Oral tolerance in disease

Preface
The induction of immunological unresponsiveness by feeding soluble antigens, termed oral tolerance, has been attracting considerable attention as a potential therapy for a variety of systemic inflammatory disorders. However, the physiological role of this phenomenon is in maintaining gastrointestinal homeostasis and preventing immunopathology in the gut. This review will discuss the mechanisms of oral tolerance in the context of the structure and function of the gut associated lymphoid tissues (GALT), outline how defects in oral tolerance can lead to immunopathology and indicate its therapeutic potential.

Introduction
The large surface area of the gastrointestinal tract is covered by a single layer of columnar epithelium which allows efficient absorption of nutrients, but also offers a potential portal of entry for microbial pathogens. Thus, a highly structured immune network, the GALT has evolved as an integral part of the intestine to provide protection against pathogenetic microorganisms. However, the intestinal immune system is faced with the dilemma of discriminating between antigens with no pathogenetic potential (dietary proteins and commensal organisms) and antigens associated with potentially harmful microbes. It has long been recognised that the encounter of dietary antigens by the immune system normally leads to antigen specific tolerance. This phenomenon has been termed oral tolerance and can be induced by most soluble antigens.

The consequences of the apparent breakdown of oral tolerance to dietary antigens are exemplified by diseases such as gluten sensitive enteropathy (coeliac disease) and other food sensitive enteropathies. The intestinal immune system must also maintain stringent control over responses to its resident bacterial flora. These organisms are essential for normal intestinal function, yet they constitute a potential challenge to the integrity of the host. Recent evidence suggests that the breakdown of intestinal immunoregulation of responses to endogenous flora may be the underlying cause of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease. Understanding the mechanisms responsible for oral tolerance may lead to new treatment strategies for such conditions. In addition, antigen feeding may provide a potential immunotherapy for a number of systemic inflammatory disorders.

Immunological tolerance
The ability of the vertebrate immune system to generate antigen specific responses to a plethora of foreign antigens depends on the expression of a large number of unique antigen receptors by lymphocytes. However, inherent in this strategy is the danger of recognition of self or useful exogenous antigens and the possibility of autoimmunity or immunopathology. Therefore, a number of mechanisms exist to prevent this by the induction of immunological tolerance.

The best understood mechanism of immunological tolerance is central tolerance established in the primary lymphoid organs, thymus and bone marrow via the physical deletion of self reactive T and B cells. However, not all self antigens are represented in the primary lymphoid organs, whereas others may be restricted to certain peripheral tissues or particular periods of life after antigen specific lymphocytes have already seeded the periphery (puberty, pregnancy, lactation, aging). Therefore, central tolerance must be complemented by additional mechanisms of peripheral tolerance. Unresponsiveness of T cells in the periphery may be achieved in a number of ways. Firstly, the T cell may never encounter the antigen in an immunologically relevant form and thus be ignorant of it. Secondly, the T cell may encounter the antigen in circumstances that result in the subsequent functional or actual elimination of the cell (anergy or deletion). Finally, regulatory cells or mediators may be induced that can modify the immune response.

Whether antigen induces tolerance or immunity in the periphery is probably dependent on costimulatory signals provided to the T cells by antigen presenting cells (APC). Early studies established that the same antigen can be either tolerogenic or immunogenic depending on its form. Administration of a soluble, monomeric antigen in the absence of coadministered adjuvant usually results in unresponsiveness to subsequent exposure to the same antigen.

Oral tolerance is a special case of peripheral immunological tolerance. The precise immunological basis remains controversial, reflecting complex interactions between digestion, specialised antigen uptake and a variety of immunoregulatory mechanisms.

Immune regulation in the intestine
BARRIER FUNCTION AND FILTRATION
Clonal ignorance of T cells would result if immunologically relevant forms of antigens did not gain access to the immune system via the gut. This might simply result from the destruction of immunological epitopes by the non-specific effects of digestion, or because the physical barrier of the intestinal epithelium and its associated mucus layer prevents antigen access to APC. However, this possibility is argued against by the fact that immunologically relevant protein can be detected in serum within minutes of ingestion and indeed this material may be essential for the development of oral tolerance. It seems more likely that the barrier effect, if any, of the gut is to filter aggregated antigens resulting in monomeric forms being available systemically. The physical barrier may also hinder uptake of

Abbreviations used in this paper: GALT, gut associated lymphoid tissue; APC, antigen presenting cell; TNF, tumour necrosis factor; IL, interleukin; LPS, lipopolysaccharide; TcR, T cell receptor; OVA, ovalbumin; MBP, myelin basic protein; TNFR, tumour necrosis factor receptor; TGF, transforming growth factor; CTL, cytotoxic T lymphocytes; IL-1, intraepithelial lymphocyte; CMI, cell mediated immune; IFN, interferon; IBD, inflammatory bowel disease; SCID, severe combined immunodeficiency; KLH, keyhole limpet haemocyanin; IDDM, insulin dependent diabetes melitus; TNBS, trinitrobenzene sulphonic acid.
bacteria with their associated adjuvant activity, an effect enhanced by the ability of the liver to remove remaining “free” adjuvant, such as lipopolysaccharide (LPS). Thus, antigens passing across the gut wall into the circulation may be in a highly soluble, monomeric form in the absence of the inflammatory signals required for costimulation. Based on knowledge from other models of peripheral tolerance, it would be expected that presentation of this form of antigen would result in T cell anergy. That this occurs in oral tolerance is supported by findings that the impaired ability of cells to respond to antigen in vitro is restored by exogenous IL-2, indicating the continued presence of antigen reactive T cells. More recent work has confirmed this idea more directly by showing that adoptively transferred antigen specific T cell receptor (TcR) transgenic T cells persist in vivo following feeding, but are hyporesponsive to antigen when restimulated in vitro. Antigen specific T cells could also be inactivated by apoptosis mediated clonal deletion. This occurs in other systems when peripheral tolerance is induced by exogenous superantigens or after administration of conventional antigens to TcR transgenic animals. Under these conditions, T cell apoptosis may result from massive release of toxic cytokines, growth factor deprivation, or direct induction of programmed cell death. Clonal deletion of CD4+ T cells via apoptosis in vivo has been demonstrated following oral administration of soluble chicken ovalbumin (OVA) to TcR transgenic mice, although this was not observed after feeding myelin basic protein (MBP) to appropriate transgenic mice. Furthermore, the physiological relevance of studying immune responses in fully transgenic animals is unclear. The more physiologically relevant system in which small numbers of identifiable TcR transgenic T cells are adoptively transferred into normal syngeneic recipients has yielded conflicting results with one study finding T cell deletion after feeding very 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 (Garside, Steel and Mowat, unpublished observations) argues against a role for fas dependent apoptosis in the intact animal, although recent studies indicate a possible role for TNF receptor (TNFR) mediated apoptosis in oral tolerance (Mowat, Viney and Garside, unpublished observations).

Regulatory T cells
An alternative possibility to clonal inactivation of individual specific T cells is the induction of regulatory cells. Many early studies found that tolerance induced by feeding could be transferred by cells that were generally found to be CD8+, and it was proposed that oral tolerance was a result of the activation of antigen specific CD8+ suppressor T cells. It has also been proposed that regulatory CD8+ T cells induced in this way may mediate “bystander suppression” via the production of inhibitory cytokines such as transforming growth factor (TGF)-β which can exert non-specific suppressive effects on other antigen reactive cells in the vicinity, irrespective of their specificity. In recent years, the existence of a discrete population of regulatory CD8+ cells has been doubted and an alternative way in which classic CD8+ T cells could act in tolerance is suggested by findings that MHC class I restricted cytotoxic T lymphocytes (CTL) can modulate antigen specific immune responses via the lysis of antigen bearing APC or idiotype bearing CD4+ T cells. Studies designed to assess the role of CD8+ CTL in oral tolerance have provided conflicting results, with tolerance, priming or ignorance of these cells in response to fed antigen all being reported. One further possibility is that the regulatory CD8+ T cells induced by feeding antigen may belong to the γδ TcR expressing subset of lymphocytes. CD8+γδ+ T cells are relatively abundant within the intraepithelial lymphocyte (IEL) compartment and their depletion with monoclonal antibodies or by genetic manipulation has shown that these cells may also be essential for the development of oral tolerance with a range of doses of fed antigen. Regulatory γδ T cells have also been implicated in tolerance induced by the intranasal route. The nature of the antigen recognised and how these cells act is unclear, but one recent report suggests that they may interact with αβ T cells. A further complication to understanding the role of γδ cells in regulating mucosal immune responses is their putative homeostatic role in maintaining epithelial integrity.

The requirement for CD8+ T cells in oral tolerance has been examined using genetically engineered CD8 or beta-2-microglobulin knockout mice and in mice treated with anti-CD8 antibodies. In all these studies, oral tolerance is induced normally, indicating that there is no absolute requirement for CD8+ T cells in the induction or maintenance of systemic tolerance. In contrast to the contentious role of CD8+ T cells, most workers agree that CD4+ T cells are essential for oral tolerance. Oral tolerance is abrogated if CD4+ T cells are depleted in vivo and CD4 knockout mice do not become tolerant to contact sensitising agents, even when the effector response is mediated by CD8+ T cells. It has also been reported that CD4+ T cells can transfer oral tolerance in vivo. The original theory to explain these results was that oral tolerance reflected the downregulation of Th1 helper 1 (Th1) CD4+ cells by Th2 helper 2 (Th2) cells, an idea compatible with the fact that it is easier to induce and maintain oral tolerance of cell mediated immune (CMI) responses in comparison with humoral responses. However, oral tolerance to OVA can suppress both Th1 and Th2 responses quite adequately and normal oral tolerance can be induced in the absence of Th2 cells in both IL-4 knockout mice and in mice treated with anti-IL-4 antibodies, even when tolerance is associated with increased production of IL-4. In addition, the highly Th2 dependent production of IgE is amongst the most sensitive of effector functions to the induction of oral tolerance. It seems reasonable to expect that Th2 cells would be susceptible to tolerance, given the fact that Th2 mediated responses may be equally as pathogenic as Th1 responses. 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 helper 3 (“Th3”) or T regulatory 1 (“Tr1”) cells which produce suppressive cytokines (see later) and which can inhibit Th1 cell mediated immunopathologies such as experimental autoimmune encephalomyelitis and colitis.

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 which have received most interest are IL-4, IL-10, interferon (IFN) γ, and TGF-β.

As we have noted, IL-4 does not seem to be essential, despite some evidence for its preferential upregulation. As IL-10 suppresses Th1 activity via downregulation of the expression of costimulatory molecules and IL-12 production by APC, it was an attractive candidate as a mediator of oral tolerance, particularly if this was mediated by Th2 cells. Initial reports suggested that the production of...
IL-10 was enhanced in oral tolerance,\textsuperscript{19, 20, 24, 56} whereas IL-10 producing “Th3” clones can be isolated from animals tolerised by feeding MBP.\textsuperscript{44} More recently, 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.\textsuperscript{5} However, we have found notable suppression of this cytokine in mice fed OVA\textsuperscript{50} and normal oral tolerance occurs in mice depleted of IL-10.\textsuperscript{51} Thus, the role of this cytokine in oral tolerance remains unclear.

The CD8+ T cells which transfer nasal tolerance to soluble OVA produce IFN-\(\gamma\) and enhanced IFN-\(\gamma\) production is a feature of some models of mucosal tolerance, in which there is suppression of IL-4 mediated IgE production.\textsuperscript{27} Also, priming of IFN-\(\gamma\) production has been noted very early after feeding (Steel, Mowat and Garside, unpublished observations). These results are consistent with the cytostatic properties of IFN-\(\gamma\),\textsuperscript{52} the preservation of its production in mice tolerised by parenteral injection of staphylococcal enterotoxin B\textsuperscript{53} and with its central role in some models of T cell anergy in vivo.\textsuperscript{54} However, most workers find that the production of IFN-\(\gamma\) is highly susceptible to the suppressive effects of feeding antigen and our recent work indicates that oral tolerance can be induced in IFN-\(\gamma\) depleted and IFN-\(\gamma\)R knockout mice (Steel, Garside and Mowat, unpublished observations). Similar findings have been made by others.\textsuperscript{55}

TGF-\(\beta\) is a mediator receiving most current attention. Abundant in the normal intestine,\textsuperscript{56} it is produced by cells of both haemopoietic and epithelial origin and is important in regulating epithelial homeostasis and IgA switching.\textsuperscript{57} TGF-\(\beta\) also has well documented suppressive effects on many aspects of the immune response.\textsuperscript{58} Increased production of this cytokine has been reported in a number of models of oral tolerance\textsuperscript{59} and TGF-\(\beta\) secreting T cell clones can be produced from animals tolerised in this way.\textsuperscript{60, 61} Furthermore, the bystander suppressive effects exerted by these cells in vitro and in vivo can be prevented with anti-TGF-\(\beta\).\textsuperscript{62} Local TGF-\(\beta\) production has also been associated with the prevention of experimental colitis in a number of models\textsuperscript{63} and the recently described regulatory “Th3” and “Tr1” cells (see earlier) are a potent source of this cytokine.\textsuperscript{64, 65} Recent studies suggest that TGF-\(\beta\) and the IL-12/IFN-\(\gamma\) pathway play opposing roles in intestinal immune regulation and that modulation of these cytokines may be the key to induction of mucosal immunity or tolerance, or both.\textsuperscript{66} However, increased TGF-\(\beta\) production is not a universal finding in all models of mucosal tolerance\textsuperscript{67} and the role of this cytokine requires further investigation.

Consequences of the loss of immunoregulation in the gut
The importance of oral tolerance for preventing hypersensitivity to dietary proteins is evidenced by the presence of unusual immune responses to gluten in patients with coeliac disease and by experimental findings that abrogation of oral tolerance allows the development of enteropathy after oral antigen challenge.\textsuperscript{68, 69} More recently, it has become apparent that inflammatory bowel diseases (IBD) such as Crohn’s disease and ulcerative colitis may reflect a breakdown in an analogous state of tolerance to commensal bacteria.\textsuperscript{70, 71} This is consistent with the frequent usefulness of antibiotics in the treatment of human IBD and with the fact that all of the experimental models of IBD can be prevented by antibiotics or a germ-free environment. However, in most cases, no specific pathogen can be identified. As there is evidence in mice and man for tolerance to autologous flora in normal individuals and a breakdown of this tolerance occurs in IBD,\textsuperscript{72} it may be that potentially pathological responses to components of the intestinal flora do occur under physiological conditions but these are suppressed by the immunoregulatory mechanisms outlined earlier.

Experimental models confirm that IBD can occur as a result of defective immunoregulation in IL-2, IL-10 and TcR knockout mice\textsuperscript{73, 74} and in severe combined immunodeficiency (SCID) mice adaptively transferred with CD45RB\textsuperscript{75} CD4+ T cells.\textsuperscript{76} The nature of the regulatory defect revealed in these spontaneous models of IBD in knockout mice is unknown, but in SCID mice adaptively transferred with CD45RB\textsuperscript{77} CD4+ T cells IBD is prevented by IL-10 dependent TGF-\(\beta\) production by a subset of CD45RB\textsuperscript{78} CD4+ T cells.\textsuperscript{79} An analogous population of IL-10 dependent “Tr1” cells which produce IL-10 and TGF-\(\beta\) have been shown to prevent CD45RB\textsuperscript{80} CD4+ T cell induced colitis in a non-specific bystander manner.\textsuperscript{81} TGF-\(\beta\) is also required for the ability of feeding hapten to prevent colitis induced by rectal administration of trinitrobenzene sulphonic acid (TNBS).\textsuperscript{82}

Thus, a simple lack of regulatory mediators or cells in the gut might lead to a breakdown in oral tolerance due to unlimited inflammation in a previously normal gut. Alternatively, a breakdown in barrier function might allow access of inflammatory bacterial products to the local immune system, overwhelming normal regulation. This possibility is supported by experimental models in which the epithelial barrier has been disrupted. Early studies showed that direct disruption of the epithelial barrier using a variety of chemical treatments (e.g. carrageenan) resulted in the induction of a local inflammatory response similar to that seen in human IBD.\textsuperscript{83} Compromising epithelial integrity by expressing a dominant negative mutant of the N-cadherin molecule\textsuperscript{84} or by knockout of the epithelial cytoskeletal structural protein keratin 8\textsuperscript{85} produces spontaneous IBD, and mice lacking intestinal trefoil factor are unusually susceptible to colitis induced with dextran sodium sulphate.\textsuperscript{86} Bacteria could also contribute to the breakdown of oral tolerance and induction of IBD by disturbing barrier function. For instance, it has been proposed that excessive hydrogen sulphide production by the sulphate reducing bacteria within the colonic flora of patients with ulcerative colitis (see Sartor and colleagues\textsuperscript{87} for review) may contribute to the pathology.

It is easy to imagine that once the initial breakdown in tolerance has been established, the inflammatory state will be perpetuated by bacteria invading the mucosa, permitting normally tolerogenic responses to most luminal antigens to be modified into immunogenic responses. Persistent inflammation would further damage the epithelium and drive the vicious cycle (fig 1). We would predict that a combination of defects in barrier function, immunoregulation and/or inflammatory mediator production will be required for a breakdown in gastrointestinal homeostasis to occur. This is consistent with the polygenic nature of IBD and also suggests that the most successful immunotherapy requires a combination of anti-inflammatory therapy, antibiotics and reinduction of tolerance. The
recent demonstration of bystander suppression of experimental colitis by nominal antigen specific regulatory T cells extends the prospects of devising such regimens considerably.

**Oral tolerance as a therapy for systemic immunopathology**

A number of experimental models have shown the efficacy of oral tolerance in preventing or treating systemic inflammation. Although all of the mechanisms we have outlined might be potentially useful, an active suppressor mechanism would have most advantages, as it might allow suppression of responses to an unrelated “bystander” antigen. Conversely, antigen induced inactivation of potentially reactive lymphocytes due to deletion or functional anergy might imply a more stable, long-lasting tolerance which is less susceptible to modification by other immune responses. However, how the latter mechanisms would maintain unresponsiveness throughout the animal, particularly in the face of repopulation with naive cells from the thymus, remains unclear and repeated administration of the specific tolerising antigen might be necessary. Studies in experimental animals suggest it may be possible to induce either of these mechanisms selectively by varying the antigen feeding regimen. Thus repeated low doses of antigen are proposed to induce active suppressor mechanisms, whereas higher doses may favour induction of anergy/deletion. Tailored immunotherapy may be feasible in this way.

Many studies have demonstrated the effectiveness of orally administered antigens in rat and mouse models of autoimmune disease, including experimental allergic encephalomyelitis, arthritis induced by collagen, adjuvant or pristane, uveoretinitis, myasthenia gravis, thyroiditis and insulin dependent diabetes (table 1). In addition, it has been possible to suppress allograft rejection by feeding allogeneic cells or MHC peptides. A number of features of these studies are of clinical significance. Firstly, it has been possible to inhibit established disease by feeding antigen, although it is often necessary to administer large amounts of antigen on repeated occasions to achieve this. Secondly, the induction of bystander suppression has been shown frequently, allowing the use of antigen which is not necessarily the autoantigen which initiates the disease. Finally, several groups have achieved disease suppression by feeding defined epitope peptides, raising the possibility of using engineered small molecules for this purpose.

**Oral tolerance in humans**

Oral tolerance has been demonstrated experimentally in human volunteers fed repeated doses of keyhole limpet haemocyanin (KLH). Under these conditions, antibody responses to subsequent parenteral challenge were not altered, but the KLH fed individuals had suppressed cell mediated immune responses. Nasal KLH has also been reported to induce tolerance in humans. Formal clinical trials have also been carried out in patients with multiple sclerosis, rheumatoid arthritis, uveitis, and insulin dependent diabetes mellitus (IDDM), with initially encouraging results and no side effects reported. In addition, one study indicated that feeding bovine myelin to patients with multiple sclerosis resulted in the preferential appearance of TGF-β secreting Th3-like cells in peripheral blood, rather similar to findings reported in analogous rodent experiments. It should be noted, however, that only a small subset of HLA-DR2+ male patients with multiple sclerosis responded to antigen feeding and a more recent phase III trial in multiple sclerosis did not show statistically significant differences between placebo and myelin fed groups (AutoImmune Inc., Lexington, MA, USA). Similarly, despite early reports that feeding chicken collagen led to statistically significant clinical improvement in adult and juvenile rheumatoid arthritis, a subsequent European trial found no evidence of a significant difference between test and placebo groups in patients receiving larger doses of bovine type II collagen (1–10 mg). This discrepancy may reflect dose dependent effects, as a recently completed multicentre double blind phase II trial of feeding a range of doses of chicken type II collagen showed statistically significant positive effects in the group treated with the lowest dose (20 µg). These results are consistent with animal studies of orally administered type II collagen in which protection against adjuvant and antigen induced arthritis and bystander suppression was observed only at the lower doses. In uveitis, no significant effects of feeding bovine uveal S-antigen were observed. Currently, other trials are underway feeding recombinant human insulin to new-onset diabetic patients, as well as in patients at risk of developing IDDM. The results of all these trials are anticipated with great interest, as they will give important pointers to the future potential of the approach. Given the notable dose and antigen dependent effects which have been described in animals, it is perhaps not surprising that more clear cut results have not been obtained from the early clinical studies, which were obliged to use somewhat arbitrary doses and preparations of antigen. Important issues will be the conditions required for induction of bystander suppression and whether the phenomenon proves to be a universal phenomenon, as causative autoantigens cannot be identified with certainty in most human disease. Finally, for practical use in humans, it may be essential to exploit approaches which seem to enhance the induction of tolerance after mucosal administration of small doses of antigen, including the use of the nasal route, or conjugation to the B subunit of cholera toxin. Alternatively, it may be feasible to obtain large amounts of mucosally delivered tolerogen by production in transgenic plants.

**Conclusions**

Thus, oral tolerance is an important physiological phenomenon with potential therapeutic applications. The interactions between barrier, immunoregulation and local antigen also provides an intriguing system to study the biology of mucosal immune responses.


