thought to have fed their children *Rhus* leaves to prevent them from becoming sensitized to poison ivy (42). Investigators have shown that exposure of a contact sensitizing agent via the mucosa prior to subsequent skin challenge led to unresponsiveness in a portion of patients studied (43). Orally administered keyhole limpet hemocyanin (50 mg) given daily for 2 weeks over a 3-week period to human subjects has been reported to decrease subsequent cell-mediated immune responses, although antibody responses were not affected (4).

Based on the long history of oral tolerance and the apparent safety of the approach, human trials have been initiated in multiple sclerosis (8), rheumatoid arthritis (9), and uveitis (44). These initial phase I/II trials have involved a relatively small number of patients, and the clinical efficacy of oral antigen in these diseases must await the results of large-scale trials that are currently in progress (Table 1). What can be said from the initial trials is that there was no apparent toxicity or exacerbation of disease. In patients with multiple sclerosis, a decrease in MBP-reactive cells was observed in the peripheral blood, and in patients with rheumatoid arthritis, joint swelling was decreased. In multiple sclerosis, there is presently a 500-patient double-blind phase III trial in which patients are randomized by sex and DR type, which may be linked to the response. In rheumatoid arthritis, a 280-patient double-blind phase II dosing trial is in progress in which doses ranging from 5 μg to 2500 μg are being tested. In uveitis, a double-masked trial of S-antigen and an S-antigen mixture is currently in progress. In addition, trials are being planned both in juvenile and in new-onset diabetes in which oral insulin, insulin derivatives, or other islet-cell antigens will be administered.

The report by Sun *et al.* (22) demonstrates that active immunization with CTB via the gut enhances oral tolerance, presumably by more efficient presentation of antigen to GALT and the generation of regulatory cells. Thus, recombinant CTB enhances tolerance by serving as a selective mucosal adjuvant. If effectively applied to human disease states, it could have an important impact on using oral tolerance to treat autoimmunity.

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