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Oral tolerance: Therapeutic implications for autoimmune diseases

ANA M. C. FARIA[1] & HOWARD L. WEINER[2]

[1]*Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627, Belo Horizonte, MG 31270-901, Brazil, and* [2]*Harvard Medical School, Center for Neurologic Diseases, Brigham and Women's Hospital, 77 Avenue Louis Pasteur, Boston, MA 02115, USA*

Abstract
Oral tolerance is classically defined as the suppression of immune responses to antigens (Ag) that have been administered previously by the oral route. Multiple mechanisms of tolerance are induced by oral Ag. Low doses favor active suppression, whereas higher doses favor clonal anergy/deletion. Oral Ag induces Th2 (IL-4/IL-10) and Th3 (TGF-β) regulatory T cells (Tregs) plus CD4+CD25+ regulatory cells and LAP+T cells. Induction of oral tolerance is enhanced by IL-4, IL-10, anti-IL-12, TGF-β, cholera toxin B subunit (CTB), Flt-3 ligand, anti-CD40 ligand and continuous feeding of Ag. In addition to oral tolerance, nasal tolerance has also been shown to be effective in suppressing inflammatory conditions with the advantage of a lower dose requirement. Oral and nasal tolerance suppress several animal models of autoimmune diseases including experimental allergic encephalomyelitis (EAE), uveitis, thyroiditis, myasthenia, arthritis and diabetes in the nonobese diabetic (NOD) mouse, plus non-autoimmune diseases such as asthma, atherosclerosis, colitis and stroke. Oral tolerance has been tested in human autoimmune diseases including MS, arthritis, uveitis and diabetes and in allergy, contact sensitivity to DNCB, nickel allergy. Positive results have been observed in phase II trials and new trials for arthritis, MS and diabetes are underway. Mucosal tolerance is an attractive approach for treatment of autoimmune and inflammatory diseases because of lack of toxicity, ease of administration over time and Ag-specific mechanism of action. The successful application of oral tolerance for the treatment of human diseases will depend on dose, developing immune markers to assess immunologic effects, route (nasal versus oral), formulation, mucosal adjuvants, combination therapy and early therapy.

Keywords: *Oral tolerance, nasal tolerance, autoimmune diseases, bystander suppression*

Autoimmunity and oral tolerance

"Immunological tolerance" has often been defined as a mechanism by which the immune system prevents pathologic autoreactivity against self and thus prevents autoimmune diseases. This definition of tolerance as a negative counterpart of immunity comes from the classical work of Burnet who first proposed self/non-self discrimination as a major principle driving the operation of the immune system and tolerance to self-components as a deletional event taking place at early periods of development (Burnet 1959). According to Burnet's "Clonal Selection Theory", self tolerance was based on the blindness of the mature immune system to body components. The description of the thymic selection of T lymphocytes, of the subsets of T cells and their distinct actions, as well as the demonstration of auto-reactive B and T cells in normal individuals contributed to change this scenario (Avrameas 1991; Kerlero de Rosbo et al. 1993; Zhang et al. 1994; Lacroix-Desmazes et al. 1998). It became clear to several researchers that natural tolerance to auto-components is a more complex phenomenon. Physiological autoimmunity, as a self-assertion process that generates immune regulation and pathological auto-immunity has to be distinguished as different outcomes of self-recognition (Cohen and Young 1991;

Modigliani et al. 1996). Studies derived from this new approach clearly demonstrated that natural tolerance to self is an active process that depends on the activity of non-inflammatory/regulatory auto-reactive T lymphocytes present at a stable frequency in the normal repertoire (Cohen and Young 1991; Modigliani et al. 1996; Sakaguchi 2004).

In analogy to the natural non-inflammatory reactivity that the immune system mounts to self components, the name "oral tolerance" has been given in the seventies (Vaz et al. 1977) to the immunological tolerance to antigens (Ag) that access the body via the oral route. Oral tolerance has been classically defined as the specific suppression of cellular and/or humoral immune responses to an Ag by prior administration of the Ag by the oral route. Since most of the natural contact with foreign Ag occurs via the mucosal surfaces, tolerance induction to commensal bacteria and dietary proteins represents the major immunological event taking place in the gut in physiological conditions.

Oral tolerance is of unique immunologic importance since it is a continuous natural immunologic event driven by exogenous Ag. Due to their privileged access to the internal milieu, commensal bacteria and dietary Ag that continuously contact the mucosa represent a frontier between foreign and self-components. Thus, oral tolerance is a form of peripheral tolerance that evolved to treat external agents that gain access to the body via a natural route as internal components that then become part of self.

Within the view of self tolerance as an active process, auto-immune diseases would arise from defects in immunoregulatory processes. The usual therapies for these pathological conditions rely on non-specific immunossupression with several undesirable side effects. Since oral tolerance is such a potent way of inducing regulatory cells towards specific Ag, the idea of using the oral route to trigger tolerance to Ag involved in autoimmune diseases comes as an important clinical application of the phenomenon. Although the idea was already present as a theoretical possibility in the seventies, it was only successfully tested in the classical studies of four groups of researchers working on experimental models of arthritis (Nagler-Anderson et al. 1986; Thompson and Staines 1986) and multiple sclerosis (Bitar and Whitacre 1988; Higgins and Weiner 1988) in the eighties.

Mechanisms of oral tolerance induction

A concept has arisen in oral tolerance studies that there are two primary effector mechanisms of oral tolerance: low doses of Ag favor the generation of regulatory cell-driven tolerance whereas high doses of Ag favor either clonal deletion (Chen et al. 1995; Marth et al. 1996; Meyer et al. 2001) or anergy of specific T cells (Mowat et al. 1982; Whitacre et al. 1991; Melamed and Friedman 1993; Friedman and Weiner 1994; Inada et al. 1997). Low doses of Ag would preferentially induce regulatory T cells (Tregs) that secrete down-modulatory cytokines such as TGF-β, IL-10 and IL-4 (Miller et al. 1992). TGF-β secreting CD4$^+$ and CD8$^+$ T cells have been isolated and cloned from the PP and MLN of orally tolerized mice (Chen et al. 1994, 1996; Santos et al. 1994; Wang et al. 1994) and from the peripheral blood of humans fed MBP (Fukaura et al. 1996). T cells cloned from tolerized mice have been ascribed to a unique subset of CD4$^+$ T cell, the Th3 cell (Chen et al. 1994; Fukaura et al. 1996; Faria and Weiner 2005). This population appears to be dependent on IL-4, rather than IL-2 for its growth and some Th3 clones produce IL-4 and/or IL-10 together with TGF-β (Chen et al. 1994; Faria and Weiner 2005). In addition, in TCR transgenic mice, oral adminstration of Ag also resulted in a relative increase of CD4+CD25+ Tregs expressing either CTLA-4 (Zhang et al. 2001) or FoxP3 (Mucida et al. 2005). These cells have suppressive properties *in vitro* and can transfer tolerance to naive recipients (Zhang et al. 2001; Nagatani et al. 2004). They either secrete high levels of IL-10 and TGF-β (Zhang et al. 2001) or are dependent in TGF-β for their development (Mucida et al. 2005). In addition to the secreted form of TGF-β, murine CD4+CD25+ or CD4+CD25 − regulatory cells have been reported to express latency-associated peptide (LAP) and TGF-β on the surface after activation and exert regulatory function by the membrane-bound TGF-β *in vitro* (Nakamura et al. 2001; Oida et al. 2003). Recombinant latency-associated peptide (rLAP) was also shown to reverse suppression by mouse CD4+CD25+ T cells as well as their human counterparts, CD4+CD25^{high} T cells (Nakamura et al. 2004). Thus, surface expression of the latent form of TGF-β can be another mode of action for TGF-β.

The generation of Ag-specific regulatory cells seem to be a result of Ag presentation by gut-associated Antigen-presenting cells (APC) (Viney et al. 1998; Simioni et al. 2004) that are particularly involved in the induction of TGF-β-producing T cells (Simioni et al. 2004). These Ag-specific regulatory cells migrate to lymphoid organs suppressing immune responses by inhibiting the generation of effector cells and to target organs, suppressing disease by releasing Ag-non specific cytokines (bystander suppression).

Since these pioneer studies, evidence has been reported that the two forms of tolerance are not mutually exclusive and they may overlap. Recent studies on the properties of Tregs also describe them as "anergic" (Takahashi et al. 1998; Taams et al. 2002; Sakaguchi 2004) and deletional events taken place in the gut mucosa (Ramachandran et al. 2000) result local TGF-β secretion by macrophages

(Fadok et al. 1998) and dying T cells (Chen et al. 2001). This cytokine acts as a growth factor for the generation of Th3 cells (Paul and Seder 1994; Weiner 2001; Faria and Weiner 2005) and of CD4+FoxP3+ Tregs (Chen et al. 2003; Horwitz et al. 2003; Sakaguchi 2004). Thus, anergy/deletion and active regulation may be different aspects of the same tolerogenic processes that are triggered by antigenic contacts in the intestine.

Major issues involved in the use of oral tolerance as a therapy

Oral tolerance has been already successfully tested in a number of experimental models for autoimmune diseases as well as other inflammatory conditions such as allergy and transplantation (Faria and Weiner 2005). After this intensive research work, three major issues emerged on the therapeutic use of oral tolerance for autoimmune diseases: (1) the choice of a target Ag; (2) the limiting therapeutic situation of treating already sensitized subjects; and (3) the large doses that are sometimes required for its efficient induction.

First, the rationale for the use of oral tolerance as a therapeutic tool in autoimmune conditions is mainly that it would provide a type of specific suppression avoiding the side effects of non-specific immunosuppression. One potential problem in its use is the choice of the target Ag. It is not always clear which are the autoantigens involved in autoimmune pathogenesis. Fortunately, as we will discuss below, oral tolerance is associated with a bystander suppression event that spreads the regulatory activity triggered by oral tolerance to other Ag presented in the same context. Cross-suppression (Vaz et al. 1981) or bystander suppression (Miller et al. 1991) of a non-related inflammatory event by mucosally administered Ag is now a well described phenomenon that provides a way to circumvent the need of unique target for oral tolerance induction.

Second, it has been already reported that oral tolerance is easily induced in naïve animals but primed animals are more resistant to its suppressive effects (Yoshino et al. 1995; Conde et al. 1998; Faria and Weiner 2005). Indeed, immunized mice are susceptible to oral tolerance induction when the Ag is administered up to 7 days after immunization. Then, they become progressively resistant to tolerance induction and 14 days later they are completely refractory (Conde et al. 1998). As a way to circumvent this problem, there are a number of adjuvants and regimens of feeding already described that modulate positively the mechanisms involved in oral tolerance induction (see section on modulation of oral tolerance).

Third, to achieve effective suppression with the oral tolerance regimen, sometimes large doses of Ag are necessary. This requirement may hinder the use of oral tolerance to human subjects. As we will discuss below, nasal tolerance may be an alternative in many cases since the nasal mucosa is also an effective route to induce suppression and the doses required are usually 10-fold lower.

Bystander suppression (Table I)

Bystander suppression is a concept that regulatory cells induced by a fed Ag can suppress immune responses stimulated by different Ag, as long as the fed Ag is present in the anatomic vicinity (Faria and Weiner 2005). It was described during an investigation of the regulatory cells induced by oral administration of low doses of MBP (Miller et al. 1991). It solves a major conceptual problem in the design of Ag- or T-cell-specific therapy for inflammatory autoimmune diseases such as MS, type 1-diabetes and rheumatoid arthritis (RA) in which the autoantigen is unknown or where there are reactivities to multiple autoantigens in the target tissue. During the course of chronic inflammatory autoimmune processes in animals, there is intra- and interantigenic spread of autoreactivity at the target organ (Lehmann et al. 1992; Cross et al. 1993; Tisch et al. 1993). Similarly in human autoimmune diseases, there are reactivities to

Table I. Models of autoimmune and other diseases that demonstrate bystander suppression.

Disease	Immunizing Ag	Oral Ag	Target organ
Arthritis	BSA, mycobacteria	CII	Joint
EAE	PLP	MBP	Brain
EAE	MBP peptide 71–90	MBP peptide 21–40	Brain
EAE	MBP	OVA	Lymph node, DTH response
Diabetes	LCMV	Insulin	Pancreatic islets
IBD	CD4 + CD45RB^{high} T cell transfer	OVA	Intestine
Stroke	None	MBP, MOG	Brain
Nerve injury	None	MBP	Brain
Atherosclerosis	None	Hsp65	Aortic arch

Abbreviation: BSA, bovine serum albumin; DTH, delayed-type hypersensitivity; EAE, experimental allergic encephalomyelitis; LCMV, lymphocytic choriomeningitis virus; MBP, myeline basic protein, OVA, ovalbumin; PLP, proteolipid; and IBD, inflammatory bowl disease.

multiple autoantigens in the target tissue. For example, in MS, there is immune reactivity to at least three myelin Ag: MBP, proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) (Kerlero de Rosbo et al. 1993; Zhang et al. 1994). In type 1 diabetes, there are multiple islet-cell Ag that could be the target of autoreactivity including glutamic acid decarboxylase (GAD), insulin and heat shock proteins (HSP) (Harrison 1992). Because regulatory cells induced by oral Ag secrete non-specific cytokines after being triggered by the fed Ag, they suppress inflammation in the microenvironment where the fed Ag is localized. Thus, for a human organ-specific inflammatory disease, it is not necessary to know the specific Ag that is the target of an autoimmune response, but only to feed an Ag capable of inducing regulatory cells, which then migrate to the target tissue and suppress inflammation.

The mechanisms by which this process could occur are usually assumed to reflect the production of inhibitory cytokines by the tolerized Treg, with resulting suppression of the T cells with other specificities in the vicinity. IL-10 and TGF β are the most favored cytokines in this respect. This idea is supported by several studies that showed TGF-β-dependent suppression in experimental allergic encephalomyelitis (EAE) studies using different myelin Ag (Miller et al. 1991, 1993). On the other hand, the role of IL-10 in the phenomenon is supported by the findings that OVA-specific Tr1 cells can prevent IBD (induced by gut bacteria), providing OVA is present in the intestinal environment (Groux et al. 1997).

Inhibitory mediators or molecules on the surface of Tregs could act directly on the responding T cells or they could act by "deactivating" the APC which is attempting to stimulate the third party T cell. Ag-presenting dendritic cells (DCs) can act as "temporal bridges" to relay information from orally tolerized memory T cells to naive CD4 + T cells (Lanzavecchia 1998). Since APC, specially Dcs, recirculate, this idea is consistent with all the data available on the phenomenon including reports showing that a type of bystander suppression occurs with Ag at different anatomic sites or injected days apart from each other (Carvalho et al. 1994, 1997).

Bystander suppression has been demonstrated in a number of autoimmune disease models. For instance, PLP-induced EAE can be suppressed by feeding MBP (al-Sabbagh et al. 1994) or by administering TGF-β-secreting MBP-specific T-cell clones from orally tolerized animals (Chen et al. 1994). In the Lewis rat model of EAE, disease induced by MBP peptide 71–90 can be suppressed by feeding peptide 21–40 (Miller et al. 1993). In arthritis models, adjuvant- and Ag-induced arthritis can be suppressed by feeding type II collagen (CII) (Yoshino et al. 1995). In a virus-induced model of diabetes, whereby a lymphocytic choriomeningitis virus (LCMV) protein is expressed on the insulin promoter and the animal is then infected with the LCMV, diabetes can be suppressed by feeding insulin (von Herrath et al. 1996).

In theory, bystander suppression could be applied for the treatment of organ-specific inflammatory conditions that are not classic autoimmune diseases, such as psoriasis, or could be used to target anti-inflammatory cytokines to an organ where inflammation may play a role in disease pathogenesis even if the disease is not primarily inflammatory in nature. For example, oral MBP decreased stroke size in a rat stroke model, presumably by decreasing inflammation associated with ischaemic injury (Becker et al. 1997). Induction of nasal tolerance in mice to a peptide of the myelin Ag MOG also reduces ischemic injury following stroke. Regulatory cells are generated in tolerant wild type but not in IL-10-deficient mice. Moreover, IL-10-producing MOG specific CD4+ T cells can transfer tolerance to naïve recipients (Frenkel et al. 2003, 2005). Bystander suppression achieved by oral Ag in non-immune pathological conditions may mimic a physiological protective reaction to self Ag in response to inflammatory insult as observed in the experimental model of central nervous system (CNS) axonal injury in EAE-susceptible and resistant rat strains. Oral treatment with low-dose MBP is beneficial for post-traumatic survival of retinal ganglion cells in Lewis rats following optic nerve injury (Monsonego et al. 1996). HSP, known to be up-regulated in inflammatory situations, are also a suitable target for bystander suppression strategies. Oral as well as nasal administration of hsp65 in LDL-R deficient mice fed a high colesterol diet downmodulates IL-2 and IFN-γ production and aortic plaque development. Production of IL-10 but not TGF-β is up-regulated in hsp-tolerized mice (Harats et al. 2002; Maron et al. 2002a).

Modulation of oral tolerance (Table II)

Data on animal models showed also that a number of factors have been reported to modulate oral tolerance. As oral tolerance has usually been defined in terms of Th1 responses, anything that suppress Th1 and/or enhances Th2 or Th3 cell development would enhance oral tolerance. Th3 cells appear to use IL-4 and TGF-β itself as one of its growth/differentiation factors. Thus, IL-4 administration i.p., oral IL-10 and IL-4 can also enhance oral tolerance when co-administered with Ag and cytokines have also been administered by the nasal route (Inobe et al. 1998; Slavin et al. 2001). Oral but not subcutaneous lipopolysaccharide (LPS) enhances oral tolerance to MBP and is associated with increased expression of IL-4 in the brain (Khoury et al. 1992). In the uveitis model, intraperitoneal IL-2 potentiates oral tolerance and is associated with increased production of TGF-β, IL-10 and IL-4 (Rizzo et al. 1994). Oral Ag delivery

Table II. Modulation of oral tolerance

Enhances	Prevents
IL-2	IFN-γ
IL-4, IL-10	IL-12, IL-18
Helminth Ag	CD80 over-expression
Anti-CD4	Anti-TGF-β Ab
Anti-IL-12	CT
CTB	Anti-MCP-1 (CCL2)
LPS	IBD
Flt3L	Anti-γδ Ab
Type 1 IFN (β and τ)	GVHR
Multiple emulsions	CY, 2′-dGuo
Lipossomes	Oestradiol
PLGA	
Continuous feeding	
TGF-β	
Pooled IgG	

Abbreviations: CT, cholera toxin; CTB, cholera toxin B subunit; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MCP, monocyte chemotactic protein 1; GVHR, Graft-versus-Host Reaction; CY, cyclophosphamide; 2′-dGuo, 2′-deoxyguanosine; TGFβ, transforming growth factor? β; and PLGA, poly lactic-co-pucolic acid.

using either a multiple emulsion system (Kim et al. 2002; Pecquet et al. 2000) or liposomes (Masuda et al. 2002) or poly-lactic-co-pucolic acid (PLGA) (Kim et al. 2002) also enhances oral tolerance. In the arthritis model, administration of TGF-β or dimaprid (a histamine type 2 receptor agonist) i.p., both of which are believed to promote the development of immunoregulatory cells, enhances the induction of oral tolerance to collagen II even after the onset of arthritis (Thorbecke et al. 1999). Coupling Ag to recombinant cholera toxin B subunit (CTB) enhances their ability to induce peripheral immune tolerance (Holmgren et al. 2003). A recently reported vaccine consisting of a fusion protein composed of CTB subunit and insulin produced in the hemolymph of silkworm showed ability to enhance oral tolerance induction in non-obese diabetic mice (Gong et al. 2005). On the other hand, cholera toxin (CT) is one of the most potent mucosal adjuvants and feeding CT abrogates oral tolerance when fed with an unrelated protein Ag. Large doses of IFN-γ given intraperitoneally abrogate oral tolerance (Zhang et al. 1990a), anti-IL-12 enhances oral tolerance and is associated both with increased TGF-β production and T cell apoptosis (Marth et al. 1996) and subcutaneous administration of IL-12 reverses mucosal tolerance (Eaton et al. 2003). Oral IFN-β and IFN-τ (tau) synergizes with the induction of oral tolerance in SJL/PLJ mice fed low doses of MBP (Nelson et al. 1996; Soos et al. 2002). Other exogenous agents which have been reported to enhance oral tolerance when given orally include parasite Ag from *H. polygyrus* (Shi et al. 2000), polysaccharide AZ9 from *Klebsiella oxitoca* (Sugihara et al. 2002) and *Schistosoma Mansoni* egg antigens (SEA) (Maron et al.

1998). Antibody (Ab) to chemokine monocyte chemotactic protein 1 (MCP-1) abrogates oral toleran (Karpus et al. 1996). Intraperitoneal co-adminstration of normal IgG to mice orally treated with Ag leads to a sustained and intense immunological tolerance including those of lupus-prone NZB X NZW lineage (Mengel et al. 2005). We also found recently that not only agents but certain regimens of feeding interfere with oral tolerance induction. Continuous and serial feeding regimens of the Ag administration enhances significantly oral tolerance by up-regulating IL-10 and TGF-β (Faria et al. 2003).

Nasal tolerance (Table III)

In the nasal and upper respiratory tract of human and animals, there is an intact mucosal lymphoid tissue containing two structures: nasal associated lymphoid tissue (NALT) and the bronchus associated lymphoid tissue (BALT). In rodents, there is a lymphoid tissue that surrounds the nasal cavity whereas in humans and certain other species, there is an oropharyngeal lymphoid tissue (Waldeyer's ring) that includes the adenoid and the bilateral tubule, palatine and lingual tonsils (Goeringer and Vidic 1987). The bronchial mucosas resemble the gut mucosa with a network of Dcs and lymphocytes scattered in the airway epithelium. One relevant difference between the absorption of Ag in the upper respiratory tract and in the gut is the absence of digestion in the former. This feature may explain why minute doses of Ag are effective in inducing tolerance via the nasal route as compared to the high doses usually required for oral administration.

Nasal adminstration of Ag has been shown to suppress a number of experimental autoimmune diseases including diabetes in nonobese diabetic (NOD) mice (Daniel and Wegman 1996; Harrison et al. 1996; Tian et al. 1996), pristane- and collagen-induced arthritis (Staines et al. 1996; Myers et al. 1997; Garcia et al. 1999; Lu and Holmdahl 1999), EAE in mice (Metzler and Wraith 1993; Liu et al. 1998) and rats (Bai et al. 1997; Li et al. 1998), experimental autoimmune myasthenia gravis (EAMG) (Ma et al. 1995; Shi et al. 1999), experimental

Table III. Autoimmune and inflammatory disease models suppressed by nasal tolerance.

Model	Administered protein
EAE	MBP, MBP peptides
EAMG	AChR
EAU	Retinal Ag
Diabetes	Insulin, GAD65
EAN	Myelin peptide
Arthritis	Hsp65, collagen II and IX, CII peptide
Atherosclerosis	Hsp65
Stroke	MOG peptide

autoimmune uveitis (EAU) (Dick et al. 1993) and experimental autoimmune neuritis (EAN) (Zhu et al. 1998).

The mechanisms involved in the inhibition of disease development after nasal administration of Ag involve induction of CD4+T cell unresponsiveness or Treg development (Harrison et al. 1996; Shi et al. 1999; Akbari et al. 2001; Samsom et al. 2004). These Tregs are described as γβ CD8+ T cells (Harrison et al. 1996) or ab CD4+T cells that secrete high amounts of IL-10 (Akbari et al. 2001; Samsom et al. 2004). Akbari et al. (2001) suggest that IL-10-producing DCs from the lungs are particularly effective in inducing the development of Tr1 cells (CD4+ cells that produce IL-10) upon nasal administration of Ag. Some authors also found nasal tolerance to be associated with the induction of TGF-β-producing T cells (Shi et al. 1999; Ostroukhova et al. 2004). Interestingly, bystander suppression in experimental models of atherosclerosis (Maron et al. 2002a,b) and stroke (Frenkel et al. 2003, 2005) is effectively induced by nasal administration of Ag and suppression it is mediated by IL-10-producing T cells.

Therapeutic applications of oral tolerance in autoimmune and inflammatory diseases in animals (Table IV)

Several studies have demonstrated the effectiveness of oral administered autoantigens in animal models of autoimmune and inflammatory diseases (Faria and Weiner 2005).

Experimental allergic encephalomyelitis

The first studies to show that orally administered myelin Ag could suppress EAE were performed in the Lewis rat. EAE was suppressed by low doses of oral MBP and MBP fragments (Higgins and Weiner 1988)

Table IV. Experimental models of autoimmune diseases suppressed by oral tolerance.

Model	Protein fed
EAE	MBP, PLP, MOG, copaxone
Arthritis (CIA, AA, AIA, PIA, SCW)	Collagen II, Hsp65, BSA
Diabetes (NOD mouse)	Insulin, insulin β-chain, GAD, OVA
Colitis	Haptenized or normal colonic proteins, OVA
EAU	S-Ag, IRBP
EAMG	AchR
Anti-phospholipid syndrome	β2-Glycoprotein
Experimental allergic neuritis	PNS-myelin
Thyroiditis	Thyroglobulin
Atherosclerosis	Hsp65
Stroke	MOG
Nerve injury	MBP

and by high doses of MBP given in bicarbonate (Bitar and Whitacre 1988). High doses of MBP can suppress EAE via the mechanism of T cell clonal anergy (Javed et al. 1995) whereas multiple lower doses prevent EAE by transferable active cellular suppression (Miller et al. 1993). In the nervous system of low-dose-fed animals, inflammatory cytokines such as TNF and IFN-γ are down-regulated and TGF-β is up-regulated (Khoury et al. 1992). Administration of myelin to sensitized animals in the chronic guinea pig model or larger doses of MBP in the murine EAE model is protective and does not exacerbate disease (Brod et al. 1991; Meyer et al. 1996) and long term (6 month) administration of myelin in the chronic EAE model was beneficial (al-Sabbagh et al. 1996a,b).

A number of studies have demonstrated suppression of EAE in murine models. Both conventional and T cell receptor transgenic animals have been used and both oral MBP and oral PLP have been administered although the majority of studies have used MBP. In these models, MBP regulatory clones have been described and such cells have also been induced in MBP T cell receptor transgenic mice. Both CD4 and CD8 cells have been shown to mediate active suppression and anergy/deletion have also been demonstrated in oral tolerance to EAE.

The latest approach in animal models has been to utilize glatiramer acetate (Cop1, Copaxone), a drug approved for therapy of multiple sclerosis which is given to patients by injection. Teitelbaum et al. (1999) have found that oral glatiramer acetate suppresses EAE in both the mouse and rat models and we have found that oral glatiramer acetate suppresses EAE in MBP T cell receptor transgenic animals and induces the upregulation of TGF-β when given orally (Maron et al. 2002b). Our working hypothesis is that glatiramer acetate is acting as an altered peptide ligand and is immunologically active in the gut (Weiner 1999).

Arthritis models

There are several animal models of arthritis including collagen induced arthritis (CIA), adjuvant arthritis (AA), pristane induced arthritis (PIA), antigen-induced arthritis (AIA), silicone induced arthritis and streptococcal cell with arthritis.

Immunization with heterologous or homologous species of CII produces autoimmune responses to CII that lead to development of arthritis in susceptible mouse strains (Courtenay et al. 1980). CIA has been used as an animal model for RA and is characterized by chronic inflammation within the joints, associated with synovitis and erosion of cartilage and bone (Trentham et al. 1977). The first experiments of oral tolerance using rat CIA was done by Thompson and Staines (1986) and Nagler-Anderson et al. (1986) in WA/KIR rats and DBA/1 mice, respectively by

immunizing with CII either in complete or in incomplete Freund's adjuvant (CFA). Feeding of CII prior to immunization delayed the onset and suppressed the incidence of CIA.

Collagen peptides are also capable of inducing CIA. Immunodominant collagen peptides have been used to suppress CIA by oral administration. Khare et al. (1995) induced CIA to DBA/1 mice by immunizing with human CII peptide (250–270) in CFA. Oral tolerance with human peptide CII (250–270) abolished anti-human and anti-mouse CII Ab and markedly reduced the disease severity both at early and effector phases.

Another major model for RA is AA, which is a well-characterized and fulminant form of experimental arthritis. Oral administration of chicken CII consistently suppressed the development of AA in Lewis rats and the suppression of AA could be adoptively transferred by T cells from CII fed animals (Zhang et al. 1990b).

HSP plays an important role in the AA model (van Eden et al. 1988). It was recently reported that oral administration of mycobacterial 65-kDa HSP suppressed the development of AA in rats (Haque et al. 1996). Suppression of AA was adoptive transferred by spleen cells from orally tolerized rats.

Oral tolerance is also effective in PIA (Thompson et al. 1993). Immunizing mice twice with 2, 6, 10, 14-tetramethylpentadecane (pristane) twice leads to arthritis after $100 \sim 200$ days. Increasing doses of oral CII lowered both the incidence and severity of PIA.

Other animal models of arthritis that have been successfully treated by mucosal tolerance include streptococcal cell wall arthritis (Chen et al. 1998) and silicone induced arthritis (Yoshino 1995).

Methotrexate is a widely used drug in RA. We found that there is a synergistic effect between methotrexate and orally administered Ag such as MBP (al-Sabbagh et al. 1997) and a synergistic effect with oral methotrexate was also observed in the AA model (Weiner and Komagata 1998).

Diabetes

Oral insulin has been shown to delay and in some instances, prevent diabetes in the NOD mouse model. Such suppression is transferable (Zhang et al. 1991), primarily with $CD4^+$ cells (Bergerot et al. 1999). Immunohistochemistry of pancreatic islets of Langerhans isolated from insulin fed animals demonstrates decreased insulitis (Hancock et al. 1995). Oral insulin suppressed diabetes in a viral induced model of diabetes in which LCMV was expressed under the insulin promoter and animals infected with LCMV to induce diabetes (von Herrath et al. 1996). Protection was associated with protective cytokine shifts (IL-4/IL-10, TGF-β) in the islets. It has also been shown that expression of TGF-β in the pancreatic

islets protects the NOD mouse from diabetes (King et al. 1998). Oral administration of β-chain of insulin, a 30-amino-acid peptide slowed the development of diabetes and prevented diabetes in some animals (Polanski et al. 1997). This effect was associated with a decrease IFN-γ and an increase in IL-4, TGF-β and IL-10 expression. Oral dosing of bacterial stimulants such as LPS and *E. Coli* extract OM-89 in NOD mice induces a Th2 shift in the gut cytokine gene expression and concomitantly, improves diabetes prevention by oral insulin administration (Bellmann et al. 1997). Oral administration of recombinant GAD from plant sources suppressed the development of diabetes in NOD mouse as does oral administration of a plant-based CTB-insulin fusion protein (Arakawa et al. 1998).

Colitis

TGF-β appears to play a crucial role in the development of animal models of colitis, including TNBS-colitis, colitis in the IL-2-deficient animal model following systemic administration of TNP-KLH in adjuvant and in the model of Th1 colitis in SCID mice. It has been shown that TNBS colitis can be prevented by oral administration of TNBS, which acts via the induction of TNBS-specific TGF-β responses (Neurath et al. 1996). TNBS-induced colitis in rats can also be prevented by feeding either human colon epithelial cells or rat colonic epithelial extracts, but not human fibroblasts nor rat small intestine extracts, showing that tolerance is organ specific. Protected animals had low IFN-γ and high TGF-β levels and tolerance could be transferred by mesenteric lymph nodes cells (Dasgupta et al. 2001). In addition, colitis can be suppressed via bystander suppression by transfer of OVA transgenic CD4+-CD45^{high} T cells (from DO.11.10 mice) into SCID mice (Zhou et al. 2004).

Uveitis

Oral administration of S-Ag, a retinal autoantigen that induces EAU, or S-Ag peptides prevents or markedly diminishes the clinical appearance of S-Ag-induced disease as measured by ocular inflammation (Nussenblatt et al. 1990). S-Ag-induced EAU can also be suppressed by feeding an HLA peptide (Wildner and Thurau 1994). Feeding interphotoreceptor binding protein (IRBP) suppresses IRBP-induced disease and is potentiated by IL-2 (Rizzo et al. 1994). Oral feeding of retinal Ag not only can prevent acute disease but also can effectively suppress second attack in chronic-relapsing EAU, demonstrating that oral tolerance may have practical clinical implications in uveitis, which is predominantly a chronic-relapsing condition in humans (Thurau et al. 1997a,b). Other investigators have found that oral administration of bovine S-Ag

peptides is very efficient in preventing EAU but could only inhibit mild disease if feeding was delayed until after immunization and relatively high feeding doses were required (Ma et al. 1998; Torseth and Gregerson 1998).

Myasthenia

Although myasthenia gravis is an Ab-mediated disease, oral administration of the Torpedo acetylcholine receptor (AchR) to Lewis rats prevented or delayed the onset of myasthenia gravis. The levels of anti-AchR antibodies in the serum were lower in orally tolerized animals than in control animals. The effect was dose dependent and large doses of Ag (at least 5 mg of AchR) plus soybean tripsin inhibitor (STI) (Wang et al. 1993) were required, suggesting that anergy may be the primary mechanism. Purified AchR was found more effective than an unpurified mixture (Okumura et al. 1994).

Other autoimmune diseases

The antiphospholipid syndrome is characterized by the presence of high titers of IgG anticardiolipin antibodies and/or lupus anticoagulant antibodies. Oral administration in BALB/c mice of low doses of β2 glycoprotein prevented the serologic and clinical manifestation of experimental antiphospholipid syndrome upon immunization with the autoantigen. Decreased T cell responses, Ab responses and increased expression of TGF-β which mediated the suppression was demonstrated. Tolerance was transferred by CD8 positive class I restricted TGF-β secreting cells (Blank et al. 1998).

Immune complex disease can be suppressed both following administration of a single large dose of Ag (Browning and Parrott 1987) or by placing Ag in drinking water (Devey and Bleasdale 1984). These studies were performed before oral tolerance was applied to autoimmune diseases. It has also been raised whether defective oral tolerance may be associated with experimental IgA nephropathy (Gesualdo et al. 1990). A recent report shows that feeding of Ro 274 peptide or Ro 60 to BALB/c mice with Sjogren's syndrome-like disease (immunized with Ro 60 previously) drastically reduces salivary infiltrates and specific Ab production (Kurien et al. 2005).

Thyroiditis has been effectively suppressed following oral administration of either porcine or human thyroglobulin (Guimarães et al. 1995; Peterson and Braley-Mullen 1995). In the murine model, CD8 positive regulatory cells which produce IL-4 and TGF-β mediated the suppression (Guimaraes et al. 1995). These cells also appear to induce bystander suppression upon triggering with the fed Ag. Other investigators (Zhang and Kong 1998) reported

that tolerance induced by IV administration of deaggregated thyroglobulin in experimental autoimmune thyroiditis (EAT) is dependent on CD4+ T cells but independent of IL-4/IL-10.

Experimental allergic neuritis is the counterpart of EAE and can be suppressed both by oral administration of peripheral nerve proteins (Gaupp et al. 1997).

Link's group has experimented with administering Ag associated with more than one autoimmune disease and immunizing with a mixture of Ag. They have demonstrated that oral administration of AChR plus MBP suppresses EAMG and EAE (Wang et al. 1995). Thus, it appears there is no interference by one autoantigen versus another when they are from different target organs.

Treatment of autoimmune diseases in humans using oral tolerance (Table V)

Based on the long history of oral tolerance and the safety of the approach, human trials have been initiated in autoimmune diseases, MS, RA, uveitis and diabetes. These initial trials suggest that there has been no systemic toxicity or exacerbation of disease, although clinical efficacy resulting in an approved drug has yet been demonstrated. Results in humans however, have paralleled several aspects of what has been observed in animals.

Table V. Human studies of oral tolerance application in autoimmune diseases.

A. Diabetes
Preliminary report: Preserved beta-cell function as measured by endogenous C-peptide in new onset diabetics over 20 years old fed 1 mg;
Oral insulin not beneficial in new onset disease fed 2.5 or 7.5 mg oral insulin for 1 year;

B. Multiple sclerosis
Oral myelin decreased MRI lesions in DR2 + males, no effect on clinical relapse;
Increased TGF-β secreting myelin cells after oral myelin;

C. RA
Oral collagen ameliorates RA;
Oral collagen benefits juvenile RA in open label trial;
20 μg best in double blind oral dosing trial of CII;
Oral bovine collagen at higher doses without positive effect;
60 μg oral collagen best in composite analysis of dosing trials;
no different than placebo in phase III trial;
Bovine collagen (0.5 mg) beneficial in placebo controlled trial;
Oral collagen II beneficial in JRA clinically and immunologically in pilot trial;

D. Uveitis
Oral S-Ag appeared to allow medication taper, retinal mixture appeared to worsen uveitis;
Oral HLA peptide allowed steroid taper;

E. Thyroid disease
Decreased cellular immunity to thyroglobulin in patients receiving oral thyroglobulin

Diabetes

Six different trials are currently underway or completed in testing mucosally administered recombinant human insulin as a tolerizing agent in type 1 diabetes: (1) a multicenter double-blind study in the US evaluating oral insulin therapy versus placebo in adults and children with new-onset disease. Preliminary analysis suggests preserved beta cell function as measured by endogenous C-peptide insulin responses in patients diagnosed over age 20 years and fed 1 mg. versus placebo (Ergun-Longmire et al. 2004); (2) a double-blind study in France to compare oral insulin therapy and parenteral insulin therapy versus placebo in patients during the remission phase. Evaluation criteria include duration of remission, measures of insulin secretion/sensitivity and immunological parameters; no effect of treatment with 2.5 or 7.5 mg oral insulin was observed (Chaillous et al. 2000); (3) a multicenter double-blind study in Italy to evaluate whether the addition of oral insulin is able to improve the integrated parameters of metabolic control and modify immunological findings compared to placebo in patients with recent onset disease treated with intensive insulin therapy (IMDIAB VI). No effect of subcutaneous insulin was observed but a positive effect of 7.5 mg of oral insulin in a high risk cohort was observed and a repeat trial of 7.5 mg of oral insulin is being planned at patients at risk. (Skyler, J., Oral communication. Immunology of Diabetes Society Meeting, Cambridge, England, 2004); (4) a multicenter double-blind study in the US to determine if diabetes can be prevented by subcutaneous insulin therapy or oral insulin therapy in subjects at risk for diabetes (DPT-1); (5) a double-blind study in Australia evaluating aerosolized insulin versus placebo in patients with new-onset disease; and (6) a double-blind study in Finland evaluating nasally administered insulin versus placebo in patients with new-onset disease. A recent report shows that oral insulin treatment of patients with type 1 diabetes for 12 months was able to deviate the pattern of response of their peripheral mononuclear cells towards an increased production of TGF-β (Monetini et al. 2004).

Multiple sclerosis

In MS patients, MBP- and PLP-specific TGF-β-secreting Th3-type cells have been observed in the peripheral blood of patients treated with an oral bovine myelin preparation and not in patients who were untreated (Fukaura et al. 1996). These results demonstrate that it is possible to immunize via the gut for autoantigen-specific TGF-β-secreting cells in a human autoimmune disease by oral administration of the autoantigen. However, a completed 515 patient, placebo-controlled, double-blind phase III trial of single-dose bovine myelin in relapsing-remitting MS did not show differences between placebo and treated groups in the number of relapses; a large placebo effect was observed (AutoImmune Inc., Lexington, MA, USA). The dose of myelin was 300 mg given in capsule form and contained 8 mg MBP and 15 mg PLP. Preliminary analysis of magnetic resonance imaging data showed significant changes favoring oral myelin in certain patient subgroups. Trials in MS have been undertaken with the MBP analogue, glatiramer acetate, which is currently given by injection to MS patients but has been shown to be effective orally in animals and to induce regulatory cells that mediate bystander suppression (Teitelbaum et al. 1999; Weiner 1999). A phase III trial of oral glatiramer acetate given daily at 5 and 50 mg versus placebo found no clinical, MRI, or immunologic effects. Phase II trials testing oral GA at doses of 300 and 600 mg are currently in progress.

Rheumatoid arthritis

In RA, a 280 patient double-blind phase II dosing trial of CII in liquid doses ranging from 20 to 2500 μg per day for six months demonstrated statistically significant positive effects in the group treated with the lowest dose (Barnett and Kremer 1998). Oral administration of larger doses of bovine CII (1–10 mg) did not show a significant difference between tested and placebo groups, although a higher prevalence of responders was reported for the groups treated with CII. These results are consistent with animal studies of orally administered CII in which protection against adjuvant- and Ag-induced arthritis and bystander suppression was observed only at the lower doses (Zhang et al. 1990b; Yoshino 1995). An open-label pilot study of oral collagen in juvenile RA gave positive results with no toxicity (Barnett et al. 1996). This lack of systemic toxicity is an important feature for the clinical use of oral tolerance, especially in children for whom the long-term effects of immunosuppressive drugs is unknown.

Five phase II randomized studies of oral CII have been performed and based on the results obtained, a multicenter double-blind phase III trial study of oral CII (Colloral®) was undertaken (AutoImmune Inc.). In the five double-blind phase II studies a total of 805 patients were treated with oral CII and 296 treated with placebo. Two of the studies have been published (Trentham et al. 1993; Barnett et al. 1998). The other three studies were included in a integrated analysis that led to the decision to carry out a phase III trial. A dose refinement study tested doses of 5, 20 and 60 μg. Colloral at 60 μg was found to be the most significant dose compared to other doses. Safety analysis demonstrated that Colloral was extraordinarily safe with no side effects. The magnitude of the clinical responses of Colloral appears to be on the same level

as NSAIDS for the majority of patients. However, there is a sub-group of patients that appear to have a more significant response to the medication. Based on these data, a 760-patient phase III trial was performed comparing 60 μg of Colloral to placebo. However, no differences were observed. There was a large placebo effect in the control group. Subsequently, a placebo controlled trial of bovine collagen showed significant effects in those receiving 0.5 mg, but not in groups receiving 0.05 or 5 mg (Choy et al. 2001). Oral CII in juvenile RA was associated with clinical improvement and decreased CII specific IFN-γ and increased TGF-β (Myers et al. 2001). Clinical trials are underway to determine whether withholding NSAIDS and prednisone will allow OT to be induced and whether oral CII has meaningful clinical efficacy in RA (Postlethwaite 2001).

Uveitis

In uveitis, a pilot trial of S-Ag and an S-Ag mixture was conducted at the National Eye Institute (Bethesda, MD, USA) and showed positive trends with oral bovine S-Ag but not the retinal mixture (Nussenblatt et al. 1997). Feeding of peptide derived from patient's own HLA Ag appeared to have effect on uveitis in that patients could discontinue their steroids because of reduced intraocular inflammation mediated by oral tolerance (Thurau et al. 1997b).

Thyroid

Thirteen patients receiving thyroid hormone replacement with synthetic thyroxin were randomly assigned to receive oral porcine thyroid or remain on synthetic T4 (Lee et al. 1998). Humoral and cellular immune responses were measured over the course of a year. A decrease in cellular immunity to thyroid peptides was observed in the fed versus the control group. No changes between groups were observed in autoantibody levels.

Other

Positive effects were reported in an open label pilot study of oral type I collagen in patients with systemic sclerosis (McKown et al. 1997, 2000).

Future directions

In spite of the negative results to date of phase III human trials of mucosal tolerance, many lessons have been learned from them as well as from the sucessful experiments with animal models. Based on the results of oral tolerance in uveitis in humans (Nussenblatt et al. 1997) and in animal models of myasthenia (Okumura et al. 1994) and EAE (Benson et al. 1999),

it appears that protein mixtures may not be as effective oral tolerogens as purified proteins.

Although it is clear that oral Ag can suppress autoimmunity and inflammatory diseases in animals, it is now clear from all these studies that several factors need to be carefully considered to improve the effectiveness of oral tolerance in human disease: (1) the dose of Ag is crucial; (2) a clear immunological marker or immunological effect has to be established as a parameter for the follow-up of each disease; (3) some mucosal adjuvants that enhance induction of mucosal have been described and may need to be used; (4) purified proteins are more effective oral tolerogens than protein mixtures; (5) frequency of oral administration interferes with efficiency of suppression achieved; (6) combination therapy using conventional anti-inflammatory and immunosuppressive drugs may yield a better result; and (7) early therapy is an important factor since oral tolerance is mostly effective before or shortly after disease onset.

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