The role of the gut lymphoid tissue in induction of oral tolerance

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The gut-associated lymphoid tissue (GALT) maintains a balance between immunological tolerance to dietary proteins and induction of active immune responses to pathogenic microorganisms. The oral administration of soluble protein antigens induces a state of systemic immunological unresponsiveness specific to the fed protein, termed oral tolerance. The two major mechanisms to explain oral tolerance are anergy/deletion of autoreactive lymphocytes and active suppression. This review will discuss the mechanisms of therapeutic oral tolerance in relation to events occurring at the site of antigen entry.

Keywords Gut-associated lymphoid tissue, oral tolerance, Peyer's patch, T-lymphocyte

Introduction

Oral tolerance (OT) refers to the oral administration of protein antigens (Ag), which induces a state of systemic immunological non-responsiveness specific for the fed antigen. Orally administered Ag first encounters the gut-associated lymphoid tissue (GALT), which is a well-developed immune network that evolved not only to protect the host from ingested pathogens, but also to prevent the host from reacting to dietary proteins. The GALT is composed of villi that contain epithelial cells, intraepithelial lymphocytes (IEL), lamina propria lymphocytes (LPL) and Peyer's patches [1]. The intestine contains more T-lymphocytes than the rest of the body combined. These cells comprise mesenteric lymph nodes, Peyer's patches, isolated lymphoid follicles or remain as isolated T-cells in the epithelium and lamina propria [2•]. Administering Ag via a mucosal route has been recognized as a means to induce immunological tolerance, as measured by unresponsiveness following subsequent challenge with the same antigen in an immunogenic form.

The oral administration of allergens, when compared to the more traditional subcutaneous route, is a more convenient and safer route of antigen administration [3]. Several animal models have been used to study human autoimmune diseases (Table 1). Animal models of both spontaneous and induced disease have contributed greatly to our knowledge of disease pathogenesis and are increasingly used to assess various treatment modalities. These model systems are employed to study oral tolerance: experimental autoimmune encephalomyelitis (EAE) [4,5•,6-8], adjuvant- or antigen-induced arthritis (AIA) [9-10], collagen-induced arthritis (CIA) [11-14], experimental autoimmune uveitis (EAU) and the non-obese diabetic (NOD) mouse [15-17]. As shown in Table 1, feeding proteins which represent prominent components of specific organs results in suppression of organ-specific autoimmune disease. For example, feeding any of the three major myelin proteins, myelin basic protein (MBP), proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG) decreases clinical and histopathological changes of EAE [4,5•,6-8]. Importantly, feeding MBP even after disease is already established is effective in suppressing the clinical signs of EAE [6]. These animal models have permitted in-depth exploration of tolerance mechanisms and have served as important preludes to human clinical trials in multiple sclerosis (MS) [18•], rheumatoid arthritis (RA) [19-20], uveitis [21] and Type I diabetes [22].

The mechanisms of oral tolerance

The two major mechanisms to explain OT are deletion/ anergy of antigen-reactive lymphocytes and active suppression, whereby suppressive cytokines downregulate specific antigen responses. Anergy occurs under conditions of feeding high doses of Ag (20 to 500 mg), while active suppression is a feature of low doses/ repeated feeding regimens (1 to 5 mg). Our early studies of OT in EAE in the Lewis rat indicated that feeding high doses of MBP induced clonal anergy [5•]. In these studies, the impaired ability of cells from MBP-fed rats to respond to Ag *in vitro* was restored by exogenous interleukin (IL)-2, indicating the continued presence of Ag-reactive T-cells [5•]. This mechanism was confirmed in studies showing that adoptively transferred T-cell receptor (TCR) transgenic T-cells persist *in vivo* following feeding, but are hyporesponsive to Ag when restimulated *in vitro* [23]. Using this system and administering relatively high doses of Ag [5•,24-26], Ag-specific cells display impaired functional properties such as diminished Th1 cytokine production (IL-2 and interferon

Table 1. Suppression of autoimmune diseases by oral administration of protein.

Animal model	Protein fed (References)	Human disease trials	Protein fed (References)
EAE	MBP, PLP, MOG [4,5•,6-8]	Multiple sclerosis	Bovine myelin [18•]
Arthritis (CIA, AIA)	CII [9,10]	Rheumatoid arthritis	Chicken type II collagen [19-20]
Uveitis (EAU)	S-Ag, IRBP [11-14]	Uveitis	Bovine S-Ag [21]
Diabetes (NOD mouse)	Insulin, GAD [15-17]	Type I diabetes	Human insulin [22]

AIA adjuvant- and antigen-induced arthritis, **CIA** collagen-induced arthritis, **CII** type II collagen, **EAE** experimental autoimmune encephalomyelitis, **EAU** experimental autoimmune uveitis, **GAD** glutamic acid decarboxylase, **IRBP** inter-photoreceptor retinoid-binding protein, **MBP** myelin basic protein, **MOG** myelin oligodendrocyte glycoprotein, **NOD** non-obese diabetic, **PLP** proteolipid protein, **S-Ag** S antigen.

(IFN)γ) and decreased Ag-specific proliferation [27]. Clonal deletion of CD4+ T-cells via apoptosis *in vivo* was demonstrated following oral administration of ultra high doses (500 mg) of soluble ovalbumin (OVA) to TCR transgenic mice [28•], and high doses (100 mg) of MBP to MBP-specific TCR transgenic mice [29]. The pathways leading to apoptosis are reported to be dependent on Fas-FasL [28•,29] and tumor necrosis factor receptor (TNFR)-TNF interactions [30].

To explain the mechanisms underlying active suppression, a number of studies have implicated CD4 T-cells as the principal regulatory cell population. These have been reported variously to be Th3, Tr1, CD38+CD45RB^{low} or CD25+CD45RB^{low}CD4+ T-cells, which can produce Th2 cytokines and lead to inhibition of Th1-mediated immunopathologies, such as EAE and colitis [31,32•,33,34]. Regulatory T-cells mediate bystander suppression by exerting non-specific suppressive effects on other Ag-reactive cells in the vicinity, irrespective of their specificity [22,35••,36,37], through production of inhibitory cytokines [38-44], including IL-4, transforming growth factor (TGF)β and IL-10 [35••,36,37,40]. The chemokine, monocyte chemoattractant protein (MCP)-1, was recently reported to regulate oral tolerance induction by inhibition of Th1 cytokines [43]. As shown in Table 2, a deficit of Th1 cytokines or chemokines (IL-12, IFNγ, IFNγR, macrophage inflammatory protein (MIP)-1α, and RANTES) does not affect OT [38,43]. In contrast, knockout (KO) of some Th2 cytokines (IL-4 and IL-10) or the chemokine, MCP-1, can abrogate OT [40,43]. TGFβ KO mice are difficult to work with as they die from severe and widespread inflammation soon after losing access to TGFβ from mother's milk. One study of OT in these mice utilized treatment with anti-leukocyte function-associated antigen (LFA)-1 to prevent inflammation, and found that OT was normally induced [41]. It is thought that non-specific bystander suppression of organ-directed autoimmune responses could prove beneficial and result in protective immunity. The advantage to active suppression mechanisms is that the precise identity of the autoantigen is not necessary. Mucosal administration of a prominent antigen contained within the organ of interest should establish the suppressive environment throughout the organ.

A model for oral tolerance induction

We and others [45-47] have recently begun to explore a highly physiologically relevant system to dissect the initial events in oral tolerance. Small numbers of MBP TCR transgenic T-cells are labeled with CFSE (5,6-carboxy-succinimidyl-fluorescein-ester) and adoptively transferred into normal syngeneic recipients. Using this technique, one is able to trace and follow the function of T-cells during OT induction. As shown in Figure 1, MBP at the gut mucosa may take one of two paths. Ag can traverse the epithelium, enter the portal circulation and be transported to the peripheral circulation and lymphoid tissue. Alternatively, Ag can be taken up by M cells in the Peyer's patch and given to antigen presenting cells (APCs), most likely dendritic cells (DCs), which present MBP to Ag-specific T-cells.

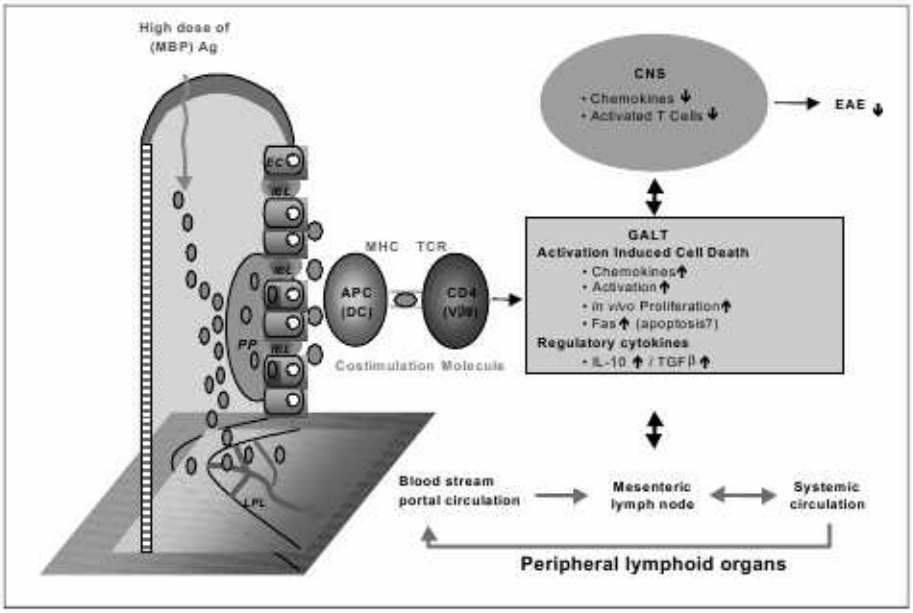
Recently, it was reported that induction of tolerance to soluble orally administered Ag does not require Peyer's patch and an organized GALT, but is induced efficiently by gut-conditioned DCs [48•]. The importance of DCs in OT induction was recently reinforced by studies using the protein growth factor Flt-3L (FL) [49•,50]. In these experiments, animals received multiple injections *in vivo* of FL and exhibited a massive increase in the number of DCs in all lymphoid organs. The expanded DC populations in FL-treated mice exhibit a resting phenotype. FL-treated mice showed enhanced OT to OVA compared with animals fed OVA alone [49•]. We have obtained similar results in EAE studying OT to MBP. Mice receiving FL and oral MBP displayed enhanced protection from EAE, relative to feeding MBP alone, and protection was achieved with a much lower dose of oral MBP in the presence of FL [50]. Moreover, FL and oral MBP administration proved successful in suppressing already established EAE [50]. It was reported that DCs from Peyer's patches under the influence of the mucosal cytokine milieu, present oral antigen inducing preferentially Th2 cytokines and/ or regulatory cells (producing

Table 2. Regulation of oral tolerance by cytokines and chemokines.

Mouse	Ag	Dose of Ag fed	Oral tolerance	Disease outcome
IL-12 KO	OVA	25 mg	Normal [38]	-
IFNγR KO	OVA	25 mg	Normal [38]	-
Anti-IFNγ Ab	OVA	25 mg	Normal [38]	-
IL-4 KO	OVA	25 mg	Normal [39]	-
IL-4 KO	IRBP	3x feeding + IL-2	Abrogate [40]	EAU Develops
IL-4 KO	IRBP	5x feeding	Normal [40]	↓ EAU
TGFβ null	OVA	1 to 20 mg	Normal [41]	-
IL-10 KO	IRBP	3x feeding + IL-2	Abrogate [40]	EAU Develops
IL-10 KO	IRBP	5x feeding	Normal [40]	↓ EAU
Anti-IL-10 Ab	OVA	20 mg (drinking)	Normal [42]	-
Anti-MCP-1 Ab	PLP139-151	2 mg	Abrogate [43]	EAE Develops
Anti-MIP-1α Ab	PLP139-151	2 mg	Normal [43]	↓ EAE
Anti-RANTES Ab	PLP139-151	2 mg	Normal [43]	↓ EAE
Stat 4 & 6 KO	OVA	25 mg	Normal [44]	-

EAE experimental autoimmune encephalomyelitis, **EAU** experimental autoimmune uveitis, **IRBP** inter-photoreceptor retinoid-binding protein, **KO** knockout, **OVA** ovalbumin, **PLP** proteolipid protein.

Figure 1. A model for the mechanisms of oral tolerance in EAE.



Ag antigen, **APC** antigen presenting cells, **CNS** central nervous system, **DC** dendritic cells, **EAE** experimental autoimmune encephalomyelitis, **EC** epithelial cells, **GALT** gut-associated lymphoid tissue, **IEL** intraepithelial lymphocytes, **LPL** lamina propria lymphocytes, **MBP** myelin basic protein, **MHC** major histocompatibility complex, **PP** Peyer's patch, **TCR** T-cell receptor.

IL-10 and TGFβ) [51•]. In contrast, if antigen is encountered in the context of a microbial stimulus [51•,52,53], then the DCs are primed to secrete IL-12 and promote the production of Th1 cytokines (IFNγ).

The T-cells become activated and proliferate in the Peyer's patch and mesenteric lymph nodes, and express high levels of apoptosis markers such as Fas. Apoptosis of T-cells in the Peyer's patch and mesenteric lymph nodes may occur at later timepoints after feeding. Also the activated regulatory cells produce regulatory cytokines such as IL-10 and TGFβ in the Peyer's patch after feeding. The oral administration of high doses of MBP induces chemokine production (MCP-1, MIP-1α and RANTES) in the gut of recipient mice. Local chemokine production leads to T-cell trafficking to the GALT [54]. Therefore, the protection from EAE may result from a decrease in the number of activated T-cells trafficking to the CNS. This model proposes that T-cells are preferentially attracted to the gut in response to orally fed MBP (Figure 1). The MBP-specific T-cells either remain in the GALT becoming regulatory cells producing regulatory cytokines, such as IL-10, or appear to promote T-cell deletion by a mechanism of activation-induced cell death. Thus, the model suggests that the mechanisms of OT are more complex than any one mechanism and that both anergy/ deletion and regulatory cells are involved at different timepoints after oral administration of high doses of Ag.

Clinical application of OT to autoimmue diseases

Table 1 summarizes the human trials of oral tolerization in autoimmune diseases: MS, RA, uveitis and Type I diabetes. Initial results were encouraging with no side effects reported [22]. The first clinical trial of oral tolerance in MS [18•] showed that patients fed bovine myelin had fewer exacerbations of their disease than patients fed a placebo antigen. It is notable that HLA-DR2+ males showed clinical improvement when compared with HLA-DR2- males or all females, although the numbers of patients tested were small. Importantly, feeding bovine myelin was reported to induce the appearance of TGFβ-producing cells in the peripheral blood, similar to observations in rodents [55•]. A subsequent larger multicenter trial using prospective stratification on the basis of sex and HLA-DR showed no statistically significant difference between the placebo and myelin-fed group. Our recent studies in chronic relapsing EAE demonstrate that homogeneous Ag (MBP) is more effective at inducing OT than heterogeneous Ag (myelin) in already established disease [56]. Thus, employment of a more well-defined antigen or different dose of orally administered antigen could possibly have led to a different outcome in the MS trial.

A similar series of events has taken place in the application of OT to RA. An early report with small numbers of patients showed that feeding chicken collagen resulted in significant clinical improvement in joint tenderness, joint pain and walk times in RA patients [21]. A follow-up, larger double-blind phase II dosing trial (20 to 2500 μg) of type II collagen by the same group showed a significantly positive effect at the lowest feeding dose when compared to the placebo group [57]. Another trial testing larger doses of orally administered collagen (1 to 10 mg) showed no significant differences between the collagen-treated and placebo groups [19]. These results generally agree with animal models of AIA (Table 1) and bystander suppression was observed only at the lower doses [9,10]. In uveitis, a pilot study indicated no significant effects of feeding bovine uveal S-antigen [58]. Interestingly, feeding an MHC peptide which cross reacts with S antigen to uveitis patients induced OT and resulted in clinical improvement [59]. Currently, other trials are underway, feeding recombinant human insulin to new onset diabetics, as well as in patients at

risk for developing Type I diabetes. In addition, a large multi-center trial is also ongoing feeding Copaxone (Autoimmune Inc/ Teva Pharmaceutical Industries Ltd), a random peptide which serves as a mimic to MBP, to MS patients. The results of the oral insulin and Copaxone trials are eagerly anticipated, as they will provide insight into the future applicability of this form of therapy to human autoimmune disease.

Conclusion

Based on the experience gained thus far in the clinical application of OT to autoimmune diseases, a number of lessons have been learned and important factors identified. First, it is apparent that enhancement of those cytokines and/ or chemokines which stimulate Th2 and/ or regulatory T-cell development, which are suppressive of the Th1 response, should lead to enhancement of OT [40,43]. Secondly, the use of mucosal adjuvants, such as recombinant cholera toxin B subunit conjugated to mucosally administered antigen can enhance the biological effects of antigen when administered orally [60]. Thirdly, the administration of FL together with mucosal antigen could increase the tolerance capabilities of oral antigen presentation to the immune system [50]. Finally, it is apparent that mucosal administration of any given antigen represents a unique situation and that the quantity and purity required for one antigen will be different from that required for another antigen. It is certainly possible that when the target of autoimmune attack is known, then large quantities of antigen should be administered which would result in deletion of self-reactive T-cell populations. Alternative routes of antigen administration, such as the nasal route, seem to be more efficient for antigen delivery [61•]. Therefore, where it was once thought that only antigen dose and timing of administration were important, it now appears that other factors such as mucosal route, antigen purity and modulatory factors can be added to the list of variables important in determining the efficacy of mucosally-administered antigen for the treatment of human disease.

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