THE REGULATION OF SYSTEMIC IMMUNE RESPONSES BY THE DIETARY ANTIGEN OVALBUMIN

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SUMMARY

Oral tolerance is the specific immunological unresponsiveness normally induced by feeding a soluble antigen. Though it is an obstacle to oral vaccination, it is probably the mechanism that prevents intestinal hypersensitivity reactions to food antigens and there is currently a great deal of interest in the manipulation of the phenomenon to provide a novel strategy for immunotherapy of autoimmune and inflammatory disorders. However, the mechanisms of oral tolerance and the major factors that influence them remain controversial and require to be clarified before practical application. The principal aim of my project was to assess the role for a number of the mechanisms that had been proposed to regulate peripheral immune responses to orally administered antigen.

At the time I began my study, the effects of oral tolerance were known to be influenced by the dose of antigen and the dogma was that low feeding doses could invoke active regulatory mechanisms, while high doses could inactivate T cells directly. However, this theory was based on reports from different model systems where the findings were not consistent in every aspect and in general were restricted to comparisons of only a few antigen doses. As active regulation and direct T cell inactivation have different implications for the clinical application of oral tolerance, I attempted to clarify this issue by using the dietary antigen ovalbumin (OVA) to examine the systemic effects of feeding mice a wide range of doses (100μg-25mg).

A single dose of 10-25mg fed OVA reduced every antigen-specific effector function examined after parenteral immunisation with OVA/CFA, including DTH, IgG, IgG1 and IgG2a responses *in vivo* and PLN cell proliferation and production of IL3, IL5, IL10 and IFNγ *in vitro*. That the activity associated with both Th1 and Th2 cells was reduced suggests that neither T cell subset could have been involved in modulating the other thereby ruling out this mechanism of active regulation in high dose oral tolerance.

Feeding OVA at doses below 10mg had different effects on individual effector functions, and although all responses were progressively reduced by increasing antigen doses, this appeared to follow a pattern generally consistent with individual T helper cell

subsets. Th1-despendent DTH, IgG2a and IFNγ responses were the most susceptible to inhibition and were reduced by feeding as little as 100μg OVA, while PLN cell proliferative responses became inhibited at doses ≥ 2mg fed OVA. The regulation of Th2-dependent responses was less clear cut. IL5 production was tolerised by as little as 100μg fed OVA, whereas IgG1 and IL10 responses resisted the effects of tolerance induced by feeding 100μg, 1mg, 2mg and 5mg OVA, only becoming significantly inhibited at doses of ≥10mg fed OVA. These findings indicate that although the regulatory factors mediating IL5 production may differ from those controlling the other Th2-dependent responses, it is possible that low dose oral tolerance may be mediated by regulatory Th2 cells, particularly since IgG1 and IL10 responses were preferentially activated by 100μg-1mg fed OVA.

However, I was unable to detect evidence for the non-specific bystander suppression that is reported to accompany Th2-dependent crossregulation of Th1 cells. Bystander suppression is induced in an antigen-specific manner but inhibits subsequent responses to unrelated antigen present in the same microenvironment as the original antigen. To examine for the presence of this phenomenon, I assessed the responses of cells restimulated with the antigen PPD, which had been present in the CFA used for parenteral immunisation with OVA. Under these circumstances, proliferation and cytokine production by orally tolerised cells was comparable with control responses and as this finding was irrespective of the dose of fed OVA, it suggests that bystander suppression may not have been operating in either low or high dose oral tolerance.

Feeding very low doses of antigen is known to prime systemic immune responses. As this would be useful for the development of oral vaccines but potentially hazardous for the clinical application of fed antigens as immunotherapy, I also assessed this feature in my dose response study. My results showed that OVA-specific DTH, proliferation and IFNγ responses were enhanced by 10-50μg fed OVA, while upregulation of IgG1, IL3 and IL5 responses required feeding 50μg OVA and IgG2a antibody production was not augmented by either feeding dose. These findings suggest that individual effector responses vary in their susceptibility to upregulation by fed OVA. However, these findings were observed

only in mice challenged with a suboptimal dose of OVA/CFA after feeding, indicating that the extent of oral priming may have been weak.

The capacity of certain Th2-dependent functions to remain resistant to inhibition by low doses of fed antigen indicated that oral tolerance may be the result of different mechanisms depending on the dose fed antigen administered. I therefore examined directly the role of Th2 cells in oral tolerance by examining $IL4^{-/-}$ mice, which lack functional Th2 cells. Although no OVA-specific Th2-dependent responses were detected in these animals, all other effector responses examined were reduced by feeding OVA prior to challenge. This included DTH, IgG and IgG2a responses *in vivo* and PLN cell proliferation and production of IL3 and IFNγ *in vitro*. Moreover, as my findings were similar for mice fed either 2 or 25mg OVA, a critical role for Th2 cell activity in either form of oral tolerance seems unlikely.

I also assessed the role for other T cell subsets and cytokines in mediating suppression. As the cytostatic properties of IFNγ and TGFβ have been known for some time, it seemed possible that either of these cytokines might be required for suppression. However, no role was found for endogenous IFNγ in either the induction or maintenance of oral tolerance, as normal suppression of effector responses occurred in animals depleted of IFNγ at the time of feeding as well as in $IFN\gamma R^{-/-}$ mice. Mice given neutralising anti-TGFβ antibody also developed specific tolerance of DTH responses *in vivo* and IL5, IL10 and IFNγ responses *in vitro* after feeding 2 or 25mg OVA. Furthermore, neutralisation of TGFβ *in vitro* did not reverse the inhibition of proliferation and secretion of IL3, IL5 or IL10 seen in cells from orally tolerised animals. However, some exceptions to these findings occurred, with apparently no tolerance of IgG or proliferation in anti-TGFβ treated mice and with variable effects on the *in vivo* and *in vitro* inhibition of IFNγ. Therefore, TGFβ remains a potential mediator of some aspects of oral tolerance.

Although my findings could not support a critical role for any one particular cytokine in the modulation of all aspects of oral tolerance, it remained possible that another form of active regulation might be involved. Early reports had implicated a suppressive role for $CD8^+$ T cells in oral tolerance and I examined whether this might reflect their cytotoxic

potential. However, I found that the peripheral immune responses suppressed by feeding either a high or low dose of OVA included $CD8^+$ CTL responses. These CTL were generated by parenteral immunisation with OVA ISCOMS and recognised the immunodominant $OVA_{258-276}$ epitope presented in the context of $H\text{-}2K^b$ class I MHC molecules. In addition, the cytotoxic response depended on the presence of $CD4^+$ T cells and CTL generated in a CD4-independent manner by challenge with OVA/CFA were not altered by fed OVA. As these findings indicated that a subset of $CD8^+$ T cells remain resistant to inhibition in orally tolerised mice, I assessed whether their presence was required for the induction of unresponsiveness. However, tolerance developed normally in mice depleted of $CD8^+$ cells at the time of feeding OVA. In contrast, the presence of $CD4^+$ T cells was compulsory for oral tolerance induction, suggesting that this T cell subset is the principal target of oral tolerance.

As my findings were inconsistent with a role for active regulatory mechanisms in oral tolerance, I went on to investigate whether direct T cell inactivation might be involved. I firstly showed that PLN cells removed from orally tolerised mice after parenteral immunisation exhibited compromised viability when cultured in the absence of antigen *in vitro*. This was comparable with naive cell death, occurred in mice fed either 2 or 25mg doses of OVA and affected both $CD4^+$ and $CD8^+$ T cells. Dying lymphocytes from unstimulated cultures of tolerised cells developed morphological features indicative of apoptosis, including nuclear fragmentation and membrane blebbing, and these changes were accompanied by an increased proportion of cells with hypodiploid DNA content. The cell death was probably more extensive than could be reasonably accounted for by deletion of specific clones alone, but the addition of OVA to the tolerant cultures reduced levels of apoptosis, indicating that the cell death was influenced by antigen and suggesting that some OVA-specific T cells remained present when lymph node cells were removed from tolerant animals. These cells may have been anergic as their unresponsiveness was found to be reversed, at least in part, by exposure to rIL2 before restimulation with OVA *in vitro*.

Although these results are compatible with direct inactivation of T cells in oral tolerance, this did not involve fas-dependent apoptosis either *in vivo* or *in vitro*. Entirely

normal oral tolerance of all immune functions was observed in fas-deficient MRL lpr mice and the functional defects associated with oral tolerance were not reversed by a fas-Fc fusion protein, which blocks fas-dependent apoptosis in other systems.

I also found that induction of a tolerant phenotype was preceded by transient T cell activation after feeding OVA. This was evidenced by the capacity of spleen cells from OVA fed mice to proliferate, enter into cell cycle and secrete IFNγ and IL3 when restimulated with OVA during the first 3 days after feeding, but not thereafter. As these mice were tolerant to challenge during this time, my findings support the possibility that a transient priming of specific T lymphocytes by fed antigen leads to functional inactivation and this may increase their susceptibility to apoptosis in the absence of antigen *in vitro*.

My findings thus far illustrate that the peripheral tolerance induced by feeding OVA is profound, can inhibit effector responses of both Th1 and Th2 cell subsets and may reflect T cell anergy and/or deletion. In the final part of my study, I examined the long term-effects of feeding antigen on a wide range of effector responses as this aspect has never been examined in any great detail and will be relevant for clinical practice. I found that animals fed 25mg OVA showed some degree of tolerance for virtually their entire life-span, with significantly reduced DTH responses *in vivo* and inhibited OVA-specific IL3, IL5 and IL10 production *in vitro* when challenged for up to 18 months after feeding. However, other effector responses recovered more quickly. The tolerance of OVA-specific serum IgG1 antibody production and antigen-specific proliferation did not last beyond 3 months after feeding, while OVA-specific IgG and IgG2a antibodies were only tolerant when animals had been challenged 10 days after feeding. The effects of tolerance were generally less persistent in mice fed 2mg OVA, where DTH responses were not tolerised beyond 9 months after feeding and the tolerance of OVA-specific PLN cell proliferative and cytokine responses wained after 3 months. More strikingly, the inhibition of OVA-specific IgG, IgG1 and IgG2a responses was not observed beyond 10 days after feeding.

Some evidence was provided that the persistence of tolerance may be accompanied by upregulation of certain responses. Thus, the long-lasting tolerance induced by feeding 25mg OVA was associated with a switch to enhanced IFNγ production upon immunisation 6

months later. Although transient, it is possible that this effect might have been necessary for the maintenance of unresponsiveness preferentially associated with high dose oral tolerance. An opposite effect was seen in low dose oral tolerance, since the decline of tolerance in animals fed 2mg OVA was concomitant with increased OVA-specific IL10 production, which was observed at both 6 and 9 months after feeding and was accompanied by enhanced IL5 responses at 9 months. This suggests that the loss of oral tolerance in these mice was followed by a skewing of immune responses in favour of the production of Th2-dependent cytokines and thereby provides further evidence against a suppressive role for Th2 cells in low dose oral tolerance.

In summary, these results highlight that oral tolerance can be manipulated, by altering the feeding dose, to be a long-lasting and dynamic phenomenon. However, its long-term effects on individual responses differ and therefore its use as a therapy for immunopathology will require that individual responses be assessed directly.

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Last, but by no means least, I thank my family for their love, support and encouragement - for believing in me.

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DECLARATION

These studies represent original work carried out by the author, and have not been submitted in any form to any other University. Where use has been made of material provided by others, due acknowledgement has been made.

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PUBLICATIONS

Parts of this thesis have been included in the following publications:

1. Garside, P., M. Steel, F.Y. Liew, and A.M. Mowat, *$CD4^+$ but not $CD8^+$ T cells are required for the induction of oral tolerance.* Int Immunol, 1995. **7**: p. 501-4.

2. Garside, P., M. Steel, E.A. Worthey, A. Satoskar, J. Alexander, H. Bluethmann, F.Y. Liew, and A.M. Mowat, *Th2 cells are subject to high dose oral tolerance and are not essential for its induction.* J Immunol, 1995. **154**: p. 5649-55.

3. Garside, P., M. Steel, E.A. Worthey, P.J. Kewin, S.E.M. Howie, D.J. Harrison, D. Bishop, and A.M. Mowat, *Oral tolerance in mice is associated with increased susceptibility of challenged lymphocytes to undergo apoptosis in vitro.* Am J Path, 1996. **149**: p. 1971-79.

ABBREVIATIONS

APC	Antigen Presenting Cell(s)
BALT	Bronchial-Associated Lymphoid Tissue
Bio	Biotin
BrdU	Bromodeoxyuridine
CFA	Complete Freund's Adjuvant
CMI	Cell-Mediated Immuninty
Con A	Concanavalin A
CT	Cholera Toxin
CTL	Cytotoxic T Lymphocyte(s)
DC	Dendritic Cell(s)
DEA	Diethanolamine
dH_2O	Distilled H_2O
DTH	Delayed-Type Hypersensitivity
DMSO	Dimethylsulfoxide
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylendiaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron Microscopy
FACS	Fluorescence-Activated Cell Scanner
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
FSE	Food Sensitive Enteropathy
GALT	Gut-Associated Lymphoid Tissue
^3H	Tritium
HAO	Heat-Aggregated OVA
HEV	High Endothelial Venule(s)
h fas-fc	human fas-fc fusion protein

IBD	Inflammatory Bowel Disease
IEL	Intra-Epithelial Lymphocyte
IFA	Incomplete Freund's Adjuvant
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
ISCOMS	Immune Stimulating Complexes
LP	Lamina Propria
mAb	Monoclonal Antibody
MALT	Mucosa-Associated Lymphoid Tissue
MDP	Muramyl Dipeptide
2-ME	2-mercaptoethanol
MHC	Major Histocompatability Complex
MLN	Mesenteric Lymph Node
$Na_2{}^{51}CrO_4$	Sodium ^{51}Chromate
NALT	Nasopharyngeal-Associated Lymphoid Tissue
NCS	Newborn Calf Serum
O.D.	Optical Density
OVA	Ovalbumin
PBS	Phosphate Buffered Saline
pCTL	Precursor Cytotoxic T Lymphocyte(s)
PE	Phycoerythrin
PEG	Polyethylene Glycol
PI	Propidium Iodine
PLN	Popliteal Lymph Node
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl Fluoride
PP	Peyer's Patch
SA	Streptavidin

SB	Staining Buffer
SEM	Standard Error of Mean
SLE	Systemic Lupus Erythymatosus
TcR	T Cell Receptor
TGF-β	Transforming Growth Factor-β
Th1	T-helper 1
Th2	T-helper 2
Th3	T-helper 3
TMB	3,3'5,5'-tetramethylbenzidine
TrisHCl	Tris(hydroxymethyl) Methylamine

DEDICATION

This thesis is dedicated to my parents, Margaret and Robert, without whom I could never have made it this far.

Chapter 1: Introduction

Preface

Dietary proteins form part of the enormous load of foreign antigen to which the gastrointestinal (GI) tract is continuously exposed. Although the gut-associated lymphoid tissues (GALT) represent a considerable arsenal of effector mechanisms to counter the threat of potential pathogens, this armoury is normally not directed against food antigens, partly because it would limit their uptake and metabolic usefulness but more importantly, because it might incur hypersensitivity to foods. This is illustrated by coeliac disease, in which a T-cell mediated reaction to wheat gluten causes severe enteropathy [1, 2]. The inflammatory bowel diseases, Crohn's disease and ulcerative colitis are now also thought to reflect inappropriate hypersensitivity to harmless intestinal antigens from the gut flora [3, 4]. However, these conditions are rare because the GALT somehow descriminates between non-harmful antigens and those of pathogenic importance. Whereas mucosal pathogens elicit an active primary immune response followed by memory to subsequent exposure [5], commensal gut flora reside in the intestine without invoking pathological responses [6] and oral administration of soluble antigens usually induces a suppressed response to subsequent enounter with the antigen, irrespective of the route of administration [7]. This phenomenon is termed oral tolerance. In addition to preventing food hypersensitivities, oral tolerance may provide a potent therapy for a variety of autoimmune and inflammatory disorders [8]. However, as well as being of profound physiological importance, orally induced tolerance respresents a major barrier to the development of oral vaccines utilising defined protein antigens.

1. The GI Tract

(A) Structure and function

The GI tract includes the mouth, pharynx, eosophagus, stomach, duodenum, jejunum, ileum, colon and rectum. Throughout its length, the wall of the mucosa has the general structural organization shown in Fig 1.1. From the stomach onwards, this surface is covered by a single layer of epithelium that is composed of columnar epithelial cells, mucus-secreting goblet cells, undifferentiated crypt epithelial cells, Paneth cells, enteroendocrine cells and intraepithelial lymphocytes. Just below the epithelium is a layer of connective tissue, the lamina propria, which is separated from the underlying tissues by a thin layer of smooth muscle, the muscularis mucosa. The submucosa lies beneath the mucosa and on top of another layer of smooth muscle, the muscularis externa, contractions of which provide the forces for moving the gastrointestinal contents. Finally, a layer of connective tissue surrounds the outer surface of the GI tract and this serosa is connected to the abdominal wall at various points by mesenteries.

The mucosa is highly folded and the surface of these folds is further convoluted by fingerlike projections known as villi, which are each covered with a single layer of epithelium with microvillous protrusions on the apical surface. In this way the surface area is greatly increased to facilitate absorption of nutrients. Yet the GI tract also must function as a barrier against a wide range of harmful antigens and infectious pathogens. Protecting against such agents are many nonimmunological factors including gastric acid, pancreatic juice, bile, motility, mucus, the surface glycocalyx and epithelial cell turnover. In addition to these physiological barriers, an immunological barrier is created and maintained by the specific immune system.

(B) The Gut Associated Lymphoid Tissues (GALT)

The GALT forms the major part of the mucosa-associated lymphoid tissue (MALT), which also comprises the bronchial-associated lymphoid tissue (BALT), nasopharyngeal-associated lymphoid tissue (NALT), the mammary, lacrimal and salivary glands and the lymphoid tissues of the urogenital tract and inner ear. In man, the total surface area of the

MALT is over 400 square metres, which is more than 200 fold greater than the surface area of the skin [6], highlighting the importance of this defense system in combating infection. Luminal antigens, including dietary proteins, are recognised by the GALT, which consists of both organised lymphoid aggregates, represented by the Peyer's patches, appendix, mesenteric lymph node (MLN) and lymphoid nodules. In addition, the gut villi contain intraepithelial lymphocytes (IEL), while many lymphocytes, macrophages and mucosal mast cells are scattered throughout the lamina propria of the intestine itself [9]. In addition, there may be a primary source of lymphoid cells present in cryptopatches within the lamina propria [10].

(C) Induction of Local Immune Responses in the Intestine

The Peyer's patches are the major inductive sites for intestinal immunity and are found on the wall of the small intestine, from the ileum to the colon, extending throughout the lamina propria and submucosa. They appear as mounds protruding between the intestinal villi and are covered by a single layer of columnar epithelial cells.

The Peyer's patch has a typical secondary lymphoid organ structure with T cell areas and B cell follicles containing germinal centres (Fig 1.2). The germinal centres are the major source of IgA^+ B cell precursors [11] and also contain a few T cells. However, the majority of T lymphocytes reside in the parafollicular region and, to a lesser extent, in the dome region immediately beneath the epithelium. The parafollicular region is also the site where lymphocytes enter the patch via high endothelial venules (HEV). Parafollicular T cells are virtually all mature $\alpha\beta$ T cells of both $CD4^+$ and $CD8^+$ phenotype, whereas those T cells present in the dome are predominantly $CD4^+$ cells of both Th1 and Th2 phenotype [9]. The Peyer's patch also contains many antigen-presenting cells (APC), including dendritic cells and macrophages, which are found throughout the patch in both T and B cell areas. Thus, the Peyer's patch contains all the components required for the initiation of immune responses to antigens encountered in the lumen of the small intestine. Furthermore, Peyer's patches have been shown to be sites where regulatory cells are generated [12-14] and this will be discussed in more detail later.

Although Peyer's patches have a typical secondary lymphoid structure, they differ from other secondary lymphoid organs because they lack afferent lymphatics. Instead, antigen is sampled directly from the intestinal lumen by overlying specialised epithelial cells, known as M cells because of the characterisitic irregular microfolds present on their luminal surface [15-17] (Fig 1.3). M cells do not express class II MHC antigens and thus primarily perform a transport function, allowing materials from the intestinal lumen to reach lymphocytes and macrophages enfolded in pockets formed by the basolateral membranes of the M cells [18, 19]. Antigens transported by M cells may also pass down through the basal lamina into the lymphoid follicles before being carried into the MLN via draining lymphatics. This route provides one way in which an antigen from the intestinal lumen can gain access to the systemic circulation.

A possible alternative route of access for soluble antigens is to be endocytosed across the absorptive gut epithelium [20, 21]. In this way, they may reach the systemic circulation or may be processed and presented in the underlying lamina propria where class II MHC$^+$ cells, such as macrophages, B cells or dendritic cells are abundant. MHC class II determinants present on the basolateral membrane of intestinal epithelial cells [22] may also allow these cells to process and present antigen to T cells of the lamina propria. Epithelial cells generally lack expression of costimulatory molecules and reports suggest that they can present antigen in a tolerogenic manner [23]. This function has recently been shown in oral tolerance to haptens [24] and therefore it represents a possible mechanism to downregulate local immune responses.

(D) Local Effector Sites

The lamina propria of the gut contains B cells, T cells, macrophages and dendritic cells, thereby constituting one of the main effector sites for mucosal immune responses. In addition, inflammatory cells such as eosinophils and mast cells are also present. B cells represent 15-45% of lamina propria cells [25], and there are many plasma cells, of which ~80% are plasma cells producing secretory IgA, while ~18% secrete IgM and only ~3% secrete IgG [26-29]. The same relative proportions are also seen in the ileum and colon. T

cells constitute ~50% of lymphocytes within the lamina propria and 40-60% of these are $CD4^+CD8^-$ [27, 30] with the remainder being $CD4^-CD8^+$. Several studies show that a significant proportion of $CD4^+$ T cells in the lamina propria have a memory/activated phenotype and are capable of providing helper signals to enhance IgA responses [31, 32]. This is consistent with reports that Th2 cells predominate in the lamina propria [33], although Th1 cells are also present [34]. In addition, as yet unidentified APC in the lamina propria can present antigen in a tolerogenic manner [35, 36] and therefore may play an important role in the induction of oral tolerance.

(E) IEL

The mucosal immune system also contains a unique population of T lymphocytes, known as IEL, which are interspersed between the columnar epithelial cells of the villi in the small and large intestine [37]. These cells can be subdivided into two classes based on their expression of either αβ or γδ TcR. Mouse IEL populations contain 20-80% γδ T cells and this population ranges from 13-87% in human large intestinal IEL isolates. However, human small intestinal IEL contain only ~10% γδ T cells. [38]. A characterisitic of IEL is their predominant expression of CD8. In mouse, >90% IEL are $CD8^+$ [39, 40], with ~10% of αβ IEL also expressing CD4 (double positives, DP) [41]. Human TcR αβ IEL are primarily $CD8^+$, although $CD4^+CD8^-$ and $CD4^-CD8^-$ subsets can be detected, and 50-80% human γδ IEL are $CD8^+$ [38, 42, 43]. IEL can be further subdivided depending on the form of CD8 expressed. Although the CD8 coreceptor usually exist as a dimer of αβ chains [44], the absence of CD8β on subsets of IEL has been known for some time [39] and may be associated with their extrathymic origin [45-51]. Thus, all γδ IEL and ~50% αβ IEL in mice are $CD8\beta^-$ [41]. Human CD8 γδ IEL are also predominantly $CD8\beta^-$ [38].

The function of IEL is not known, but one suggestion is that they constitute a primitive population specialised for immune surveillance of epithelial surfaces [37]. In support of this hypothesis, IEL exhibit constitutive cytotoxic activity *in vitro* [40], but as their proliferative responses are poor [52-54] and can be reconstituted in the presence of IL2 [55], these cells may be anergic *in situ*. This idea is futher supported by studies of antigen-

specific TcR transgenic mice where IEL were rendered functionally unresponsive after recognition of antigen presented by gut epithelium [56, 57]. Another possibility is that IEL may play a role in local immunoregulation via their production of a variety of cytokines, including IL2, IFNγ, IL5 and TGFβ [58, 59] and the relevance of this for oral tolerance will be discussed later.

(F) IgA

Secretory IgA is a dimeric molecule held together by a J (joining) chain, which forms disulphide bonds with the IgA monomers [60]. This polymeric molecule is secreted intact by mucosal plasma cells and transported across the epithelia into the external environment of the gut lumen. Although the overall function of secretory IgA remains unclear, it is capable of neutralising viruses [61] and toxins [62, 63]. In addition, IgA helps prevent bacterial colonisation of the mucosa by binding to the mucus layer overlying the epithelia and inhibiting the adherence of microorganisms [64] or promoting their entrapment in the mucus [65, 66] and subsequent agglutination [67]. This function is known as immune exclusion and a similar mechanism may also reduce the absorption of dietary and respiratory antigens [68], possibly accounting for the finding that patients with selective IgA deficiency show increased absorption of food antigens [69], as well as an increased susceptibility to food hypersensitivity [70]. However, most patients with this common immune deficiency have no clinical symptoms and there is no correlation between the presence of immune exclusion and oral tolerance [71]. Moreover, the absence of IgA antibodies against food antigens in normal individuals indicates that local IgA production does not correlate with systemic unresponsiveness induced by a fed antigen.

(G) Lymphocyte Recirculation

Upon encountering the appropriate antigen in the Peyer's patch or lamina propria, responding lymphocytes exit via the lymphatic network in the mucosal wall and drain into the afferent lymphatics of the MLN and thence via the efferent lymphatics and thoracic duct to the bloodstream before recirculating back to the effector sites of mucosal tissues [72, 73].

This recirculation pathway differs from the route taken by lymphocytes stimulated by antigen in a peripheral tissue (Fig 1.4) and as a result T cells primed or tolerised by mucosal antigen may not disseminate throughout the systemic immune system. This distribution of T cells is regulated by lymphocyte-specific homing antigens and by adhesion receptors expressed on the surface of endothelial cells in effector sites, with both sets of molecules being distinct for the mucosal and peripheral recirculation pathways. The adhesion receptor, mucosal addressin cell adhesion molecule 1 (MAdCAM-1) is preferentially expressed by mucosal endothelia and binds to the $\alpha 4\beta 7$ integrin molecule (LPAM-1) expressed on the surface of lymphocytes activated in mucosal tissues. This interaction is thought to play a key role in the homing of effector lymphocytes to mucosal tissues [74, 75].

2. Regulation of Immune Responses to Dietary Antigens in Oral Tolerance

(A) History

The observation that fed antigens could suppress systemic immune responses was recognized long before the era of modern immunology and was first reported in 1829 by Dakin, who described how South American Indians ate poison ivy in an attempt to prevent what we now understand to be contact hypersensitivity to the plant [76]. The first experimental evidence for oral tolerance was obtained by Wells (1911), who described a state in which anaphylaxis in guinea pigs was prevented by previous feeding of hen's egg proteins [77]. However, the immunological nature of the phenomenon was only established by the later experiments of Chase (1946), who demonstrated the antigen specificity of oral tolerance using the hapten DNFB to suppress contact hypersensitivity in guinea pigs [78]. Since then oral tolerance has been investigated widely, initially by mucosal immunologists, but now also by those interested in exploiting the phenomenon as a model of immunoregulation or as a therapy for immunopathology.

(B) Scope & Longevity of Oral Tolerance

The induction of oral tolerance has been described in many species, including humans [79-81], pigs [82, 83], dogs [84], guinea pigs [85, 86] and rabbits [87]. Species

differences do occur and oral tolerance may not be induced at all in adult ruminants [88]. However, the phenomenon has been best described in laboratory rodents, where systemic immune unresponsiveness was demonstrated with a wide range of nonreplicating antigens, including numerous proteins, contact sensitizing agents [89-93], peptides [94-96], sheep red blood cells (SRBC) [12, 97-99], allogeneic leukocytes [100] and inactivated viruses or bacteria [101-103]. In addition, more recent work has used proteins of immunopathological importance, such as myelin basic protein (MBP), uveal S antigen, insulin and collagen [94, 104-106].

Virtually all aspects of the immune response can be rendered tolerant by feeding antigen. Fed protein antigen will inhibit subsequent systemic IgM, IgG and IgE antibody responses [102, 107-113], as well as cell mediated immune (CMI) responses measured by delayed-type hypersensitivity (DTH) or contact sensitivity *in vivo* [90-93, 111, 112, 114-116] and lymphocyte proliferation [94, 102, 111, 117] and cytokine production *in vitro* [118]. However, CMI responses are generally easier to tolerize than are humoral responses, requiring less antigen and persisting longer [86, 112, 115, 116, 119, 120]. The one exception is that IgE production is relatively resistant to oral tolerance [109, 121, 122]. IgE and DTH responses are associated with pathological food hypersensitivity and therefore it would be logical that prevention of food-specific IgE and DTH responses were the most important biological role of oral tolerance.

The effect of oral tolerance on mucosal immune responses is less clear and levels of secretory IgA have been reported to be primed [102, 110], tolerised [12, 99] or unaltered [123] by different feeding regimes. In addition, fed antigen has been reported to prime Peyer's patch T cells that suppressed IgG production but aided the synthesis of IgA antibodies [124].

(C) Factors Influencing Immune Responses to Fed Antigen

(i) Nature of Antigen

Although a wide range of antigens is capable of inducing oral tolerance, certain types of antigen are more likely to provoke active immunity rather than tolerance when

administered via the oral route. Thus oral tolerance requires that the antigen is non-viable [103], thymus-dependent [101, 111] and generally soluble in form [125]. This may explain the ability of the intestinal immune system to discriminate between dietary and pathogenic material, since potential pathogens, such as bacteria, will present a large amount of thymus-independent antigen in a particulate and viable form. In addition, particulate antigens may target M cells in Peyer's patches [17, 126] and therefore be processed by the intestine more efficiently than soluble antigens, which may passively diffuse across the villus epithelium to be presented by epithelial cells in a tolerogenic manner, as described previously. Tolerance can also be induced by nasal administration of similar forms of antigen [96, 127-130], suggesting the possibility of a common mucosal phenomenon.

(ii) Frequency and Dose of Fed Antigen

The influence of antigen dose on the induction of oral tolerance has been investigated using a number of protein antigens, including OVA [131], HEL [132] and MBP [132]. Although a wide dose range of fed antigen induces oral tolerance in experimental animals [8, 131, 133] and a single feed of as little as a few milligrams of antigen can tolerise mice [134, 135], the exact dose required for optimal effect depends on the protein under study. Moreover, individual systemic responses display dose-dependent differences in their susceptibility to tolerance induction. CMI responses have been shown to be particularly easy to tolerise, with 100μg or more of fed OVA being sufficient to suppress DTH responses in mice [131, 136]. Under the same circumstances, humoral immunity is more resistant to the effects of oral tolerance, requiring at least 5-10mg of fed OVA to become inhibited [131, 136]. These differences may reflect the induction of distinct regulatory mechanisms (see below) and have important implications for the practical application of oral tolerance.

Antigen doses below those that induce oral tolerance, can prime systemic immune responses. In mice, systemic priming occurs after feeding 1-50μg protein and affects CMI responses more than humoral immunity [131]. This has also been reported in piglets, where large amounts of weaning diet produced tolerance if fed before weaning, whereas low

amounts of this diet primed the animals to develop food hypersensitivity [82]. Moreover, it has been proposed that initial exposure to low amounts of food antigens predisposes to eczema in children [137].

In addition to dose, the frequency of antigen administration can also regulate oral tolerance since multiple or continuous feeding inhibits IgG antibody production more effectively than a single feed of the same total antigen dose [138, 139].

(iii) Genetic Background

Coeliac disease is closely linked to the HLA-DQw2 locus of the human MHC [140], indicating that genetic factors may influence the regulation of immune responses to fed antigens. Consistent with this theory, early studies in mice fed OVA noted strain differences in the susceptibility to oral tolerance induced either in normal animals [71, 141, 142] or those already primed to OVA [141]. Further work showed that congenic mice carrying the H-2^d MHC haplotype were particularly susceptible to the induction of oral tolerance, whereas the H-2^b haplotype was associated with a less profound tolerance [143, 144]. However, the immunological basis of this MHC-linked effect remains to be established.

Other non-MHC-linked genes have also been implicated in oral tolerance [142] and appear to operate through the rapid clearance of absorbed antigen from the circulation [71]. Thus oral tolerance induction may be under the control of several genes that influence both specific immune responsiveness and nonspecific factors such as protein clearance and catabolism.

(iv) Host Age

Food hypersensitivities are most common in infants, particularly at or near the time of weaning, suggesting that oral tolerance is defective during these periods. Neonatal and weaning mice also exhibit defective induction of tolerance to fed protein antigens [145-147]. The ability to sensitise calves or piglets by feeding antigen during the preweaning period further supports an age-related defect in oral tolerance at this time [82, 83, 88, 148]. It has been suggested that the defect in oral tolerance during infancy may reflect an inability of the

immature immune system to respond appropriately to intestinally derived tolerogen [7]. This proposal is consistent with the finding that oral tolerance can be induced normally when infant mice are transferred with mature lymphocytes [138, 145]. In contrast, the disruption of oral tolerance associated with weaning occurs after the animal has developed an adult pattern of susceptibility to tolerance. Therefore, this phenomenon has been proposed to reflect alterations in either the intestinal microenvironment or the systemic hormone levels associated with weaning [7].

Ageing may also affect oral tolerance, as many immune functions generally decline with age [149]. However, the onset and rate of immunosenescence in GALT has been reported to occur much later and more slowly than changes in the systemic immune system [150] and this might explain why, even at 20 months of age, oral tolerance can still be induced in mice [151]. However, the duration of systemic unresponsiveness was brief in these aged mice, suggesting that the factors regulating maintenance of unresponsiveness had become defective with age. These studies assessed only humoral immunity and are similar to observations made in orally tolerised young adult mice, where suppression of humoral, but not CMI, waned within a few months after feeding [120].

(v) Intestinal Flora

As noted above, changes in the gut flora at the time of weaning may account for the defective oral tolerance associated with this period and there is ample evidence that the intestinal flora can influence local and systemic immune responsiveness. This idea is consistent with observations that germ-free mice have defective systemic immune competence [152, 153] and that the duration of oral tolerance is reduced in these animals [154]. However, the bacterial products responsible for this immunoregulation have yet to be determined.

(vi) Intestinal Absorption and Antigen Uptake

Oral tolerance to protein antigens may reflect the manner in which the protein is processed in the intestine. Serum removed from mice 1 hour after feeding OVA induces

systemic tolerance when transferred intraperitoneally to naive recipients [155-157] and similar observations have been made for other protein antigens [116]. The processing event required to generate the serum tolerogen appears to occur in the intestine, since the ability to transfer tolerance cannot be reproduced using serum from mice given equivalent doses of antigen via other parenteral routes [156]. This passage across the mucosal epithelium appears to be a critical factor, an idea supported by the fact that coupling antigen to the non-toxic, epithelial-binding cholera toxin (CT) B subunit dramatically enhances its tolerogenic capacity [158]. However, an intact lymphoid system is also required for generation of serum tolerogen, as this material is not found in OVA-fed SCID mice [159] or irradiated mice [160] unless these animals are reconstituted with lymphoid cells [159, 160].

The precise nature of the serum tolerogen remains unclear, but recent studies suggest that it consists of low molecular weight fragments (21 & 24kD) of intact OVA (43kD) [157]. Digestion by gut enzymes does not appear to generate these fragments, as they are present at a time when complete cleavage would not have occured *in vivo* and rectal administration of antigen can also induce tolerance [161, 162]. These findings suggest that the small intestine may filter soluble antigens from the lumen to produce deaggregated monomers of protein that are known to be tolerogenic in other systems [163].

(vii) Antigen Presentation

The fact that stimulation of the reticuloendothelial system (RES) can prevent the induction of oral tolerance [164-166] indicates that antigen presentation may be important in regulating the response to a fed antigen. However, both the site of antigen presentation and the APC involved remain to be elucidated.

If oral tolerance was induced at local sites, this may implicate MHC class II^+ epithelial cells or non-professional APC within either the Peyer's patches or lamina propria, as antigen presentation in both of these mucosal tissues has been associated with tolerance induction [35, 124, 167]. However, as T cells tolerised via the intestine should recirculate preferentially back to mucosal sites [72, 73], it would be difficult to explain systemic immune unresponsiveness in this way and thus antigen presentation in the periphery may be

required. This could be achieved if APC acquired antigen in the gut and then dispersed to the periphery to present antigen in a tolerogenic manner. Although dendritic cells (DC) are normally associated with T cell activation [168], they are the most potent presenters of soluble proteins [169], and recent work suggests that DC can present antigen in a tolerogenic fashion [170]. Numerous antigen-laden DC have been shown to migrate to the MLN from the gut after intra-luminal or intra-gastric challenge [171, 172] and, although these cells prime T cells when transferred into naive recipients [172], the possibility remains that the cells were activated during the isolation procedure. An alternative is that fed antigen might associate with non-professional APC, such as resting B cells or unactivated macrophages, either locally or peripherally, to be presented to T cells in the absence of costimulation or in low affinity interactions, thereby inducing anergy and/or preferential activation of Th2 cells [173-175]. Epithelial cells could also do this [23].

(viii) Immunological Status of Host

The ability to tolerise an animal that has already been primed to that antigen will be critical if oral tolerance is to be used to inhibit established autoimmune disorders. Although the induction of systemic tolerance to an antigen is more difficult if the T cells are experienced [176], several investigators have shown that oral tolerance can be induced in parenterally primed mice [141, 177, 178] and other recent studies have shown that feeding specific antigens can cure animals with ongoing EAE or collagen-induced arthritis [94, 113, 117]. Primed T cells appear to be suppressed far more easily than B cells, but compared with oral tolerance in naive animals, suppression of primed responses requires larger doses of fed antigen and more frequent feeds, which have to be administered within a short time window after systemic priming. Therefore the immunoregulatory mechanisms governing tolerance in primed mice may differ from those induced in naive animals. This may reflect differences in the costimulatory requirements of primed and naive cells, as will be discussed in more detail later.

3. Clinical and Practical Relevance of Oral Tolerance

(A) Physiological Prevention of Intestinal Hypersensitivity

Hypersensitivity to foods is probably due to a breakdown of oral tolerance [121, 179]. This idea is consistent with the findings that mucosal pathology can be induced by feeding antigen when oral tolerance is prevented experimentally by administering cyclophosphamide, by activating the reticuloendothelial system, by feeding very low doses of antigen or by using animals during the neonatal or weaning period [83, 119, 164, 166, 180]. The features of this pathology are similar to those found in the early stages of naturally occurring food sensitive enteropathies (FSE), such as coeliac disease [181, 182]. More recently, studies of transgenic and knockout rodents suggest that this IBD may also reflect a similar defect in local immunoregulation. Colitis has been found in several models, including HLA-B27/β_2 microglobulin transgenic rats [183], mice transgenic for the CD3ε chain when reconstituted with bone marrow [184, 185] and mice genetically deficient in IL2 [186], IL10 [187], either α or β chains of the TcR [188], MHC class I [189], TCRδ [189, 190] or Gi2a [191], an inhibitor of G proteins. In each case, development of IBD requires gut flora and there may normally be a stable immune suppression against luminal bacteria which is required for maintaining mucosal homeostasis and preventing enteropathy. This remains to be proven for IBD, but highlights the need to understand the basis of oral tolerance.

(B) Immunotherapy

The oral route offers a convenient and highly acceptable means of administering therapeutic agents and there are now numerous examples in which feeding antigen can prevent experimental models of antigen-specific immunopathology [94, 100, 192, 193] (Table 1.1). This work has stimulated trials of oral tolerance as an immunotherapy for human disease. Multiple sclerosis was the first condition to be treated in this way and the results of a phase II double blind trial showed that 12 out of 15 multiple sclerosis patients improved when fed daily with 300mg bovine myelin [194]. A large multi-centre trial is now underway which should provide more information. The other clinical disease for which

published information is available is rheumatoid arthritis. Phase I and II trials of feeding relatively small amounts of chicken collagen (100-500μg daily) suggested that a small proportion of patients improved when treated for 90 days and up to 15% had complete remission [195]. However, a recent report from an analogous study of rheumatoid arthritis patients fed bovine collagen for 12 weeks showed rather more ambivalent results [196]. Nevertheless, a multi-centre trial of around 300 patients is now in progress in the U.S.A.. There are also clinical trials proceeding in patients with insulin-dependent diabetes mellitus, myaesthenia gravis and uveitis, but no published data are available yet. Although encouraging, clearly more needs to be understood of the regulatory mechanisms involved in oral tolerance if widely applicable effective regimes are to be developed utilising this approach.

(C) Development of Oral Vaccines

Traditional vaccine research has focussed on the induction of systemic immunity by parenteral immunistion. While this approach may be approriate against pathogens which gain access to the body through damaged or punctured skin, the majority of pathogens infect hosts via mucosal surfaces. Parenterally administered vaccines generally do not induce the mucosal immune responses required to prevent infection with pathogens encountered at mucosal sites [197]. Orally administered vaccines, such as polio, are also favoured because, in addition to inducing local immunity in the intestine, they may also stimulate immune responses at distal mucosal surfaces, such as the breast [198], and can induce widespread systemic immunity [199-201]. Furthermore, oral vaccines have several economic and practical advantages, including ease of administration, reduced side-effects and the potential for almost unlimited boosting. For these reasons, a major goal of current vaccine research is the construction of orally active vaccines that contain protective recombinant proteins or peptides as the immunogen. Such vaccines will be unsuccessful unless the induction of oral tolerance can be overcome. Therefore an understanding of the principles involved in this phenomenon will assist the design of appropriate oral vaccines.

4. Mechanisms of Tolerance

Clearly a fuller understanding of oral tolerance will have important implications not only for the development of oral vaccines, but also for the prevention of FSE and for the manipulation of autoimmune/inflammatory disorders.

(A) Current Ideas on the Mechanisms of Peripheral Tolerance

Oral tolerance is a form of peripheral tolerance and therefore the mechanisms by which peripheral tolerance can be induced by parenterally administered antigens may clearly be relevant to oral tolerance.

Antigen Ignorance

When a naive lymphocyte meets its antigen, it can respond in one of three ways: ignore it, become activated or become unresponsive. There is evidence from transgenic models that peptides may be presented in the context of class I MHC on cells which are unable to trigger any response from T cells with the appropriate TcR because they lach expression of the costimulatory or accessory molecules required to enhance T cell avidity [202-204]. In many cases extrathymic antigens also appear to be ignored by $CD4^+$ T cells and this may be because parenchymal tissues normally express relatively few class II MHC molecules and therefore may not be surveyed efficiently by naive T cells [205, 206]. The T cells apparently ignore the presentation of antigen by these non-professional APC, as they show no evidence of activation and respond normally if the appropriate antigen is later presented by a professional APC or in the context of inflammation [206-209].

Active Regulation

In active suppression, tolerance is mediated by the regulation of one lymphocyte population by another. Evidence for this effect was initially demonstrated in adoptive transfer studies, where spleen cells from mice tolerant to SRBC could transfer unresponsiveness to naive recipients [210]. Suppression has now been described in various

forms of extrathymic tolerance [211] and is also believed to play an important role in the regulation of normal immune responses [212].

Although induced in an antigen-specific manner [213, 214], active suppression of this kind may exert its effects through a variety of antigen non-specific soluble factors, including glucocorticoids [215] and lymphokines, such as IL6 [216], IL10 [217], IFNγ [218] and TGFβ [219]. Although originally considered to be mediated by $CD8^+$ T cells, the suppressor phenomenon has recently been applied to the current paradigm of $CD4^+$ T cell regulation, in which Th1 cells are associated primarily with IL2 and IFNγ production, while Th2 cells secrete IL4, IL5, IL6 and IL10. Th1 cells are involved in DTH and inflammatory responses and support B cell production of IgM and IgG2a, while Th2 cells predominate in allergies and parasitic infections and are classical helper cells for antibody production, particularly IgE and IgG1 [220]. In addition, the Th1 and Th2 type cells mutually regulate each other. Thus Th1-dependent IFNγ downregulates production of lymphokines by Th2 cells, which in turn can inhibit Th1 cell activity via IL4 and IL10 production [220, 221]. This original model may now encompass $CD8^+$ T cells, as this population can also be divided into subsets which appear similar to Th1 and Th2 $CD4^+$ T cells in their production of distinct lymphokines [222, 223]. Type 1 cells suppress B cells and display cytolytic activity, while type 2 cells provide B cell help and suppress DTH responses [222, 223]. Therefore lymphokine regulation of tolerance could underly the suppressor activity reported of both $CD4^+$ and $CD8^+$ T cells [224-226].

Direct Inactivation

Direct T cell inactivation can occur in two different ways, either by clonal deletion via apoptosis or by functional anergy.

Clonal Deletion

Clonal deletion of T lymphocytes and possibly B cells is required for central tolerance to self antigens [227] and also occurs extrathymically when peripheral tolerance is induced by exogenous superantigens [228] or after parenteral administration of conventional

antigens to TcR transgenic animals [229]. Clonal deletion occurs via apoptosis, which begins with condensation of the cell nucleus and cytoplasm leading to membrane blebbing and a final stage of cell fragmentation into membrane-bounded bodies [230, 231]. This process reflects the action of endonucleases, which become activated in apoptosis to degrade nuclear DNA into oligosomal fragments [232, 233]. Death of mature lymphocytes by apoptosis frequently involves signalling via fas/Apo-1/CD95 [234, 235], a surface receptor belonging to the tumor necrosis factor (TNF) receptor family and constitutively expressed on T lymphocytes. Both fas and the 55kDa TNF receptor 1 (TNF-R1) share a distinctive cytoplasmic region (the "death box") essential for signalling apoptosis upon interaction with ligand. The ligand for fas (fasL) is expressed on the surface of T cells after activation and may interact with fas to trigger cell death on the same cell [236] or on different cells [237]. This proccess has been proposed as an important homeostatic mechanism for controlling the size and persistence of antigen-specific effector responses [238]. fas-fasL interactions account for the proportion of CTL-mediated cytotoxicity which is not mediated by perforin [239-242] and operates when CTL recognise self peptides either on themselves, to induce suicide [236, 243], or on other T cells, to induce fratricide [237]. Clearly this could be implicated in oral tolerance and a similar fas-dependent phenomenon has also been reported for $CD4^+$ T lymphocytes, particularly those of the Th1 cell subset, which preferentially express fas L [241, 244]. Further support that fas-fas L interactions play an important role in extrathymic tolerance comes from studies of MRL lpr/lpr and gld/gld mice, which have a genetic absence of functional fas or fas L respectively and display profound lymphoproliferation [245] and increased susceptibility to autoimmune disorders [246]. Less is known of TNF-R1-transduced death, but this has been shown to be involved in some cytolytic functions of T cells [247].

Anergy

$CD4^+$ T cells require TcR engagement by an immunogenic peptide bound to a class II MHC molecules, as well as a costimulus provided by the APC, for successful activation leading to clonal expansion [248, 249]. Although the nature of the costimulus has not been

completely elucidated, substantial evidence implicates a role for B7-CD28 interactions in Th1 cell activation [250], whereas IL1 has been shown to costimulate Th2 cells [251, 252]. It has been clearly shown that TcR engagement without costimulation does not provide adequate signalling to induce proliferation [253-255] but rather leads to profound T cell unresponsiveness upon restimulation [254-258]. This phenotype is known as T cell anergy and was first demonstrated directly when T cell clones that had been exposed to antigen on other T cells in culture were subsequently unable to respond to the same antigen presented on normal APC [253]. It is now known that anergy can be induced by costimulator-incompetent "non-professional" APC [259] or by using altered peptide ligands [260, 261]. Furthermore, anergic T cells can be characterised by their inability to proliferate or produce IL2 [262] and by their capacity to regain normal activity when provided with exogenous IL2 [263] or "parked" in the absence of antigen [264]. Although the intracellular events leading to anergy are currently under investigation, it is known that partial TcR signalling results in altered TcRζ chain phosphorylation and a subsequent lack of its association with zap 70 protein-tyrosine kinase [261].

Anergic T cells may be compromised in many ways, but they can still produce readily detectable, although reduced levels of certain cytokines, including IFNγ, IL3 and granulocyte macrophage-colony stimulating factor (GM-CSF) upon restimulation [256, 261, 265]. The regulatory activity associated with some of these cytokines might explain why anergic cells from orally tolerised mice can act as Ts when adoptively transferred into athymic and SCID mice [266]. Alternatively, this may be due to their ability to compete with naive cells for available IL2 or antigen-MHC complexes on APC [267].

(B) Mechanisms of Oral Tolerance

(i) Introduction

As with peripheral tolerance, the mechanisms of oral tolerance may also involve either active modulation or direct inactivation of responding lymphocytes. The distinct immunological properties of each of these mechanisms may have important clinical implications. Thus, a unique feature of active suppression is the phenomenon of "bystander

suppression," in which immune responses to an unrelated antigen may also be inhibited if both antigens are present simultaneously at the time of challenge. This could only occur if the active regulatory mechanism was mediated by antigen non-specific factors such as cytokines. In contrast, the functional effects of deletion or anergy should be restricted to antigen-specific lymphocytes and to be of practical use in therapy of autoimmune and inflammatory disorders, tolerance caused by direct T cell inactivation would require that the disease-inducing antigen was known. Conversely, bystander suppression would allow use of an unrelated but anatomically linked antigen. However, antigen-induced inactivation of lymphocytes might have the theoretical advantage of being stable and long-lasting, as it would be less susceptible to modification by other immune responses. Both mechanisms have been implicated in oral tolerance and it has been suggested that their induction may be dependent on the dose of antigen used to induce tolerance.

(ii) Evidence for Active Modulation

Many early studies demonstrated active suppression *in vivo* following oral administration of antigen by showing that tolerance could be transferred to naive recipients with T cells [268, 269]. Furthermore, lymphocytes from tolerised animals can actively suppress T cell proliferation and cytokine production by primed cells in co-culture *in vitro* [270, 271]. A number of mechanisms have been implicated in this phenomenon.

$CD8^+$ T Cells

Most of the initial mechanistic studies in oral tolerance proposed a role for $CD8^+$ suppressor T cells. Oral tolerance could be transferred by $CD8^+$ cells, reviewed in: [7, 8, 133] and the tolerance *in vivo* could be prevented by treatment with agents that were believed to be specifically toxic for Ts cells, such as cyclophosphamide [272] and 2'-deoxyguanosine [119]. Upon further investigation, the Ts cells were thought to be restricted by the product of "I-J" genes encoded by the class II region of the MHC which had been implicated in other Ts-mediated models of peripheral tolerance [273]. However, $CD8^+$ Ts cells and the I-J

molecule were never characterised at the molecular level and most immunologists now consider their existence unlikely.

However, a number of recent studies appear to confirm that $CD8^+$ T cells can transfer suppression to naive recipients [96, 274]. In addition, the regulatory $CD8^+$ T cells induced in this way have been shown to mediate "bystander suppression" via the release of inhibitory cytokines such as TGFβ [274]. Clearly this is an area that needs to be reexamined. In addition, the possibility that $CD8^+$ T cells may mediate suppression by conventional MHC class I-restricted lysis either of antigen-bearing APC [275] or $idiotype^+$ T cells needs to be explored.

γδ TcR^+ Cells

One further possible population of $CD8^+$ regulatory T cells may be the subset which express the γδ form of the TcR. In both murine and rat models of intranasal tolerance, transfer of as few as 500 antigen-specific $CD8^+$ γδ TcR^+ cells suppressed $CD4^+$ T cell responses and IgE production in naive recipients [129, 276]. Similarly, γδ TcR^+ cells from orally tolerised rats have been found to transfer suppression of uveitis in an antigen-specific manner [277], while the depletion of γδ TcR^+ cells either with monoclonal antibodies [278] or by genetic manipulation [279], has shown that these cells may be essential for the development of peripheral tolerance induced by fed antigen. Interestingly, the γδ TcR^+ cells identified in all of these studies were of splenic origin and, despite the relative abundance of γδ TcR^+ cells in the gut, attempts to transfer tolerance with IEL have been unsuccessful [280]. However, the functions of γδ TcR^+ cells in the immune system are still unknown and their role in oral tolerance must remain controversial.

$CD4^+$ T Cells

A possible role for $CD4^+$ T cell crossregulation in oral tolerance is suggested by the fact that $CD4^+$ T cells can transfer oral tolerance in some systems [266, 281] and by the fact that it is easier to induce and maintain oral tolerance of CMI responses in comparison with humoral responses [131, 136]. Th2 cells are said to be predominant in the mucosa and

enhancement of IL4 production and preferential outgrowth of IL4/IL10-producing T cell clones *in vitro* from the MLN of animals tolerised by feeding OVA or MBP has been described [282-284]. The prevention of EAE by feeding rats MBP has also been associated with preferential upregulation of IL4 in the brain, together with decreased IFNγ expression [285]. Although this supports a role for classical Th2 cells, it has also been suggested that there may be a unique $CD4^+$ T cell population which produces TGFβ in addition to IL4 and IL10, and referred to as Th3 cells [282]. However, it should be noted that these findings have been confined to one or two models of oral tolerance and may be very dose dependent [7, 282-284]. Moreover, IFNγ has also been implicated in mediating mucosal tolerance, although this was based on results from a very limited panel of effector responses [129]. Therefore the regulatory activity of Th1 and Th2 cell subsets in oral tolerance remains an open issue.

Cytokines

An alternative to the idea that a discrete subpopulation of lymphocytes is responsible for oral tolerance is that individual inhibitory cytokines may be produced preferentially in response to fed protein. Those which have received most interest are IL4, IL10, IFNγ and TGFβ.

IL4

Development of Th2 cells and their ability to inhibit Th1 cells is dependent on IL4 [286-288]. In several models of peripheral tolerance, IL4 is enhanced and depletion of IL4 prevents the induction of tolerance [289]. In addition, as noted above, preferential upregulation of IL4 production has been described in oral tolerance [282-284].

IL10

IL10 is a further Th2-dependent cytokine [290] which is an attractive candidate mediator of oral tolerance as it suppresses Th1 cell activity via downregulation of macrophage IL12 production [221, 291]. In addition, its absence in $IL10^{-/-}$ mice allows the

development of intestinal pathology due to hyperreactivity to components of the normal gut flora [187]. However, the role of IL10 in oral tolerance is controversial, as although initial reports showed that IL10-producing T cell clones could be isolated from animals tolerised by feeding MBP [282], normal oral tolerance occurs in mice depleted of IL10 using antibody [292].

IFNγ

As described above, immune regulation by IFNγ has been reported in some models of mucosal tolerance, including the $CD8^+$ γδ TcR^+ cells which transfer nasal tolerance to soluble OVA [128, 129]. These results are consistent with the cytostatic properties of IFNγ [293], together with the preservation of IFNγ production in mice tolerised by parenteral injection of SEB [228]. Furthermore, IFNγ plays a central role in some models of T cell anergy and cell death *in vitro* [294, 295]. However, as most workers find that IFNγ and its effects are highly susceptible to the suppressive effects of feeding antigen, once again its exact role in oral tolerance remains uncertain.

TGFβ

Abundant in the normal intestine [296-298], TGFβ is produced by cells of both haematopoietic and epithelial origin and is important in regulating epithelial homeostasis and IgA switching [299]. TGFβ also has well-documented suppressive effects on many aspects of the immune response [299] and is the cytokine mediator receiving the most attention in current studies of oral tolerance. The prevention of EAE by oral administration of MBP is associated with upregulation of TGFβ in the brain [285] and TGFβ-secreting T cell clones can be isolated preferentially from animals tolerised in this way [282]. Furthermore, protection from EAE can be transferred with $CD8^+$ T cells or clones that produce TGFβ, and the bystander suppressor effects exerted by these cells *in vitro* can be prevented with anti-TGFβ [282-285]. Consistent with an important role for TGFβ in oral tolerance, it has also been recently demonstrated that antigen-specific TGFβ production is preserved in orally

tolerised TcR transgenic animals [300] and that depletion of this cytokine *in vivo* can prevent the orally induced suppression of EAE [301].

(iii) Direct Inactivation of Antigen Specific Lymphocytes

Even in some early studies, oral tolerance was found to be non-transferrable [302] and partly insensitive to the effects of cyclophosphamide [112]. These results would be compatible with what we now understand of the mechanisms involved in direct T cell inactivation.

Clonal Deletion

Evidence for deletion of antigen-reactive cells in oral tolerance has been provided in OVA-specific TcR transgenic mice, where both local and systemic clonal deletion by apoptosis was apparent within a few days after feeding OVA [300]. However, in this study, very large doses of antigen were required to demonstrate deletion and the molecular mechanism of apoptosis was not addressed. Similar effects have yet to be documented in normal mice fed more physiologically relevant doses of antigen.

Clonal Anergy

The induction of clonal anergy has recently been proposed as a possible mechanism of oral tolerance. Studies of oral tolerance to MBP or OVA showed that T cells removed from antigen-fed mice failed to produce IL2 but did express IL2R upon restimulation with antigen *in vitro* and limiting dilution analysis indicated that this may be due to a decrease in the frequency of proliferating or IL2-producing antigen-specific cells rather than active suppression [118, 303-305]. Although similar results would also be obtained if clonal deletion occurred in oral tolerance, more direct evidence for anergy is that the impaired antigen-specific proliferation of the orally tolerised cells was restored by culture *in vitro* with exogenous IL2, indicating the continued presence of antigen-reactive T cells [304]. However, anergy in oral tolerance has yet to be shown directly using transgenic systems, as has been done in other models of peripheral tolerance.

iv) Influence of Antigen Dose on the Mechanisms of Oral Tolerance

One explanation for the apparently contradictory evidence for both active regulation and direct T cell inactivation in oral tolerance is that these mechanisms may be involved in tolerance induced by different doses of fed antigen [132]. The balance of evidence suggests that feeding high doses of antigen may induce clonal deletion or anergy, while lower doses may elicit active suppression by inhibitory cytokines or by Th2/$CD8^+$ T cells [131-133, 136, 284]. However, the majority of this work examined only two comparative doses of fed antigen [132, 284] and the few groups which assessed a more complete antigen dose range concentrated mainly on describing *in vivo* effector responses and did not determine the underlying mechanisms [131, 133, 136]. Therefore, it is possible that active regulation and direct T cell inactivation may not be mutually exclusive in oral tolerance and this requires fuller investigation, particularly since this would have important implications for the manipulation of oral tolerance as a therapeutic strategy.

5. Aims of this Study

The principal aim of this study was to examine the regulation of peripheral immune responses to a fed protein under closely defined experimental conditions. The antigen OVA was used for this purpose for the following reasons. Firstly, OVA is an immunologically well characterised protein antigen which is inexpensive and readily available from commercial sources. Secondly, it had been extensively utilised in this laboratory as an oral antigen and the techniques for inducing oral tolerance and for assessing various aspects of systemic immunity to OVA were well established.

A variety of different consequences have been reported for systemic immune responses after feeding antigen. As these are suggested to reflect the distinct doses of fed antigen administered, I thought it important to begin my study by establishing the regulatory effects of a wide dose range of fed OVA. I examined both Th1- and Th2-dependent responses to determine if their regulation was independent of each other at any particular antigen dose. The results of this work, which are detailed in Chapter 3, allowed me to

choose a high and low dose of antigen for the comparative studies detailed in the subsequent chapters examining the role of Th2 and $CD8^+$ regulatory cells.

Although high doses of fed antigen have become associated with direct T cell inactivation, most evidence for this idea was provided by studies of oral tolerance in antigen-specific TcR transgenic models or in animals fed superantigens and few studies have explored the mechanism in normal mice fed conventional antigen. Therefore, in Chapter 6, I attempted to provide evidence for cellular deletion and anergy in mice fed OVA.

Finally, since oral tolerance is currently under evaluation as a potential immunotherapy it would be important to understand the longevity of its effects. This has been addressed by few previous studies and I therefore attempted to extend their findings in Chapter 7 by comparing two doses of fed OVA and describing the persistence of tolerance for a wider range of effector responses.

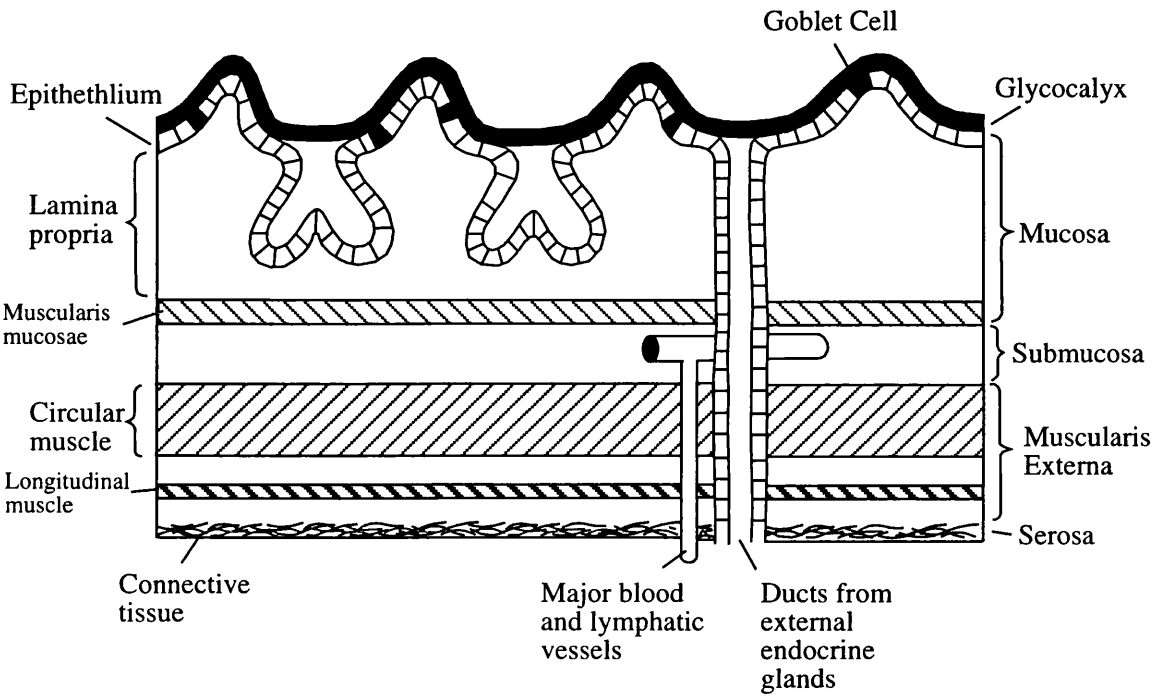

Fig 1.1

Representative longitudinal section of the wall of the gastrointestinal tract.

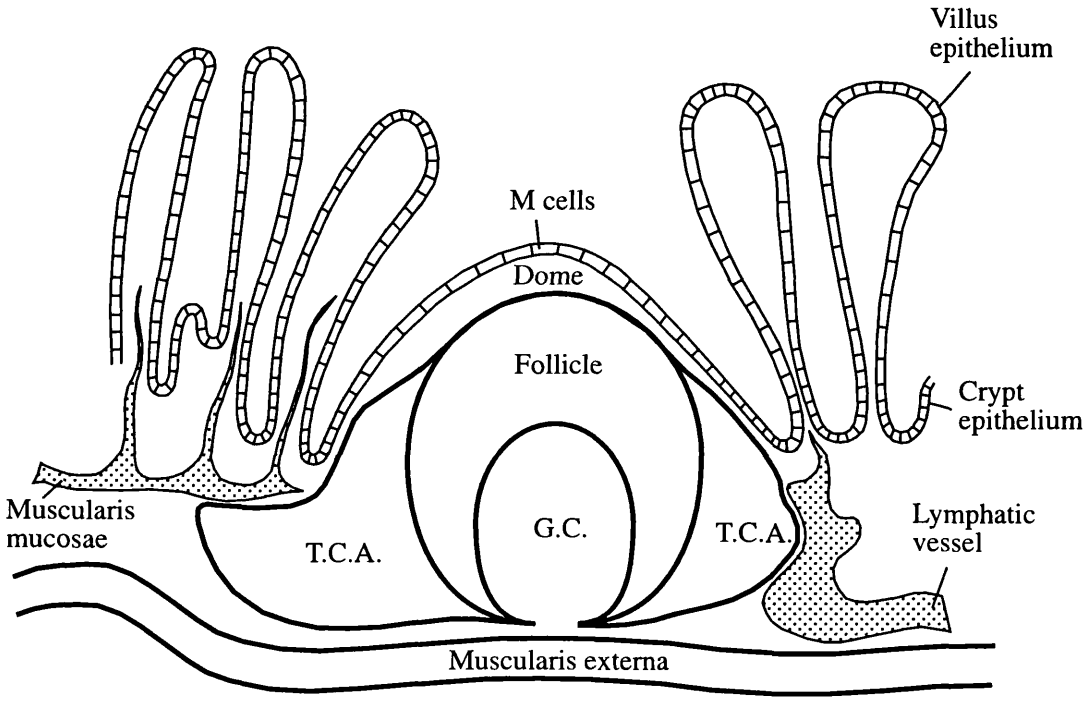

Fig 1.2

Schematic diagram of a typical Peyer's patch

G.C. = germinal centre
T.C.A. = T cell area

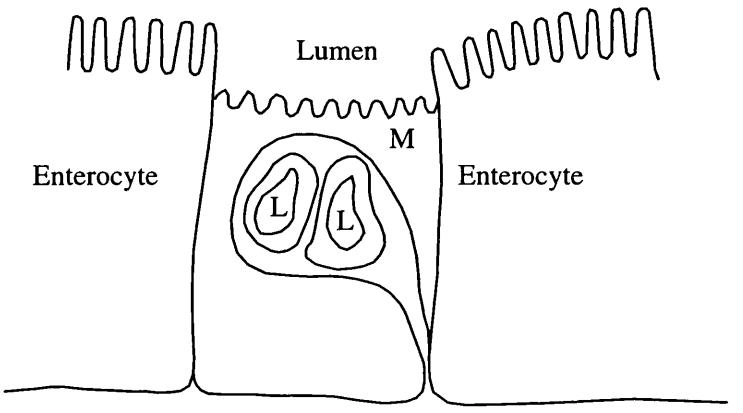

Fig. 1.3

Schematic diagram of an intestinal M Cell. Antigen is sampled from the gut lumen by M cells, passed down through the Peyer's patch and via the draining lymphatics to the MLN.

M = M cell
L = Lymphocyte

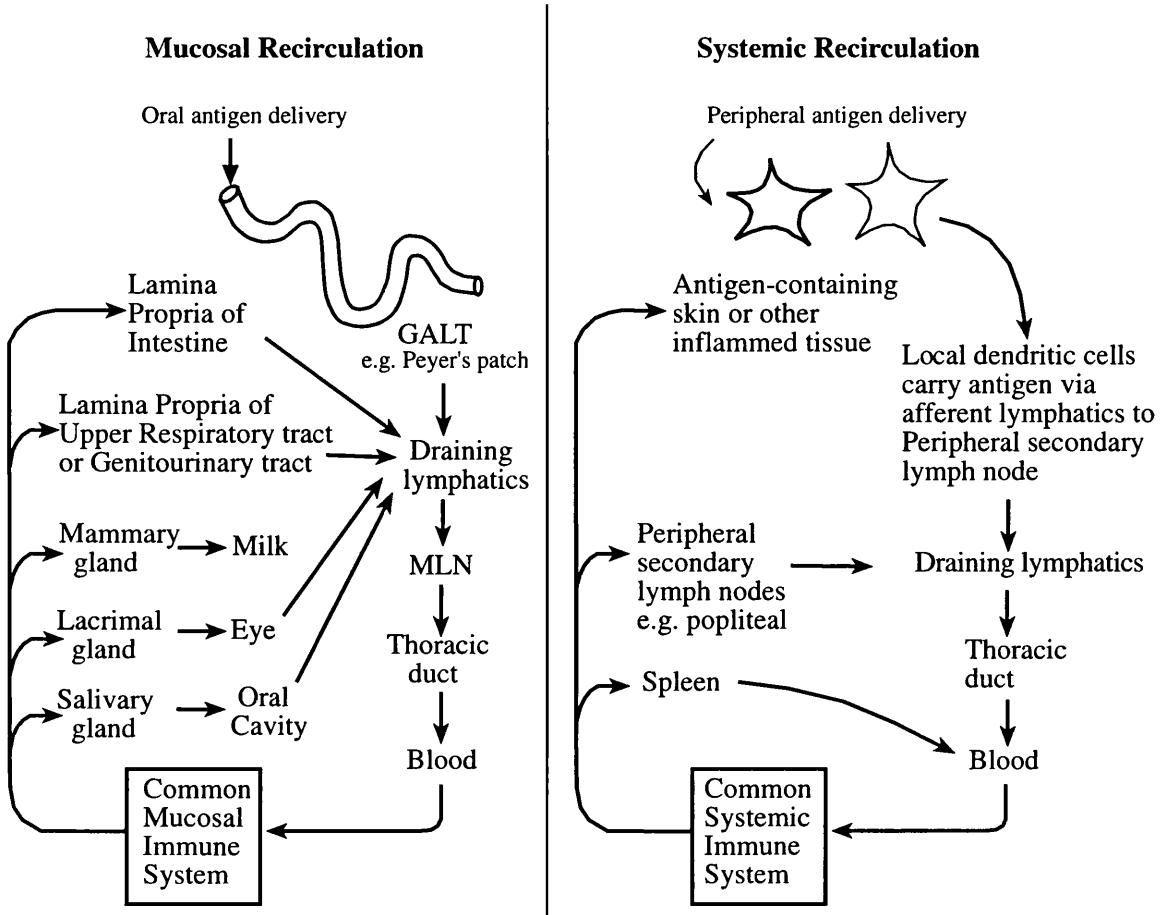

Fig. 1.4

Schematic diagram of mucosal and systemic lymphoblast recirculation pathways. After antigen recognition in a lymphoid tissue, naive T cells are induced to express particular homing molecules before leaving via the efferent lymphatics and thoracic duct to enter the bloodstream. These recirculating lymphoblasts can extravasate across post-capillary venules to entered inflammed tissue or lymphoid tissues and this is regulated by the interaction of endothelial addressins with the T cell homing molecules, both of which are distinct for mucosal and systemic tissues.

Table 1.1: Use of Oral Tolerance to Prevent Immunopathology[a]

Immunopathology	Antigen
Encephalomyelitis	Myelin basic protein
Arthritis	Collagen
Diabetes	Insulin
Uveoretinitis	Uveal S antigen
Glomerulonephritis	Various proteins
Allograft rejection	Allogeneic leukocytes/Allopeptides

[a]Feeding a range of antigens of pathological significance has been found to prevent the induction of associated immunopathological disease. See text for references.

Chapter 2: Materials and Methods

Animals

Specified pathogen-free (SPF), female BALB/c (H-2^d), C57Bl/6 (H-2^b), athymic outbred nude (H-2^{bxd}), MRL lpr/lpr$^{-/-}$ (H-2) and lpr/lpr$^{+/+}$ (H-2) mice were purchased from Harlan Olac (Bicester, Oxon, U.K.) or were bred in house (Central Research Facility, CRF, University of Glasgow) and maintained in the CRF.

Female IL4$^{-/-}$ (129Sv x C57Bl/6)F_2 (H-2^b) mice [306] were kindly provided by Drs J. Alexander (University of Strathclyde, Glasgow) and H. Bluethmann (F. Hoffman-La Roche AG, Basel, Switzerland). These mice were maintained initially in the Department of Immunology, University of Strathclyde and then in the CRF. Whenever possible, controls in these experiments were sex-matched wild-type (IL4$^{+/+}$)F_2 mice of the same strain combination, but in some cases, normal C57Bl/6 mice were used as controls. Female IFNγR$^{-/-}$ (129Sv, H-2^b) mice [307] were also obtained from Dr. H. Bluethmann and maintained in CRF.

Unless specified, all animals were housed under standard conditions with free access to both water and standard rodent pellets, containing no ovalbumin, and were first used at 6-8 weeks of age. Nude mice were housed in isolators.

Antigens and Mitogens

Ovalbumin (OVA, Fraction V), human serum albumin (HSA), Staphylococcal enterotoxin B (SEB) and concanavalin A (Con A) were obtained from Sigma (Sigma-Aldrich Company, Poole, U.K.), while purified protein derivative (PPD) from *M. tuberculosis* was obtained from Central Veterinary Laboratory, (C.V.L., New Haw, Addlestone, Surrey, U.K.) and prepared according to manufacturer's instructions.

Heat-aggregated OVA (HAO) was prepared by heating a 2% (w/v) solution of OVA in saline (Baxter Healthcare Ltd. Norfolk, U.K.) at 70°C for 60 minutes in a water bath

(Grant Instruments Ltd., Barrington, Cambridge, U.K.). The resulting suspension was centrifuged at 450g for 5 minutes and the precipitated OVA washed further by resuspending in ice cold saline and centrifuging at 450g for 10 minutes. After discarding the supernatant, the HAO was resuspended at 20mg/ml in saline and stored at -20°C until required. Before use, the HAO was diluted to 2mg/ml in saline and sonicated for 20 minutes to produce a colloidal suspension [111, 112].

Maintenance of Cell Lines and Hybridomas *in vitro*

EL4 cells (a thymoma of C57Bl/6 origin from American Type Culture Collection, ATCC, Rockville, MD. U.S.A.) were maintained in tissue culture flasks (Costar, Nucleopore, High Wycombe, U.K.) in RPMI 1640 containing 10% (FCS), 100U/ml penicillin/100μg/ml streptomycin, 1.25μg/ml amphotericin B (Fungizone), 2mM glutamine (all Gibco Life Technologies, Paisley, U.K.) and 0.05M 2-mercaptoethanol (2-ME; Sigma) (complete medium). EG7 OVA cells (H-2^b) were obtained originally from Dr M. Bevan (University of Washington, Seattle, U.S.A.). These cells were derived from EL4 cells transfected with a neomycin resistance gene and a single copy of a plasmid containing a cDNA copy of the chicken OVA mRNA (Moore et. al.) and were maintained in complete medium supplemented with 400μg/ml Geneticin (Gibco). Both cell lines were subcultured every 2-3 days by adding 1-2ml of cell suspension, containing approximately 2-5x10^6 cells, to 10ml of fresh medium and were subcultured 2 days before being used *in vitro* to ensure that the majority of cells would be in the log phase of growth.

YTS 191.1.2. (a rat anti-murine L3T4 (CD4) hybridoma) and YTS 169.4.2.1 (a rat anti-murine Lyt-2 (CD8α) hybridoma) [308] were both obtained from European Collection of Animal Cell Cultures (ECACC), Centre for Applied Microbiology and Research, Porton Down, Salisbury, U.K and were maintained at 3-9x10^5 cells/ml in either RPMI 1640 containing 20%FCS, 100U/ml penicillin/100μg/ml streptomycin, 1.25μg/ml Fungizone, 2mM glutamine and 5x10^{-5}M 2-ME or in complete medium, respectively.

Oral Administration of OVA

Mice were fasted for 18 hours prior to being fed single doses of OVA, which were dissolved in 0.2ml saline and administered via a stainless steel gavage needle (1.5 by 20 gauge, International Market Supply, Dane Mill, Broadhurst Lane, Chesire, U.K.) without anaesthetic. Control animals were fed 0.2ml saline alone.

Systemic Immunisation Procedures

In all experiments, subcutaneous (s.c.) immunisations were performed by injection of a total volume of 50µl into one rear footpad under light anaesthetic using 5% halothane (Zeneca Ltd. Macclesfield Cheshire, U.K.), while intraperitoneal (i.p.) immunisations were performed by injection of a total volume of 0.2ml without anaesthetic.

Systemic IgG, delayed type hypersensitivity and proliferative responses were induced by immunising mice s.c. with an emulsion of 100µg OVA in saline prepared at a ratio of 1:1 with complete Freund's adjuvant (CFA; Sigma).

Systemic CTL responses were induced in C57Bl/6 mice either by i.p. immunisation with 3µg OVA/ISCOMS (kindly provided by A. Donachie, Department of Immunology, University of Glasgow) prepared in saline and containing OVA and Quil A at a ratio of 10:1, as described elsewhere (Reid and Mowat), or by s.c. immunisation with 100µg OVA/CFA.

Induction of Hyperimmune Anti-OVA Antibodies

Serum IgG was induced in BALB/c mice by s.c. injection into one rear footpad with 100µg OVA/CFA and boosted with 100µg OVA in incomplete Freund's adjuvant (IFA; Sigma) one month later. Serum was collected after a further month.

Collection of Serum for Antibody Measurements

Mice under halothane anaesthesia were bled from the retro-orbital plexus using heparinised capillary tubes (Hawksley & Sons Ltd. Lancing, Sussex, U.K.). A maximum of 200µl was collected and serum was separated by centrifugation for 15 minutes at 450g and stored at -20°C until use. Alternatively, blood samples were collected from the major

blood vessels within the chest cavity of mice immediately after sacrifice, and serum separated as described above.

Preparation of Monoclonal Antibodies (Mabs) from Ascites Fluid in Athymic Mice

Nude mice were injected i.p. with 0.5ml pristane (2,6,10,14-teramethylpentadecane; Sigma) 14 and 7 days prior to i.p. inoculation with either $1x10^4$ YTS 169.4.2.1 or $1.5x10^4$ YTS 191.1.2. hybridoma cells in 1ml RPMI-1640. After a further 1-2 weeks, Mab rich-ascites fluid was harvested from the peritoneal cavity using a needle and syringe when peritoneal tumour and swelling had developed. The fluid was centrifuged at 1500g for 10 minutes at room temperature and supernatant collected for further purification of IgG.

Ammonium Sulphate Precipitation of IgG from Hyperimmune Serum or Ascites Fluid

IgG was purified from hyperimmune serum or ascites by dropwise addition of saturated ammonium sulphate solution (pH 6.5) to a final concentration of 45% w/v and the mixture left overnight at 4°C to ensure complete precipitation of IgG. Following centrifugation at 200g for 1 hour at 4°C, the supernatant was discarded and the precipitate, dissolved in 10ml PBS (Appendix 1), was placed in dialysis tubing (Medicell, Inernational Ltd. London, U.K.) and dialysed against 1l PBS for 48 hours at 4°C to remove the ammonium sulphate. The PBS was changed 6 times over this period and afterwards the dialysis tubing was removed and placed in polyethylene glycol (PEG) 2000 (Sigma) until its contents were concentrated to approximately 5ml. The solution was then removed, centrifuged at 50g for 30 minutes to clarify the supernatant and an aliquot assessed for IgG content by measuring the OD at 280nm using an Ultospec 2000 spectrophotometer (Pharmacia Biotech, Herts, U.K.). The concentration of IgG was calculated to be 1.85mg/ml on the basis that an OD_{280} of 1.43=1mg/ml IgG.

Depletion of T Cell Subsets *in vivo*

To deplete $CD4^+$ and $CD8^+$ T cells *in vivo*, mice were injected i.p. with 0.5mg of anti-CD4 mAb (YTS 191.1.2) or anti-CD8 mAb (YTS.169.4.2.1) dissolved in 0.5ml saline on days -2 and 0 before first administration of antigen. Control animals received either 0.5mg rat IgG (Sigma) or 0.5ml saline.

Depletion of Cytokines *in vivo*

To deplete IFNγ *in vivo*, mice were injected i.p. with 0.5mg hamster-anti murine IFNγ (IgG; Cell Tech Ltd. Berkshire, U.K.) in 0.2ml saline 1 day before and 1 hour prior to the first administration of antigen. Control animals received 0.2ml hamster serum diluted 1:4 to produce an approximate IgG concentration of 0.5mg (kindly provided by the Anatomy Animal Facility, University of Glasgow).

Assessment of Antigen-specific Delayed Type Hypersensitivity (DTH) Responses *in vivo*

OVA-specific DTH responses were assessed as decribed in detail elsewhere [112]. 20 days after s.c. immunisation with OVA/CFA in the rear footpad, mice were anaesthetised with halothane and the thickness of the unimmunised rear footpad measured using skinfold calipers (0-10mm in 0.1mm; Kroeplinn Langenmesstecknik, Kingston-on-Thames, Surrey, U.K.). The footpads were then injected intradermally (i.d.) with 100μg HAO in 50μl saline and after a further 24 hours, the increases in individual footpad thickness were measured. The mean increment of each group was calculated and the OVA-specific DTH responses obtained by subtracting the increment found in CFA unimmunised mice challenged with HAO.

Measurement of Antigen-specific Serum IgG Responses

Enhanced protein binding, 96-well ELISA plates (Immulon-4; Dynatech, Billingshurst, Sussex, U.K.) were coated overnight at 4°C with 100μl of a 10μg/ml solution of OVA in 0.1M carbonate buffer (pH 9.3: Appendix I). After three washes with

150µl/well PBS/0.05% Tween 20 (Sigma), 100µl aliquots of doubling dilutions of $(NH_4)_2SO_4$-purified anti-OVA hyperimmune serum IgG standard (described previously) and serum samples, both diluted 1:400 in PBS/0.05% Tween 20/1%FCS (Gibco Life Technologies, Paisley, U.K.), were added to the plates. After incubation at room temperature for 2.5 hours, the plates were washed as before, and incubated for a further 3 hours at room temperature with 100µl/well alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) diluted 1:500 in PBS/Tween 20. After a final wash step, 100µl/well of phosphatase substrate (1mg/ml in 10% diethanolamine, DEA, dissolved in distilled water; both from Sigma) was added and 5-30 minutes later, the plates read at 405nm (reference filter 510nm) using a MR5000 automatic microplate reader (Dynatech). The anti-OVA IgG concentration of test supernatants was determined with reference to a standard curve constructed using serial dilutions of the hyperimmune anti-OVA standard.

Measurement of Antigen-specific Serum IgG Isotype Antibodies

Enhanced protein binding, 96-well ELISA plates (Immulon-4) were coated overnight at 4°C with 100µl of a 10µg/ml solution of OVA in 0.05M carbonate buffer. After three washes with PBS/0.05% Tween 20, non-specific protein-binding sites were blocked with 100µl/well of a 3% solution of bovine serum albumin (BSA; Sigma) in PBS/Tween 20 for 1 hour at room temperature. After three washes with PBS/Tween 20, 50µl aliquots of test sera in doubling dilutions (beginning at 1:400 for IgG1 or 1:20 for IgG2a assays) in PBS/Tween 20 were added to the plate, as were doubling dilutions of the $(NH_4)_2SO_4$-purified anti-OVA IgG hyperimmune serum standard (described above), diluted 1:400 in PBS/Tween 20, and incubated at room temperature for 1.5 hours. After three further washes, biotinylated rat anti-murine IgG1 (Serotec Ltd. Kidlington, Oxford, U.K.), diluted 1:16000, or biotinylated rat anti-murine IgG2a (both AMS Biotechnology, Witney, Oxon, U.K.) diluted 1:1000 in PBS/Tween 20 was added at 50µl/well and incubated at room temperature for 1 hour. The plates were then washed four times and 75µl/well extravidin-peroxidase (Sigma) in PBS/Tween 20 was added at 2µg/ml. After a final incubation for 1 hour at room temperature, the plates were washed six times before 100µl of 3,3',5,5'-

tetramethylbenzidine peroxidase (TMB) substrate (Dynatech) was added to each well. The plates were read at 630nm (reference filter 405nm) using a MR5000 automatic reader. Concentrations of anti-OVA antibody isotypes in test supernatants were determined with reference to a standard curve constructed using serial dilutions of the hyperimmune anti-OVA standard.

Preparation of Lymphoid Cells

Single-cell suspensions of spleen and popliteal lymph node (PLN) were prepared in RPMI-1640 by rubbing gently through a stainless steel mesh using a syringe plunger and passed through Nitex mesh (gauge 100μm, Cadisch & Sons, London, U.K.) to remove any clumps. After washing the cells twice in RPMI 1640 by centrifugation at 4°C for 7 minutes at 450g and resuspending in 10ml RPMI 1640, viable cells were counted by phase contrast microscopy (x40 objective; Nikon Labophot microscope, Nikon House, Surrey, U.K.) using a haemocytometer (Neubauer). Cells were finally resuspended in complete medium supplemented with 25mM Hepes (Sigma).

Measurement of T Cell Proliferation *in vitro*

200μl aliquots of lymphoid cells resuspended at 10^6/ml in complete medium supplemented with 25mM Hepes were added to quadruplicate wells of flat-bottomed 96-well tissue culture plates (Costar, Northumbria Biologicals, Cramlington, Northumberland), either alone or in the presence of OVA, PPD or Con A, at concentrations previously shown (unpublished data) to be optimal for generating effector immune responses *in vitro* (1mg/ml, 50μg/ml or 10μg/ml, respectively). The plates were covered with plate sealers (FLOW ICN Biomedicals Inc., Costa Mesa, CA , U.S.A.) and incubated in a 5% CO_2 humidified incubator at 37°C. Proliferation was assessed at various times by addition of 1μCi/well [^{3}H] thymidine (West of Scotland Radionucleotide Dispensary, Western Infirmary, Glasgow) for the last 24 hours of culture. Cell bound DNA was harvested onto glass fibre filter mats and [^{3}H] thymidine incorporation measured on a 1205 Betaplate scintillation counter (both Wallac Oy, Turku, Finland).

Measurement of Cytokine Production *in vitro*

Lymphoid cells resuspended at $4x10^6$/ml in complete medium containing 25mM Hepes were added in 1ml aliquots to 24-well tissue culture plates (Costar), either alone or with OVA, PPD or Con A at the concentrations described above. The plates were covered with plate sealers and incubated in a 5% CO_2 humidified incubator at 37°C. Supernatants were harvested at various times of culture, centrifuged at 13000rpm for 5 minutes to remove non-adherent cells and stored at -20°C until assayed for cytokine content.

Cytokine production was quantified using sandwich ELISA techniques, for which optimal conditions had previously been established in the laboratory. Enhanced protein-binding 96-well plates (Immulon 4; Dynatech) were coated overnight at 4°C with 50µl of monoclonal anti-cytokine antibody (Table 1) in 0.1M $NaHCO_3$ buffer (pH8.2, Appendix 1). The plates were then washed twice with PBS/0.05% Tween 20, before non-specific binding was blocked by incubation with 200µl of PBS/10%FCS for 1 hour at 37°C. After three washes, 50µl/well of neat culture supernatant was added to quadruplicate wells, while doubling dilutions of standard recombinant murine cytokine (Table 2) in PBS/10% FCS, was added to duplicate wells. The plates were then incubated for 3 hours at 37°C and washed four times before 50µl/well biotinylated anti-murine cytokine antibody (Table 1) diluted in PBS/10% FCS was added. After incubation for 1 hour at 37°C, the plates were washed six times before addition of 100µl/well of TMB substrate. The plates were read at 630nm (reference filter 405nm) using a MR5000 automatic microplate reader. Cytokine concentrations in test supernatants were determined with reference to a standard curve, constructed using serial dilutions of the standard cytokines and analysed using Mikrotek software (Dynatech).

Generation and Measurement of Antigen-specific CTL Responses *in vitro*

a) Restimulation of Splenocytes *in vitro*

EG7 OVA cells were incubated at 10^7/ml in RPMI 1640 medium containing 50µg/ml mitomycin C (Sigma) for 75 minutes at 37°C and washed 5 times in RPMI 1640 by

centrifuging at 400g for 5 minutes. Immune spleen cells were obtained from mice immunised 10 or 14 days previously by i.p. or s.c. injection of OVA ISCOMS or OVA/CFA respectively. $3x10^7$ pooled splenocytes were restimulated for 5 or 6 days in $25cm^2$ tissue culture flasks (Costar) with $1.5x10^6$ mitomycin C-treated EG7 OVA cells in a final volume of 10ml CTL medium (RPMI-1640, containing 10% FCS, 5% NCTC 135 (Gibco), 100U/ml penicillin/100µg/ml streptomycin, 2mM glutamine and 0.05M 2-ME) in a 5% CO_2 humidified incubator at 37°C.

b) Microcytotoxicity Assay for OVA-specific CTL

i)Labelling of Target Cells with ^{51}Cr

Aliquots of $2.5x10^6$ EG7 OVA target cells were labelled with ^{51}Cr by incubation in 1ml RPMI 1640/5% newborn calf serum (NCS; Gibco BRL) containing 2 MBq $Na_2{}^{51}CrO_4$ (West of Scotland Radionucleotide Dispensary), for 60 minutes at 37°C. After washing 5 times in 10ml RPMI/5% NCS, by centrifuging at 450g for 5 minutes, the cells were recounted before being used in the microcytotoxicity assay.

ii)Microcytotoxicity Assays

After culture, the restimulated effector splenocytes were washed twice in RPMI/5% NCS and recounted, before being incubated at different effector cell:target cell (E:T) ratios in V-bottomed microtitre plates (Flow Labs Ltd.) with ^{51}Cr-labelled target cells in a total volume of 200µl/well CTL medium. After a 4 hour incubation period in 5% CO_2 at 37°C, 100µl of supernatant was removed from each well and ^{51}Cr-specific activity measured in a 1282 Compugamma counter (Wallac). Results were calculated as the percentage cytotoxicity for triplicate assays, determined using 10% Triton-X (Sigma) to obtain total release and unimmunised B6 spleen cells to obtain spontaneous release. Percentage cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{experimental release - spontaneous release}}{\text{total release - spontaneous release}} \times 100\%$$

In all assays, cytotoxicity against EL4 cells was also determined to control for non-OVA-specific activity.

Phenotypic Analysis of Lymphocytes by Flow Cytometry

10^6 lymphoid cells, either freshly prepared or removed from restimulation cultures, were resuspended in plastic conical tubes (Falcon, Cowley Oxford, U.K.) in 50µl staining buffer (SB, Appendix 1) containing primary Mab at the appropriate concentration (Table 3). The samples were incubated in the dark on ice for 30-40 minutes before the cells were washed twice in 2ml SB by centrifugation for 7 minutes at 450g. Biotinylated (Bio)-antibodies were detected by reincubating the cells in 50µl of either a 1:50 dilution of phycoerythrin (PE)-streptavidin or a 1:100 fluorescein dilution of (FITC)-streptavidin (both Vector, Bretton, Peterborough, U.K.) in SB for 30-40 minutes on ice. The cells were then washed twice in SB and any red blood cells present were lysed by resuspending in 1ml FACSLyse (diluted 1:10 in distilled water; Becton Dickson, San Jose, CA, U.S.A.) at room temperature for 10 minutes. After a final wash in 1ml PBS, cells were resuspended in 0.5ml of ice cold FACSFlow (Becton Dickson) and analysed using a FACScan IV flow cytometer (Becton Dickson). A 488nm argon ion laser was used to detect green and red fluorescence, while dead cells were excluded from analysis by gating on forward and side light scatter properties. The data were analysed using Lysis II software (Becton Dickinson). In all experiments, negative control samples were cells incubated with FITC-Streptavidin or PE-Streptavidin in the absence of any primary antibody.

Assessment of Lymphocyte Morphology

Cytospin preparations of freshly isolated or cultured lymphocytes were made by spinning 10^6 cells on to glass slides at 450g for 2 minutes using a Shandon cytocentrifuge. Slides were allowed to air dry for 15 minutes before fixing in methanol for 15 minutes. The slides were then immersed in May-Grunwald stain (BDH, Poole, Dorset, U.K.) for 4 minutes, rinsed and allowed to stand for 2-3 minutes in distilled water, before staining with

Giemsa (BDH) diluted 1:10 in distilled water for 10 minutes. Finally, the slides were washed with tap water and allowed to air dry before being mounted in DPX mountant (BDH). Cells remaining adherent to culture wells were stained *in situ.* by a similar method after removal of non-adherent cells by vigorous washing with PBS/10% FCS.

The numbers of intact and apoptotic lymphocytes on cytospin slides were assessed by light microscopy (Olympus BH-2 biological microscope, Olympus Optical Co. Ltd., London, U.K.) under oil immersion at x100 magnification. Apoptotic cells were identified as those with clearly blebbed membranes and condensed chromatin in their nucleus.

Assessment of Ultrastructural Morphology

Electron microscopy (EM) was performed by Mrs J. Hare (Department of Pathology, Western Infirmary, Glasgow). 10^6 lymphoid cells from culture plates were pelleted by centrifugation at 450g for 5 minutes in conical-bottomed centrifuge tubes (Falcon). Cell pellets were fixed overnight in 2% glutaraldehyde (Agar Scientific Ltd., Stanstead, U.K.) in Sorensen's buffer (pH7.4, Appendix 1) and after a brief rinse in 1% osmium tetroxide (Agar Scientific Ltd.), were dehydrated through graded alcohol and rinsed briefly with propylene oxide (BDH) before embedding in araldite epoxy resin (Agar Scientific Ltd.) and polymerising at 60°C overnight. Sections 2μm thick, were cut on a Reichert Jung Ultracut E microtome, stained with toludine blue (BDH) and examined by light microscopy. Ultrathin sections 90μm thick, were cut, mounted on 200-mesh copper grids (Emitek, Ashford, U.K.) and stained with uranyl acetate and lead citrate (Agar Scientific Ltd.) prior to examination using a Philips CM10 transmission electron microscope at a final magnification of 73000x.

Analysis of Cellular DNA Content *in vitro*

To assess the position of cultured cells in cell cycle, flow cytometric analysis of propidium iodide (PI; Sigma) [309] stained cells was performed. Aliquots of 10^6 cells were resuspended in 100μl of PBS and incubated for 10 minutes at room temperature with 450μl trypsin solution (0.03mg/ml; Sigma) dissolved in stock buffer (Appendix 1) to permeabilise

the cell membrane. 325µl of a solution of 0.5mg/ml trypsin inhibitor and 0.1mg/ml ribonuclease A (both Sigma) in stock buffer was then added and the mixture incubated at room temperature for a further 10 minutes. Finally, 250µl of a solution of 0.42mg/ml PI and 1mg/ml spermine tetrahydrochloride (Sigma) in stock buffer was added and incubated for 10 minutes at 4°C in the dark to allow PI incorporation into the nuclear DNA. Stained cells were analysed for PI fluorescence at an excitation wavelength of 488nm on a Coulter EPICS XL flow cytometer (Coulter, Luton, U.K.) using DNA Analysis software, where chicken erythrocyte nuclei (Beckton Dickinson) were used as a source of diploid DNA for standardisation.

Depletion of T Cell Subsets *in vitro*

$CD4^+$ and $CD8^+$ T cell subsets were depleted *in vitro* by complement-mediated lysis. PLN cells were resuspended at 10^7/ml in RPMI 1640 supplemented with 5% FCS and 25mM Hepes, and containing either anti-CD4 mAb (YTS 191.1.2) or anti-CD8 mAb (YTS.169.4.2.1) at 100µg/ml. After incubation for 1 hour at 4°C, the cells were washed in 10 ml RPMI 1640/5% FCS, by centrifuging for 7 minutes at 450g and resuspended at 10^7/ml in RPMI 1640/5% FCS containing 10% (v/v) rabbit complement (Low-Tox M, Vector). After a final incubation for 1 hour in 5%CO_2 at 37°C, the cells were washed twice in RPMI 1640/5% FCS, counted and resuspended at $2x10^6$ cells/ml in complete medium for assessment of proliferative responses and cytokine production *in vitro*. Control cells were incubated in the absence of MAb or complement under identical conditions.

Blocking Fas-dependent Apoptosis *in vitro*

To aliquots of lymphoid cells resuspended in complete medium for measurement of either proliferation or cytokine production *in vitro*, 20µg/ml hfas-Fc fusion protein (kindly provided by Dr. T. Brunner, La Jolla Institute for Allergy and Immunology, La Jolla, CA, U.S.A.) was added in an attempt to block fas-dependent apoptosis, as has been shown by previous reports [310].

Statistical Analysis

Results are represented as the mean ± 1 SEM where indicated and were analysed using Student's t-test.

IgG isotypes and IgA antibodies were not normally distributed and were compared using Wilcoxon's Rank test.

Table 2.1: Monoclonal Antibodies used in Cytokine Sandwich ELISAs

i) Capture Antibodies

Specificity	Clone	Isotype	Conc. (μg/ml)
Murine IL2	JES6-1A12	Rat IgG_{2a}	2
Murine IL3	MP2-8F8	Rat IgG_1	2
Murine IL4	BVD4-1D11	Rat IgG_{2b}	2
Murine IL5	TRFK5	Rat IgG_1	4
Murine IL10	JES5-2A5	Rat IgG_1	4
Murine IFNγ	P4–6A2	Rat IgG_1	2

ii) Biotinylated Detecting Antibodies

Specificity	Clone	Isotype	Conc. (μg/ml)
Murine IL2	JES6-5H4	Rat IgG_{2b}	1
Murine IL3	MP2-43D11	Rat IgG_{2a}	0.5
Murine IL4	BVD6-24G2	Rat IgG1	1
Murine IL5	TRFK4	Rat IgG2a	4
Murine IL10	SXC-1	Rat IgM	2
Muirne IFNγ	XMG1.2	Rat IgG1	1

All of the above monoclonal antibodies were purchased from PharMingen, San Diego, U.S.A.

Table 2.2: Recombinant Murine Cytokine Standards used in Sandwich ELISAs

Cytokine	Source
IL2	Pharmingen
IL3	Genzyme, West Malling, Kent.
IL4	Genzyme.
IL5	Genzyme.
IL10	Both gifts from Prof. F.Y. Liew,
IFNγ	Department of Immunology, University of Glasgow.

Table 2.3: Primary Antibodies used in Flow Cytometric Analysis

Specificity	Clone	Isotype
Bio-anti-murine CD3ε	145-2C11	Hamster IgG
PE-anti-murine CD4 (L3T4)	GK1.5	Rat IgG2b
FITC-anti-murine CD8α (Lyt-2)	53.6.7	Rat IgG2a
Bio-anti-murine CD25 (IL2Rα)	7D4	Rat IgM
Bio-anti-murine CD69	H1.2F3	Hamster IgG
Bio-anti-murine CD40L (gp39)	MR1	Hamster IgG

All of the above monoclonal antibodies were obtained from Pharmingen and used at 1:25 dilutions in staining buffer.

Appendix 2.1: Buffers

Phosphate Buffered Saline (PBS)

80.0g NaCl

11.6g Na_2HPO_4

2.0g KH_2PO_4

2.0g KCl.

Initially add to 7 litres distilled water (ddH_20: Purite Prestige Analyst HP water purifier, Purite Ltd., Bandet Way, Thame, Oxon). Stir and allow to dissolve, then pH to 7.0 and make up to a final volume of 10 litres.

Coating Buffer

0.1M $NaHCO_3$.

Add 8.4g $NaHCO_3$ to 1l ddH_20 and allow to dissolve, then pH to 8.2.

0.05M Carbonate Buffer

1.6g Na_2CO_3

2.95g $NaHCO_3$.

Add to 1l ddH_20 and pH to 9.3.

0.1M Carbonate Buffer

3.2g Na_2CO_3

5.9g $NaHCO_3$

Add to 1l ddH_20 and pH to 9.3.

Staining Buffer

PBS containing:

5%NCS,

0.05% (w/v) sodium azide (Sigma).

Stock buffer

20mg trisodium citrate,

1.21mg Tris,

10.44mg spermine tetrahydrochloride,

20µl Nonidet P40 (all Sigma).

Add to 100ml ddH_20 and allow to dissolve, then adjust pH to 7.6

Chapter 3: The Role of Antigen Dose in Oral Tolerance and Priming

Introduction

The principal aim of my project was to investigate the mechanisms regulating peripheral immune responses to orally administered antigen. Previous studies of systemic immunity had shown that the effects of fed antigen could depend on the dose used, with very low antigen doses preferentially priming cell mediated immune responses (CMI) *in vivo* [120, 131], slightly higher doses producing selective tolerance of CMI [7, 133] and high doses inhibiting levels of both CMI and humoral immunity [131]. As the cellular and humoral arms of the immune response are now believed to be controlled by the Th1 and Th2 subsets of $CD4^+$ T cells, respectively [220, 311, 312], it is possible that each T cell subset is differentially regulated by particular doses of fed antigen. Moreover, since each T cell subset can inhibit the activity of the other [221, 291] and this function has been proposed as a model of immunoregulation in some systems of peripheral tolerance [289], a similar mechanism may operate in oral tolerance. This possiblity is supported by observations of preferential Th2 cell activation and subsequent inhibition of Th1-dependent responses after feeding relatively low doses of antigen [313]. High doses of fed antigen are shown to inhibit both T cell subsets similarly [118], suggesting that the induction of distinct regulatory mechanisms may be associated with the dose of antigen administered.

As it is difficult to make valid comparisons between the effects of antigen dose in these different experimental models and few rigorous dose response studies have been performed, I examined the effects of feeding an extensive dose range of OVA on subsequent Th1- and Th2-dependent responses both *in vivo* and *in vitro.*

Experimental Protocol

To induce oral tolerance or priming, a single dose of 10μg-25mg OVA was administered orally to BALB/c mice 10 days prior to s.c. immunisation with OVA/CFA. 14 days later, draining PLN cell suspensions were prepared in culture with medium, OVA or PPD for assessment of proliferation and cytokine production *in vitro*. Systemic DTH responses and serum levels of OVA-specific IgG were examined 21 days after immunisation.

In all experiments, control mice were fed 0.2ml saline.

Results

Dose-dependent Effects of Fed OVA on Subsequent Effector Responses

(A) Oral Tolerance

In order to establish the range of antigen doses leading to oral tolerance, my first experiments assessed the sensitivity to inhibition displayed by individual Th cell effector responses in mice fed 100μg, 1, 2, 5, 10 or 25mg OVA prior to parenteral challenge with OVA/CFA. These doses were chosen since previous studies had demonstrated their ability to tolerise [131].

In vivo Responses

OVA-specific DTH responses were significantly inhibited by feeding mice all these doses of OVA prior to parenteral antigen challenge (Fig 3.1a), with the tolerance becoming more profound with increasing doses of fed OVA (Fig 3.1a). A similar dose-dependent effect on the levels of specific serum IgG was observed, but significant suppression found only in mice fed 2mg OVA or greater (Fig 3.1b). These findings are consistent with previous reports that cell mediated immunity is more susceptible to oral tolerance induction than the humoral limb of the immune response [133].

In order to determine how this dose-dependent immune regulation affected individual Th cell subsets, I next measured serum levels of OVA-specific IgG isotypes, using IgG1 and IgG2a to reflect Th2 and Th1 cell activity, respectively [314].

In comparsion with controls, OVA-specific IgG1 antibody production was significantly reduced in mice fed 10 or 25mg OVA before immunisation, unaltered by feeding 2 or 5mg OVA and significantly raised in immunised mice fed 100μg or 1mg OVA (Fig 3.2a). In contrast, OVA-specific IgG2a antibody production was significantly reduced in mice receiving 100μg or greater of fed OVA compared with immunised control mice (Fig 3.2b). Although the IgG2a response of mice fed 10mg OVA did not differ significantly from that of controls (Fig 3.2b), this result was not reproducible in less detailed dose response studies and therefore considered to be erroneous. Taken together, these findings show that while most doses of fed OVA tolerise IgG2a responses, there is a biphasic effect on IgG1 production, which is primed by low doses and tolerised by high doses.

In vitro Responses

In order to examine Th cell activity more directly, I assessed the dose-dependent effects of fed OVA on subsequent PLN cell proliferative responses and cytokine production *in vitro*. I measured proliferation and IFNγ production as indicators of Th1 cell activation and IL4, IL5 and IL10 production to reflect the activity of Th2 cells [220]. I also measured IL3, a cytokine secreted by both cell subsets [220].

Proliferation

The OVA-specific proliferation of PLN cells from all mice increased over the culture period, with the highest responses being found after 120h (Fig 3.3a). The level of proliferation was significantly reduced by feeding mice 2mg OVA or greater before immunisation (Fig 3.3a) and this OVA-specific tolerance became more profound with

increasing doses of fed OVA (Fig 3.3a). Feeding 100μg or 1mg OVA had no significant effect (Fig 3.3a).

In these assays of proliferative activity, I also examined the possibility that feeding low doses of antigen might induce a state of bystander suppression, in which lymphocytes from a mouse tolerised to one antigen release inhibitory cytokines which suppress the response to an unrelated antigen given together with the original antigen at the time of challenge. This has been proposed in other models of oral tolerance induced by low doses of antigen (Miller, A. et.al. 1991 J.Exp. Med 174:701-798) and to investigate if it occurred in any of my experiments, I examined proilferative responses to PPD, as this antigen is present in the CFA used to challenge mice with OVA. As expected, cells from immunised control mice proliferated well to PPD, with optimal levels being found after 120h (Fig 3.3b). The maximal PPD responses of OVA fed mice were similar to those of controls, but at 48h of culture, cells from mice fed between 100μg and 5mg OVA displayed significantly reduced proliferation compared with controls (Fig 3.3b), indicating that the kinetics of this response may have been delayed by feeding low doses of OVA. In a less detailed dose response study, where cells from mice fed 2 or 25mg fed OVA were restimulated *in vitro* with both OVA and PPD, the proliferative responses were comparable with controls at all time points examined (results not shown).

IFNγ Production

Although the OVA-specific production of IFNγ by PLN cells from all mice increased over the culture period of 120h, this response was significantly reduced in cells from mice fed all doses of OVA compared with controls (Fig 3.4a).

PPD-specific IFNγ production also increased throughout culture, but cells from mice fed OVA did not differ significantly from controls in this response (Fig 3.4b) and similar findings were made in a less detailed dose response study where cells were cultured in the presence of OVA+PPD (results not shown).

IL3 Production

The OVA-specific production of IL3 by PLN cells from all mice increased over the culture period, with the highest levels being found after 120h (Fig 3.4a). Throughout culture, this response was significantly reduced in cells from mice fed 25mg OVA compared with controls and by the end of culture, was also significantly inhibited in cells from animals fed 10mg OVA (Fig 3.4a). Feeding 100μg or 1mg OVA significantly primed the later production of IL3 *in vitro*, while feeding 2 or 5mg of OVA had no significant effect on this response (Fig 3.4a).

PPD-specific IL3 production was also found to increase over the culture period of 120h and feeding OVA had no effect on this response at any time (Fig 3.4b). Similar findings were made in a less detailed dose response study where cells from mice fed 2 or 25mg OVA were cultured in the presence of OVA+PPD (results not shown).

IL4 Production

Although there was no OVA-specific IL4 detected in any group (data not shown), this cytokine is required for the development of Th2 cells [290], and further evidence for their activity in OVA-restimulated control cultures is described below. Therefore, it seems likely that IL4 had been produced but was either used up before it could be detected in culture or was present at levels sufficient to induce Th2 cells but below the level of sensitivity for detection by ELISA.

Low levels of IL4 were found after restimulation of PLN cells by PPD and all groups displayed similar levels (Fig 3.4b).

IL5 Production

OVA-specific IL5 production by PLN cells from all mice increased over the culture period, with the highest levels being found after 120h (Fig 3.4a). This response was significantly reduced by feeding mice between 100μg and 25mg OVA before immunisation (Fig 3.4a) and the inhibition became more profound with increasing doses of fed OVA (Fig 3.4a). The only exception was that IL5 secretion by cells from animals fed 2mg OVA did not differ significantly from controls until 120h of culture, when this response was significantly tolerised.

PPD-specific IL5 production also reached optimal levels after 120h (Fig 3.4b) and cells from mice fed OVA did not differ significantly from controls in this response (Fig 3.4b). Similar findings were made in a less detailed dose response study where cells from mice fed 2 or 25mg OVA were cultured in the presence of OVA+PPD (results not shown).

IL10 Production

The OVA-specific production of IL10 by all cells reached optimal levels after 120h (Fig 3.4a). Throughout culture, this response was significantly reduced in cells from mice fed 25mg OVA compared with controls (Fig 3.4a) and by the end of culture, was also significantly inhibited in cells from animals fed 10mg OVA (Fig 3.4a). Feeding 1mg OVA significantly primed the production of IL10 at 120h, while 100μg, 2 and 5mg fed OVA had no significant effect on this response (Fig 3.4a).

PPD-specific IL10 production reached optimal levels after 120h and cells from mice fed OVA did not differ significantly from controls in this response (Fig 3.4b). Similar findings were made in a less detailed dose response study where cells from mice fed 2 or 25mg OVA were cultured in the presence of OVA+PPD (results not shown).

These findings show that oral tolerance was induced by feeding a wide range of antigen doses, where as little as 100μg fed OVA reduced specific DTH responses and IgG2a antibodies *in vivo,* as well as the production of IL5 and IFNγ by PLN cells *in vitro.* Total OVA-specific IgG antibody production and PLN cell proliferation became susceptible to inhibition at doses of 2mg OVA, while at least 10mg fed OVA was required to reduce the specific production of IgG1, IL3 and IL10. Therefore, more responses became susceptible to tolerance as progressively higher doses of fed OVA were used and each response was then reduced to a greater extent by increasing OVA doses. Th1-dependent DTH, IgG2a, proliferation and IFNγ responses were more susceptible to tolerance than the Th2-dependent IgG1 and IL10 responses, indicating that each Th cell subset may be differentially regulated by fed antigen. Consistent with this idea, was the observation that Th2-dependent IgG1 and IL10 production were actually primed by low doses of fed antigen which induced concomitant tolerance of Th1-dependent responses. However, Th2-dependent IL5 production was neither resistant to tolerance nor primed by any tolerising dose of fed OVA examined, suggesting that the dose-dependent regulation of individual Th2-dependent effector responses may be distinct.

(B) Priming of Systemic Immunity by Feeding Low Doses of Antigen

In the experiments discussed above, I found that the lower doses of fed antigen were only partly effective in inducing oral tolerance and that this effect was different for some Th1 and Th2 cell functions. As earlier reports showed that even lower doses of fed antigen (10-50μg OVA) could preferentially prime DTH responses *in vivo* [120, 131], I decided to extend my studies to study the regulation of Th1- and Th2-dependent responses by very low doses of fed OVA. I assessed individual Th cell effector responses in mice fed 10 or 50μg OVA prior to systemic immunisation and, as a comparison, included a group tolerised by feeding OVA in a dose known to reproducibly inhibit all responses (25mg OVA).

In vivo Responses

As expected, OVA-specific DTH and serum IgG responses were significantly reduced by feeding 25mg OVA prior to immunisation (Fig3.5a&b). However, the DTH and serum IgG responses of mice fed 10 or 50μg OVA were not significantly different from those of control animals after immunisation (Fig3.5a&b). Therefore, my findings are inconsistent with other reports where these doses of fed OVA enhanced subsequent DTH responses [131]. One possible explanation for this discrepency would be if the OVA/CFA immunising regime was invoking maximal responses in my experiments, so that it was difficult to observe priming. To address this possibility, I compared the systemic immune responses of mice fed 10, 50μg or 25mg OVA before s.c. immunisation with either optimal (100μg) or suboptimal (2μg) doses of OVA/CFA.

The OVA-specific DTH responses induced in mice immunised with 2μg OVA/CFA were markedly lower than those seen with 100μg OVA/CFA (Fig 3.6a). As before, the DTH responses of optimally challenged mice were significantly reduced by feeding 25mg OVA before immunisation and were not significantly altered by 10 or 50μg fed OVA (Fig 3.6a). In contrast, the DTH responses of suboptimally challenged mice fed 25mg OVA were not significantly altered by a prior feed of 25mg OVA, but were significantly enhanced by 10μg fed OVA (Fig 3.6a), suggesting that the CMI induced by immunisation with 2μg OVA/CFA was so low that it became difficult to observe tolerance and easier to detecting priming. Athough the DTH responses of mice fed 50μg OVA were also above control levels, these were not significantly different from those of the relevant control mice (Fig 3.6a), indicating that this dose was less efficient than 10μg fed OVA in priming CMI.

The level of OVA-specific IgG antibody was also dependent on the dose of antigen used for parenteral immunisation and in the experiment shown, animals challenged with 100μg OVA/CFA and fed 10, 50μg or 25mg OVA had significantly reduced IgG responses compared with controls (Fig 3.6b). However, in two repeat experiments, the IgG responses of mice fed either 10 or 50μg OVA prior to immunisation with 100μg OVA/CFA were comparable with controls, indicating that these feeding doses of OVA were not tolerogenic.

In suboptimally challenged mice, total OVA-specific IgG responses were also significantly reduced by a prior feed of 25mg OVA, but feeding 10 or 50μg OVA had no significant effect on this response (Fig 3.6b).

Therefore, extremely low doses of fed OVA did not prime the responses induced by subsequent immunisation with 100μg OVA/CFA. However, oral priming of cell mediated, but not humoral, immune responses was observed if mice were fed 10μg OVA prior to immunisation with only 2μg OVA/CFA.

In order to determine how this oral priming affected individual Th cell subsets, I next measured serum levels of OVA-specific IgG isotypes, again using IgG1 and IgG2a to reflect Th2 and Th1 cell activity, respectively.

Both IgG1 and IgG2a isotype antibodies were elicited in a dose-dependent manner to OVA/CFA immunisation. In animals challenged with 100μg OVA/CFA, a prior feed of 25mg OVA significantly reduced the levels of each isotype (Fig 3.7a&b), while 10 or 50μg fed OVA did not significantly alter these specific responses (Fig 3.7a&b). In suboptimally challenged mice, the levels of specific IgG1 in mice previously fed either 10μg or 25mg OVA were comparable with those of controls (Fig 3.7a). In contrast, feeding 50μg OVA before suboptimal challenge significantly primed this response (Fig 3.7a). However, no dose of fed OVA examined could significantly alter the subsequent levels of IgG2a generated upon suboptimal challenge (Fig 3.7b). These results suggest that IgG1 and IgG2a serum antibodies are relatively resistant to modulation by extremely low doses of fed antigen, although some priming of IgG1 may be feasible.

In vitro Responses

In order to assess Th cell activity more directly, I examined PLN cells from mice fed 10 or 50μg OVA prior to normal or suboptimal challenge for specific proliferative responses and cytokine production *in vitro,* as before.

Proliferation

The OVA-specific proliferative responses of PLN from mice immunised with 2μg OVA/CFA were markedly lower than those seen with 100μg OVA/CFA (Fig 3.8). This response was significantly enhanced if mice received a prior feed of 10 or 50μg OVA prior to suboptimal, but not optimal, challenge (Fig 3.8).

Cytokine Production

The OVA-specific production of IL3, IL5 and IFNγ by PLN cells from suboptimally challenged mice was significantly lower than observed for cells from optimally challenged animals with little or no IL3 and IL5 detected (Fig 3.9). Moreover, cytokine production by optimally challenged mice was never augmented by feeding either 10 or 50μg OVA before immunisation (Fig 3.9). Instead, IL5 production was significantly decreased by each dose of fed OVA and all other cytokines were produced at levels comparable to controls (Fig 3.9). In contrast, the levels of IL3, IL5 and IFNγ produced by cells from suboptimally challenged animals were significantly enhanced by feeding mice 50μg OVA before immunisation (Fig 3.9). In addition, cells from mice fed 10μg OVA before suboptimal challenge produced levels of IFNγ significantly higher than control cells (Fig 3.9). However, this was the only cytokine found to be primed by feeding 10μg OVA, as IL3 and IL5 were produced at levels comparable to controls (Fig 3.9).

Taken together, these findings provide evidence that feeding mice 10μg OVA preferentially augments Th1-dependent DTH responses and PLN cell IFNγ production, while a 50μg dose of fed OVA primes both Th1 and Th2 cell activity, as evidenced by raised levels of IgG1, IL3, IL5 and IFNγ.

Summary and Conclusions

The results presented in this chapter show that systemic immune responses can be primed or tolerised by the same antigen, depending on the dose at which it is fed. Moreover, the activity of Th1 cells appears more sensitive to these effects than do Th2 cells, which show a varied response to both the priming and tolerising effects of orally administered OVA.

Tolerance of systemic immune responses was achieved by feeding between 100μg-25mg OVA and was found to improve with each increasing antigen dose in terms of both the extent of inhibition and the range of responses affected. Although the threshold dose of antigen required to induce tolerance differed for individual effector responses, Th1-dependent functions were generally most susceptible to inhibition. OVA-specific DTH responses and IgG2a antibodies *in vivo*, as well as PLN cell IFNγ production *in vitro* were tolerised by as little as 100μg fed OVA, while PLN cell proliferative responses became inhibited at doses $\geq$ 2mg fed OVA. The regulation of Th2-dependent responses was less clear cut. Levels of OVA-specific IL5 were tolerised by as little as 100μg fed OVA, while serum IgG1 and PLN cell IL10 production resisted the effects of tolerance induced by feeding between 100μg-5mg OVA, only becoming significantly inhibited at doses of $\geq$10mg fed OVA. These findings indicate that the regulatory factors mediating IL5 production may differ from those controlling the other Th2-dependent responses and are consistent with a recent study of the functional diversity of T lymphocytes, which showed that the T cell production of IL5 required different costimulation from that required to induce IL4 production [315, 316]. Moreover, since the Th2-dependent IgG1 and IL10 responses were preferentially activated by low doses of fed antigen which tolerised most Th1-dependent effector functions, this finding is partially consistent with the current dogma that low dose oral tolerance is mediated by regulatory Th2 cells. The precise role of these cells in oral tolerance will be addressed in the following chapter.

Priming of systemic immunity could be elicited by feeding mice extremely low doses of OVA, where 10μg fed OVA enhanced subsequent Th1-dependent PLN cell proliferation and IFNγ production *in vitro*, while 50μg fed OVA primed these responses in addition to increasing Th2-dependent IgG1 levels *in vivo* and PLN cell IL3 and IL5 secretions *in vitro*. Therefore, although Th1-dependent responses were most sensitive to oral priming, the activity of both Th1 and Th2 cell subsets could be upregulated by feeding an appropriate dose of OVA. However, it should be noted that priming could only be demonstrated if mice were challenged systemically with suboptimal doses of antigen in CFA, indicating that systemic priming by soluble antigen may not be particularly efficient and therefore of limited use as an oral vaccination strategy.

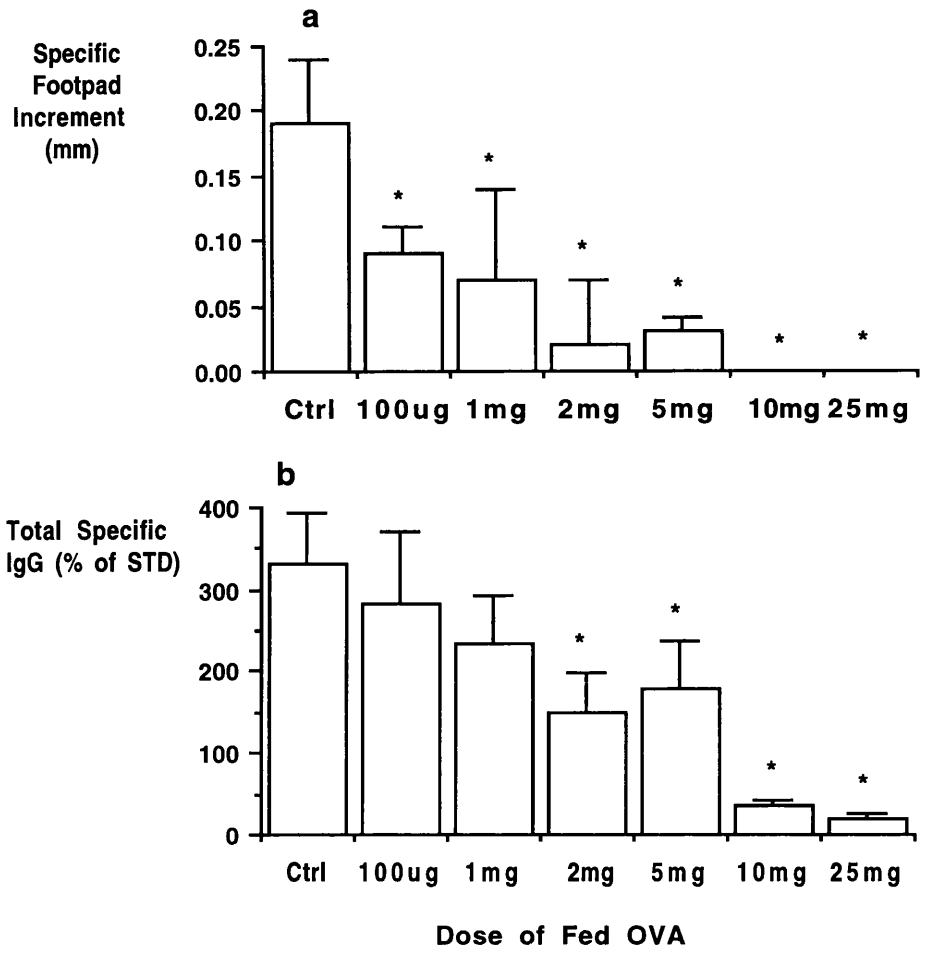

Figure 3.1 Effects of Feeding Different Doses of OVA on Subsequent Systemic Immunity to OVA *in vivo*

a) OVA-specific DTH responses in mice s.c. immunised 21 days before with OVA/CFA. Results shown are mean specific increments in footpad thickness ± 1 SEM for 6 mice per group in animals fed saline (Ctrl), 100μg, 1, 2, 5, 10 or 25mg OVA 10 days prior to immunisation. (*$p<0.05$ versus Ctrl). b) Total OVA-specific IgG responses 21 days after immunisation with OVA/CFA. Results shown are % hyperimmunised control serum and are the means ± 1 SEM for individual sera from 6 mice per group. (*$p<0.05$ versus Ctrl). Similar results were obtained in 2 replicate experiments.

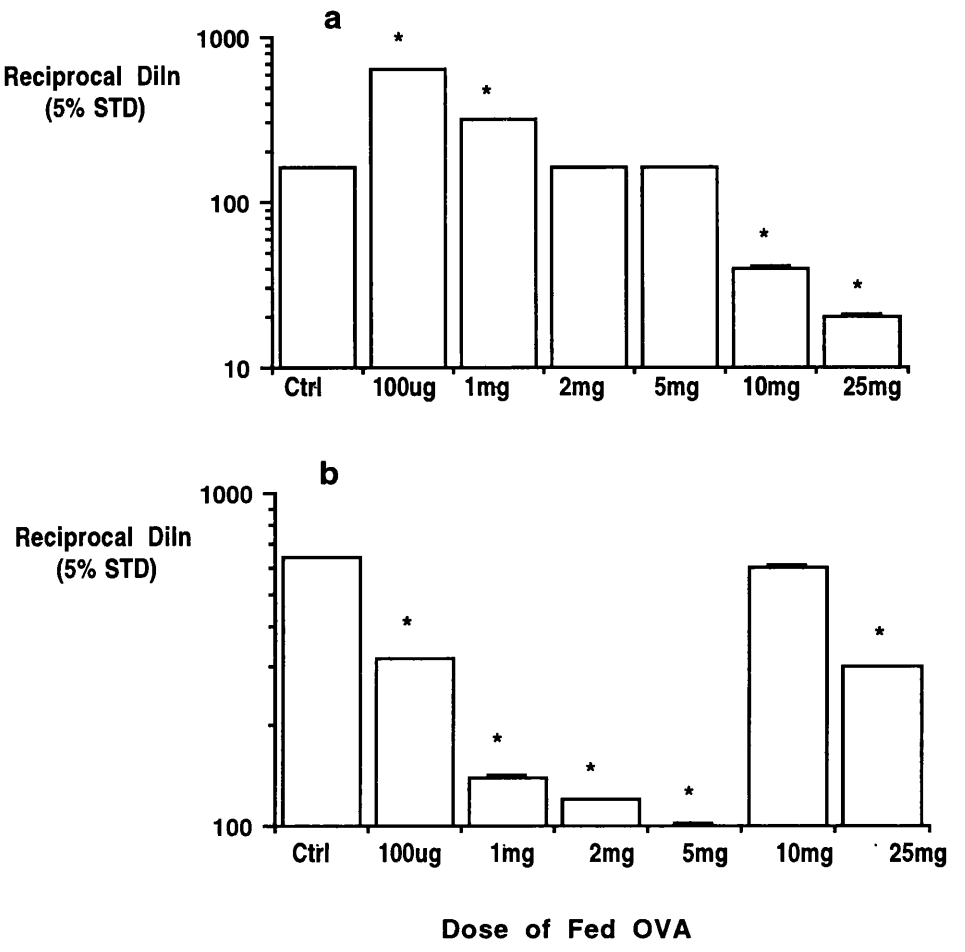

Figure 3.2 Effects of Feeding Different Doses of OVA on Specific Serum IgG Isotypes

Primary OVA-specific a) IgG1 and b) IgG2a responses 21 days after immunisation with OVA/CFA. The results shown are reciprocal dilutions giving an OD value equivalent to 5% hyperimmunised control serum and are the means ± 1 SEM for individual sera from 6 mice per group in animals fed saline (Ctrl), 100μg, 1, 2, 5, 10 or 25mg OVA 10 days prior to immunisation. (*$p<0.05$ versus Ctrl). Similar results were obtained in 2 replicate experiments.

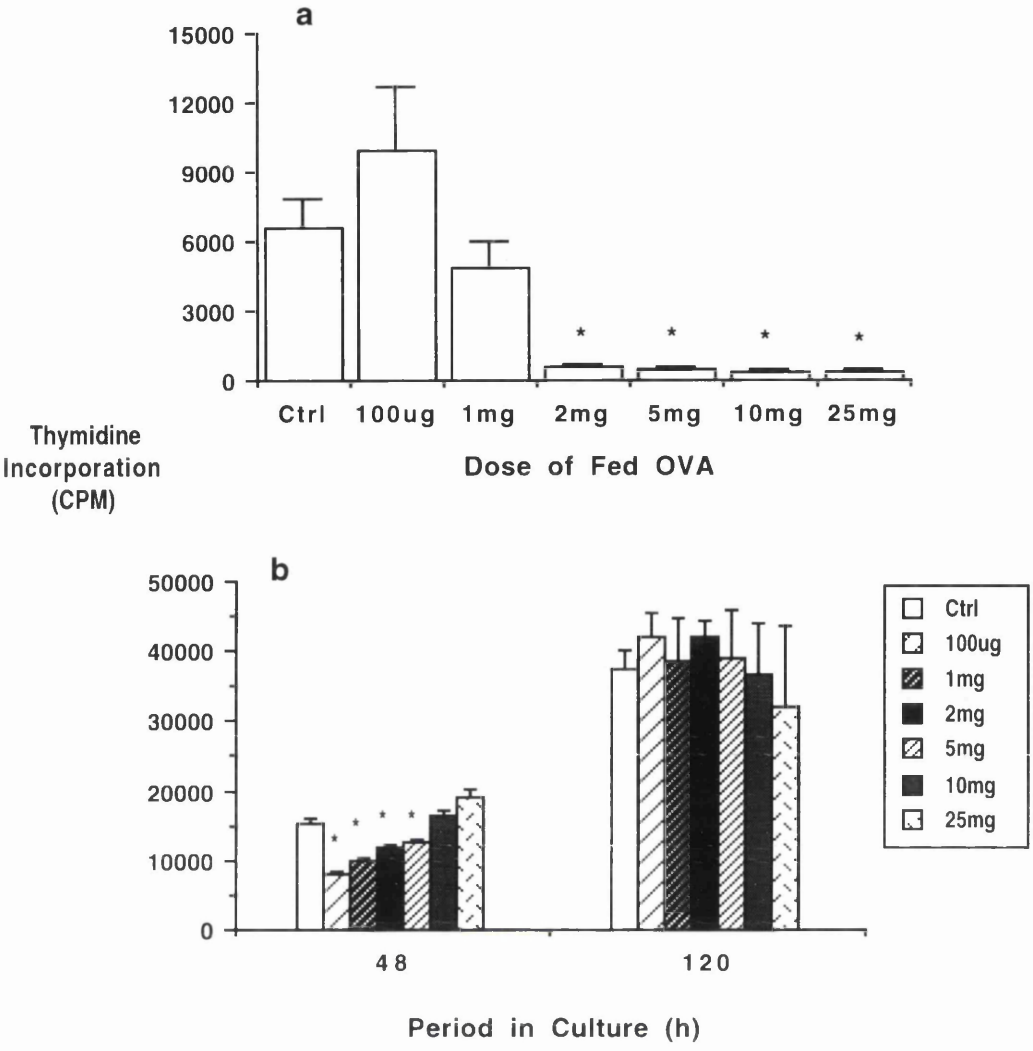

Figure 3.3 Effects of Feeding Different Doses of OVA on Proliferative Responses *in vitro*.

Proliferative responses of PLN cells removed 14 days after s.c. immunisation of mice with OVA/CFA and cultured a) with 1mg/ml OVA for 120h or b) with 50µg/ml PPD for 48 and 120h. Results shown are mean ^{3}H-TdR incorporation (CPM) ± 1 SEM in quadruplicate cultures of cells pooled from 5 animals per group in mice fed saline (Ctrl), 100µg, 1, 2, 5, 10 or 25mg OVA 10 days prior to immunisation. (*$p<0.05$ versus Ctrl). Similar results were obtained in 2 replicate experiments.

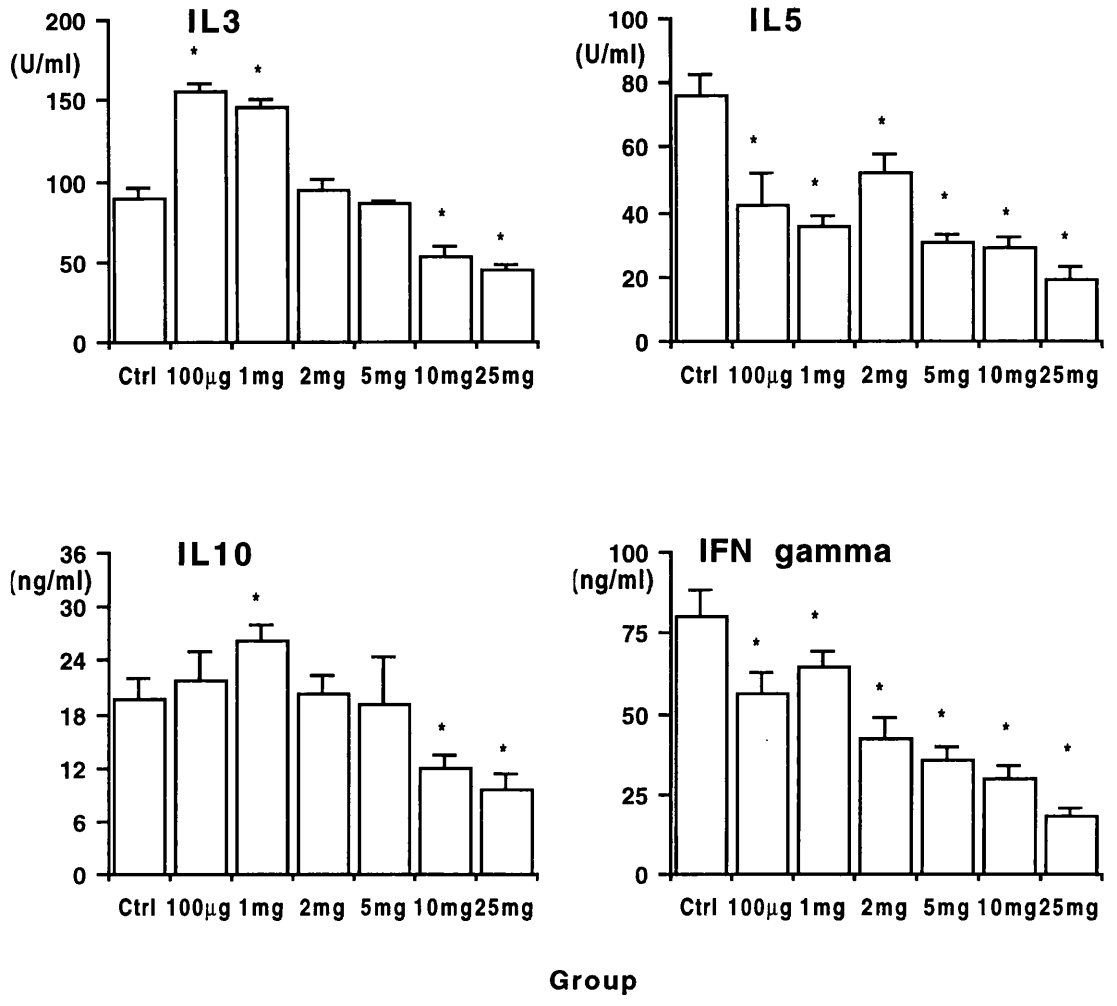

Figure 3.4a Effects of Feeding Different Doses of OVA on Cytokine Production *in vitro*.

OVA-specific production of IL3, IL5, IL10 and IFNγ by PLN cells removed 14 days after immunisation of mice with OVA/CFA and restimulated for 120h with 1mg/ml OVA. Results shown are mean cytokine levels (U/ml or ng/ml) ± 1 SEM in culture supernatants of cells pooled from 5 animals per group in mice fed saline (Ctrl), 100µg, 1, 2, 5, 10 or 25mg OVA 10 days prior to immunisation. (*$p<0.05$ versus Ctrl). Similar results were obtained at 48 and 72h in culture and in 2 replicate experiments.

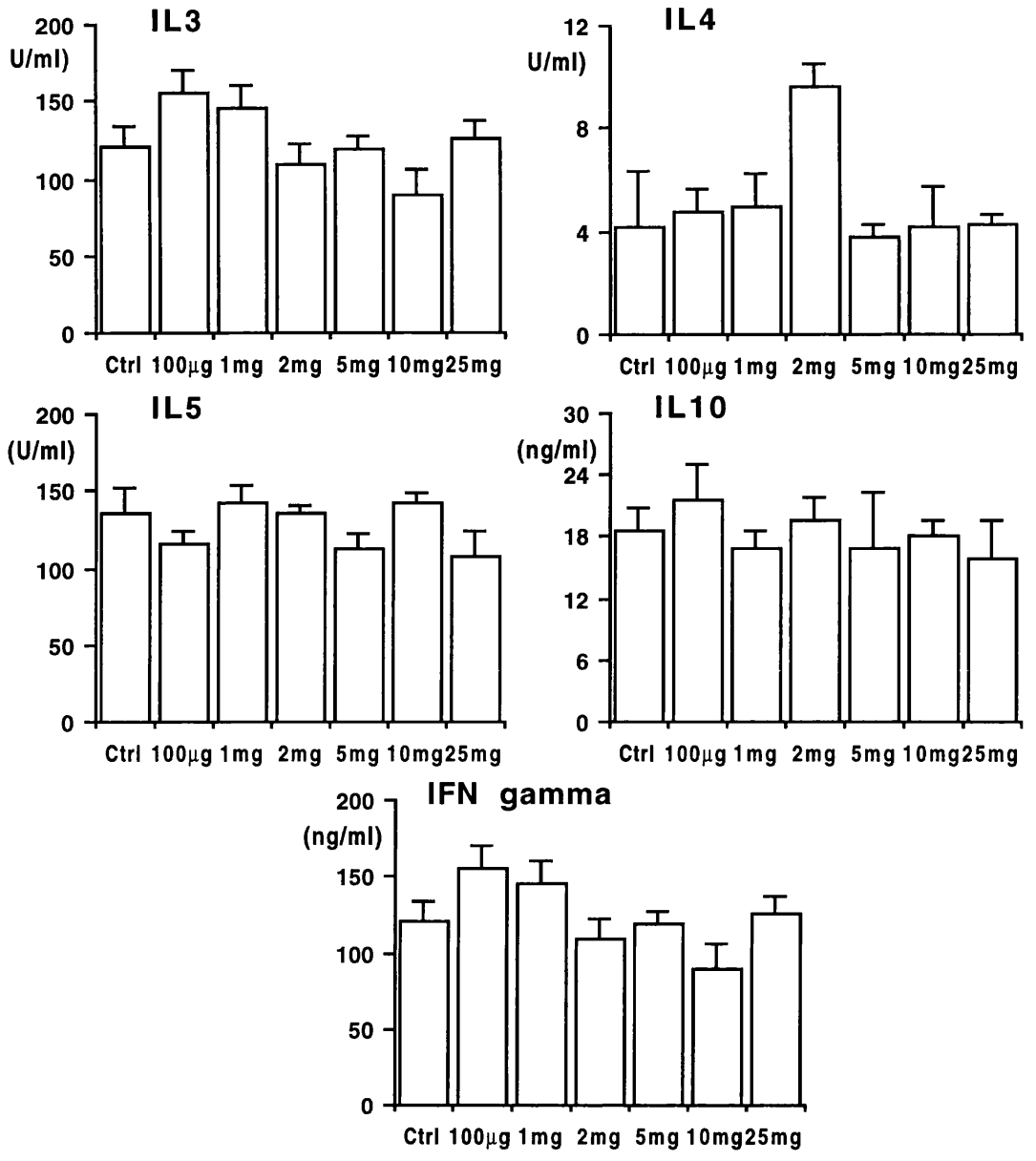

Figure 3.4b Effects of Feeding Different Doses of OVA on Cytokine Production *in vitro*.

PPD-specific production of IL3, IL4, IL5, IL10 and IFNγ by PLN cells removed 14 days after immunisation of mice with OVA/CFA and restimulated for 120h with 50µg/ml PPD. Results shown are mean cytokine levels (U/ml or ng/ml) ± 1 SEM in culture supernatants of cells pooled from 5 animals per group in mice fed saline (Ctrl), 100µg, 1, 2, 5, 10 or 25mg OVA 10 days prior to immunisation. (*$p<0.05$ versus Ctrl). Similar results were obtained at 48 and 72h in culture and in 2 replicate experiments.

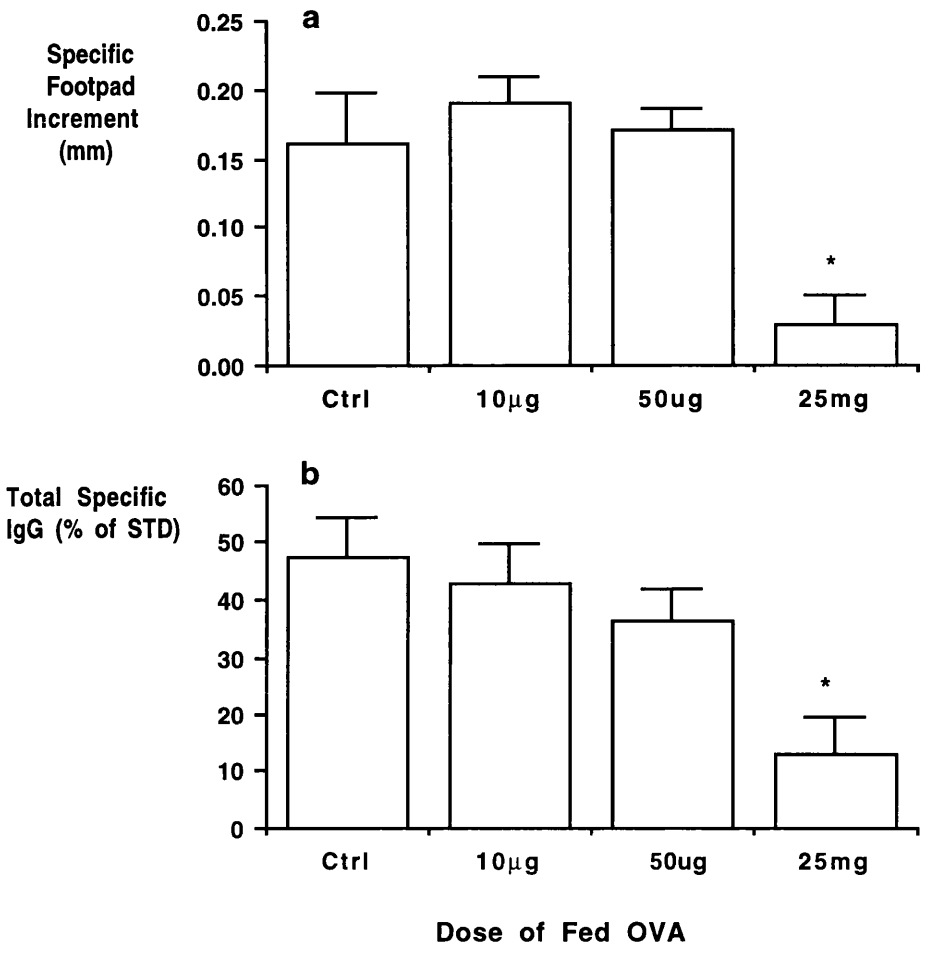

Figure 3.5 Effects of Extremely Low Doses of Fed OVA on Subsequent Systemic Immunity *in vivo*.

a) OVA-specific DTH responses in mice s.c. immunised 21 days before with OVA/CFA. Results shown are mean specific increments in footpad thickness ± 1 SEM for 6 mice per group in animals fed saline (Ctrl), 10, 50μg or 25mg OVA 10 days prior to immunisation. (*$p<0.05$ versus Ctrl). b) Total OVA-specific IgG responses 21 days after immunisation with OVA/CFA. Results shown are % hyperimmunised control serum and are the means ± 1 SEM for individual sera from 6 mice per group. (*$p<0.05$ versus Ctrl).

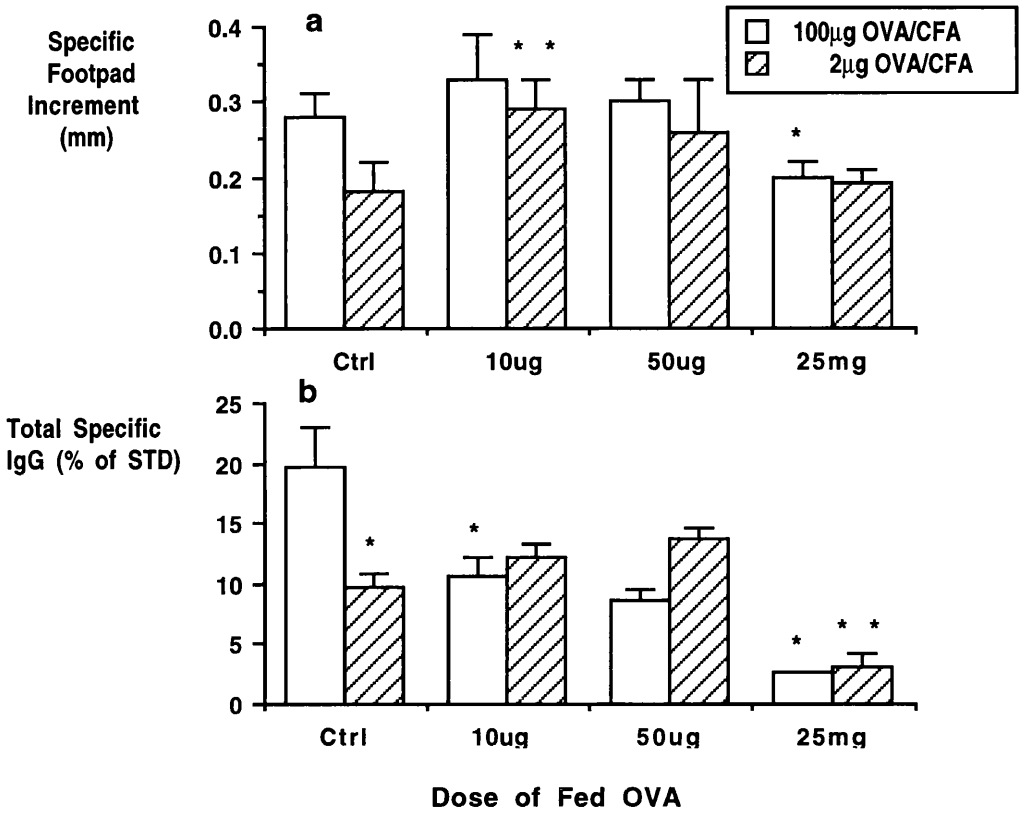

Figure 3.6 Effects of Extremely Low Doses of Fed OVA on Suboptimal and Optimal Systems.

a) OVA-specific DTH responses in mice s.c. immunised 21 days before with 100µg (Optimal Challenge) or 2µg (Suboptimal Challenge) OVA/CFA. Results shown are mean specific increments in footpad thickness ± 1 SEM for 6 mice per group in animals fed saline (Ctrl), 10, 50µg or 25mg OVA 10 days prior to immunisation. (*$p<0.05$ versus optimally challenged Ctrl; **$p<0.05$ versus suboptimally challenged Ctrl). b) Total OVA-specific IgG responses 21 days after immunisation with 100µg (Optimal Challenge) or 2µg (Suboptimal Challenge) OVA/CFA. Results shown are % hyperimmunised control serum and are the means ± 1 SEM for individual sera from 6 mice per group. (*$p<0.05$ versus optimally challenged Ctrl; **$p<0.05$ versus suboptimally challenged Ctrl). Similar results were obtained in a replicate experiment.

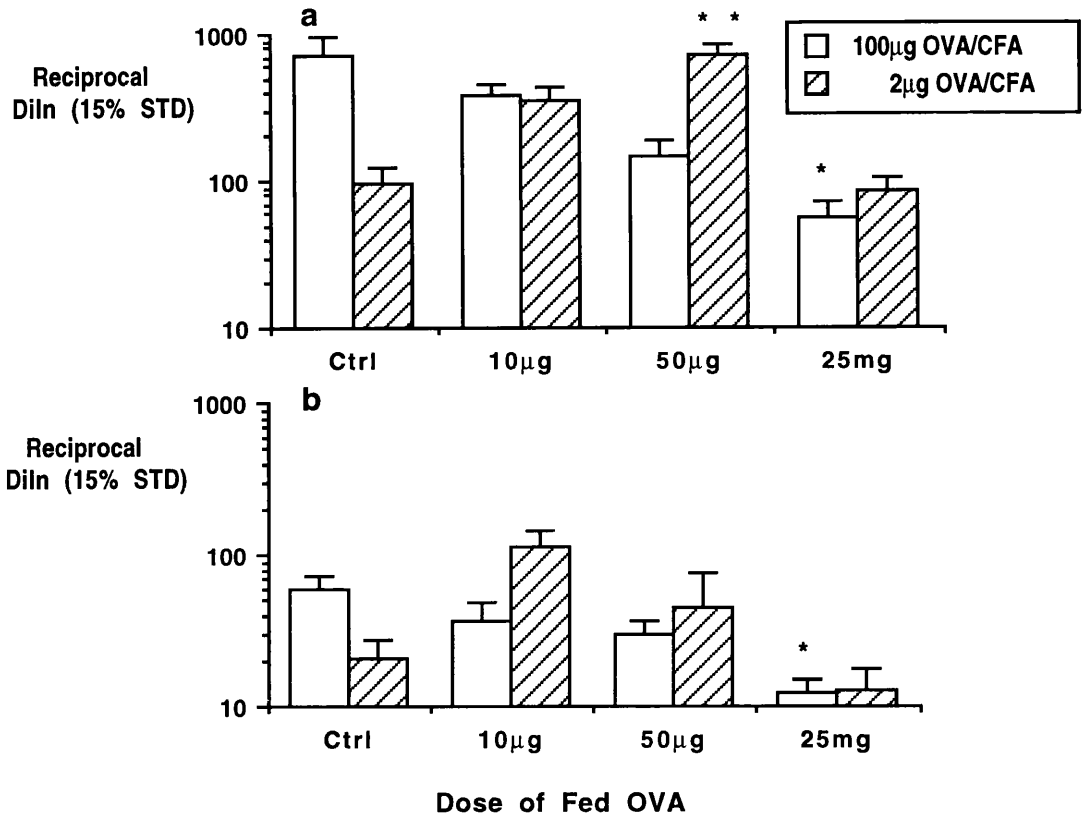

Figure 3.7 Effects of Extremely Low Doses of Fed OVA on Serum IgG Isotypes in Suboptimal and Optimal Systems.

Primary OVA-specific a) IgG1 and b) IgG2a responses 21 days after immunisation with 100μg (Optimal Challenge) or 2μg (Suboptimal Challenge) OVA/CFA. The results shown are reciprocal dilutions giving an OD value equivalent to 15% hyperimmunised control serum and are the means ± 1 SEM for individual sera from 6 mice per group in animals fed saline (Ctrl), 10, 50μg or 25mg OVA 10 days prior to immunisation. (*$p<0.05$ versus optimally challenged Ctrl; **$p<0.05$ versus suboptimally challenged Ctrl). Similar results were obtained in a replicate experiment.

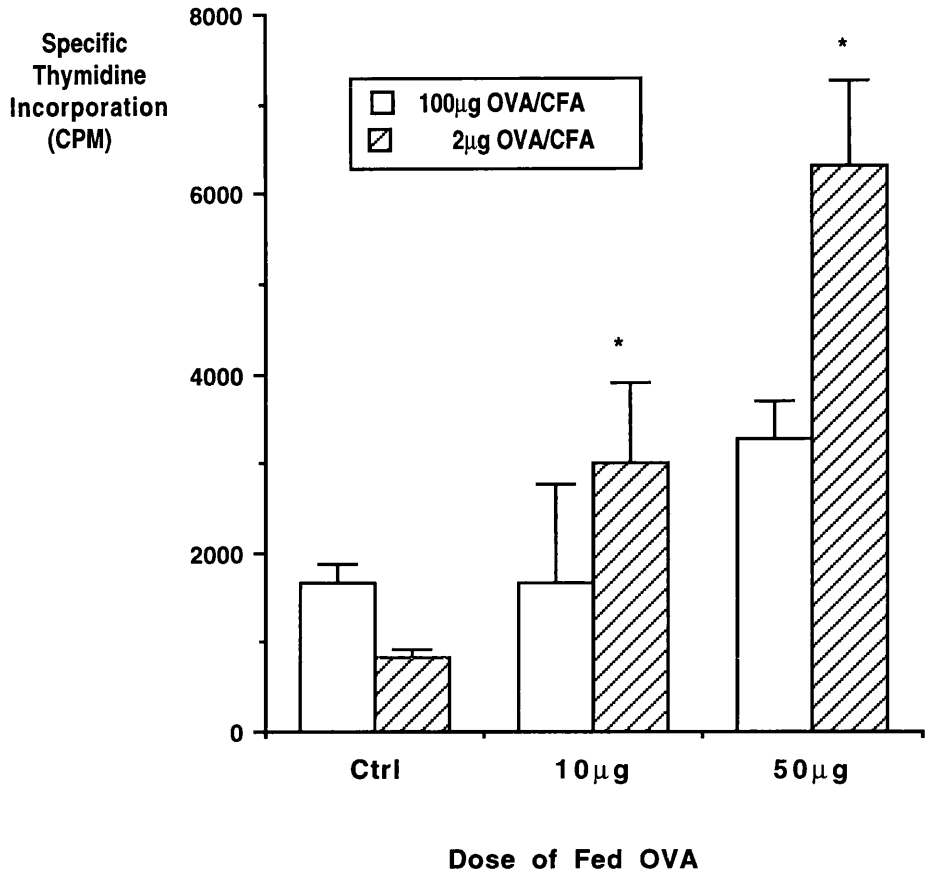

Figure 3.8 Effects of Extremely Low Doses of Fed OVA on OVA-specific Proliferative Responses in Suboptimal and Optimal Systems.

OVA-specific proliferative responses of PLN cells removed 14 days after s.c. immunisation of mice with 100µg (Optimal Challenge) or 2µg (Suboptimal Challenge) OVA/CFA and cultured with OVA (1mg/ml) for 120h. Results shown are mean ^{3}H-TdR incorporation (CPM) ± 1 SEM in quadruplicate cultures of cells pooled from 5 animals per group in mice fed saline (Ctrl), 10 or 50µg OVA 10 days prior to immunisation. (*$p<0.05$ versus suboptimally challenged Ctrl). Similar results were obtained in a replicate experiment.

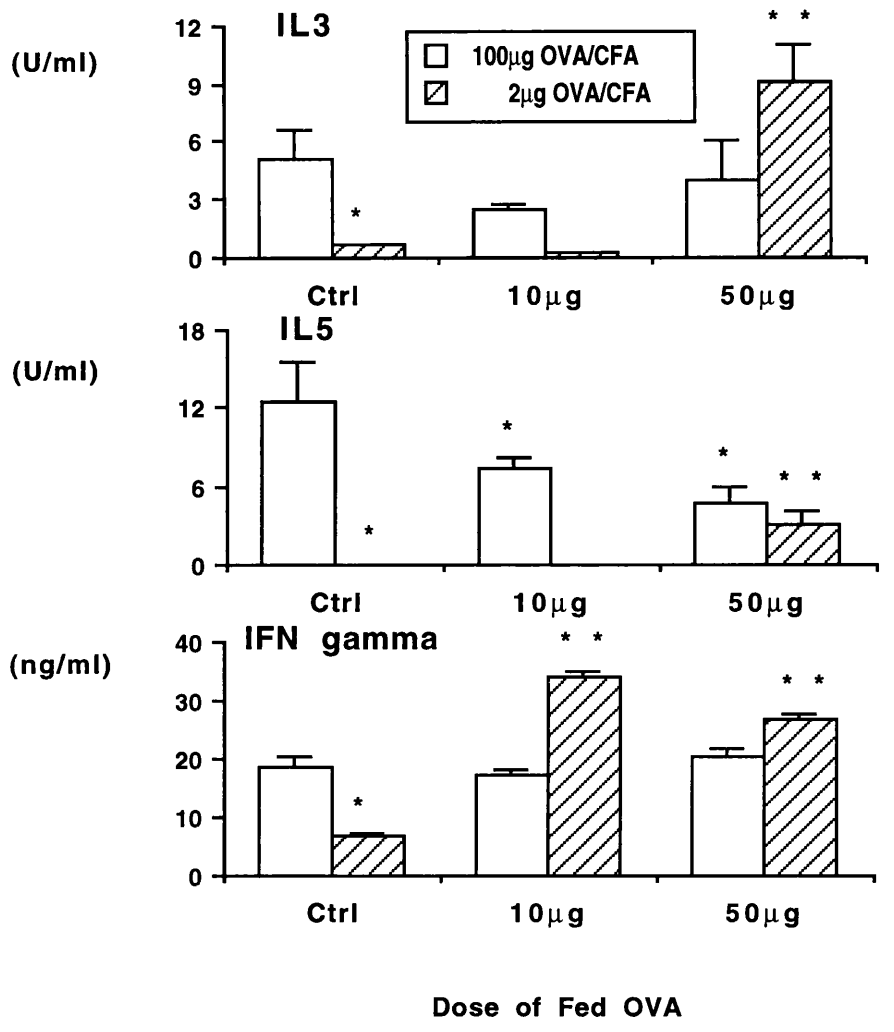

Figure 3.9 Effects of Extremely Low Doses of Fed OVA on OVA-specific Cytokine Production in Suboptimal and Optimal Systems.

OVA-specific production of IL3, IL5 and IFNγ by PLN cells removed 14 days after immunisation of mice with 100μg (Optimal Challenge) or 2μg (Suboptimal Challenge) OVA/CFA and restimulated for 120h with OVA (1mg/ml). Results shown are mean cytokine levels (U/ml or ng/ml) ± 1 SEM in culture supernatants of cells pooled from 5 animals per group in mice fed saline (Ctrl), 10 or 50μg OVA 10 days prior to immunisation. (*$p<0.05$ versus optimally challenged Ctrl; **$p<0.05$ versus suboptimally challenged Ctrl). Similar results were obtained in a replicate experiment.

Chapter 4: The Role of Cytokines in Oral Tolerance

Introduction

The results presented in the previous chapter indicated that although all immune functions could be tolerised by appropriate doses of fed antigen, certain effector responses appeared to be more resistant to oral tolerance than others. When OVA was fed at low doses, antigen-specific responses characteristic of a Th2 phenotype, including serum IgG1 antibodies *in vivo* and IL10 production *in vitro*, were generally the most difficult to tolerise. These findings are consistent with reports that $CD4^+$ T cells of the Th1 subset are more susceptible to the induction of tolerance than Th2 cells *in vivo* and *in vitro* [254, 317]. As a result, it has been suggested that certain forms of tolerance, including oral tolerance induced by low doses of fed antigen, reflect the preferential activation of Th2 cells, with subsequent down-regulation of Th1-dependent, cell-mediated immune responses via the production of inhibitory cytokines such as IL4, IL10 [271, 282, 289, 318], TGFβ [274], or a combination of these [285]. Conversely, IFNγ has also been implicated in certain models of nasal tolerance [128] and may regulate immune responses via its cytostatic properties [293, 319].

In this chapter, I investigated whether these cytokines played a direct role in oral tolerance to OVA using animals depleted of individual cytokines either by antibody or genetic manipulation. I also examined the influence of the dose of fed antigen, as it has been suggested that only low dose regimes stimulate active suppressor mechanisms mediated by Th2 cells.

Experimental Protocol

Mice were tolerised by feeding either 2 or 25mg OVA 10 days before s.c. immunisation with OVA/CFA. 14 days later, draining PLN cells were prepared for assessment of antigen-specific proliferative responses and cytokine production *in vitro,* while systemic DTH responses and serum levels of OVA-specific IgG were measured 21 days after immunisation. Control mice were fed 0.2ml saline.

To examine the role of IL4 and IFNγ, $IL4^{-/-}$ and $IFN\gamma R^{-/-}$ mice were used and IFNγ was also neutralised *in vivo* in normal BALB/c mice by i.p. administration of 0.5mg anti-IFNγ mab given two days before and two days after feeding antigen. A similar protocol was used to examine the role of TGFβ. C57Bl/6 mice were used as controls for the $H\text{-}2^b$ knock-out mice, while isotype-matched antibodies were used as controls for neutralising antibodies.

The role of TGFβ was also investigated by addition of a neutralising antibody during restimulation of PLN cells with OVA *in vitro.*

Results

A) Oral Tolerance does not Require Th2-dependent Cytokines

In the previous chapter I found that IL10 production *in vitro* was somewhat resistant to the effects of oral tolerance, with occasionally enhanced levels found using some low doses of fed antigen which induced tolerance of other effector functions. Since this cytokine can not only suppress Th1 cell activity via down-regulation of macrophage IL12 production [221], but its absence in $IL10^{-/-}$ mice allows the development of intestinal pathology due to hyperreactivity to components of the normal gut flora [187], I thought it important to clarify whether my findings were consistent with the growing evidence for a role for Th2-dependent crossregulation in low dose oral tolerance [271, 282, 289, 318]. Therefore, I

examined the effects of feeding either 2 or 25mg OVA to IL4$^{-/-}$ mice, which have a genetically engineered lack of IL4 and hence are impaired in all Th2-dependent responses, including IL10 [306].

In vivo Responses

The DTH responses of saline fed IL4$^{-/-}$ mice were significantly lower than those of control WT mice after immunisation with OVA/CFA (Fig 4.1a). Despite this, both WT and IL4$^{-/-}$ mice fed either 2 or 25mg OVA exhibited significantly reduced DTH responses compared with saline fed WT and IL4$^{-/-}$ mice, respectively (Fig 4.1a). Saline fed IL4$^{-/-}$ mice also displayed significantly lower total OVA-specific IgG antibody levels compared with control WT mice and again, this response was reduced in WT mice by a prior feed of either 2 or 25mg OVA and also in IL4$^{-/-}$ mice fed 25mg OVA before immunisation (Fig 4.1b). Although the IgG levels of IL4$^{-/-}$ mice fed 2mg OVA were not significantly different from their unfed controls in this instance, similar experiments showed that OVA-specific IgG responses could be significantly tolerised by feeding IL4 mice$^{-/-}$ 2mg OVA (data not shown). Therefore, the absence of IL4 *in vivo* does not prevent the induction of oral tolerance of OVA-specific DTH or serum IgG responses.

To investigate the scope of oral tolerance in these animals, I measured OVA-specific IgG isotypes. Consistent with previous reports [306], the serum levels of IgG1 were virtually neglible in all IL4$^{-/-}$ mice (Fig 4.2a), making it difficult to determine the effect of feeding OVA and perhaps explaining why I had observed particularly low total OVA-specific IgG responses in these mice. In contrast, IgG1 antibodies were readily detectable in control WT animals and were significantly reduced by feeding 25mg, but not 2mg OVA (Fig 4.2a). Saline fed IL4$^{-/-}$ produced significantly more OVA-specific serum IgG2a than WT controls (Fig 4.2b), suggesting that Th2-dependent crossregulation of Th1 cell activity was important for modulating this response. Both IL4$^{-/-}$ and WT IgG2a responses were significantly

reduced by feeding either 2 or 25mg OVA before immunisation (Fig 4.2b), demonstrating that oral tolerance of this isotype did not involve Th2-dependent suppression.

In vitro Responses

The OVA-specific proliferation of PLN cells from saline fed IL4$^{-/-}$ mice after immunisation was significantly higher than that of WT control cells (Fig 4.3). The proliferative responses of cells from both WT and IL4$^{-/-}$ mice fed 2 or 25mg OVA prior to immunisation were significantly reduced compared with their respective controls (Fig 4.3). All proliferative responses were negligible in medium alone (results not shown).

The OVA-specific cytokine profile of saline fed IL4$^{-/-}$ mice differed from that of WT controls with more IFNγ, less IL5 and equivalent levels of IL3 being produced *in vitro* (Fig 4.4). The production of both IL3 and IFNγ was significantly suppressed in WT and IL4$^{-/-}$ mice by feeding 2 or 25mg OVA prior to immunisation (Fig 4.4). The high levels of IL5 production in WT mice were also significantly reduced by feeding OVA and the very low IL5 response in IL4$^{-/-}$ animals was completely abrogated by prior feeding of either dose of OVA (Fig 4.4). OVA-specific IL4 and IL10 production was also measured in these experiments, but levels were negligible in all groups (results not shown). All cytokine responses were also negligible in medium alone (results not shown). Therefore, the Th2-depleted cytokine profile of IL4$^{-/-}$ animals was still susceptible to tolerance in mice fed either a low or high dose of OVA.

Together, these findings are not consistent with a role for IL4 or Th2 cells in tolerance induced by feeding either a high or a low dose of OVA.

(B) Oral Tolerance is Induced in the Absence of IFNγ

Since the findings above suggested that Th2 cells were not important for oral tolerance, I next investigated if the characteristic product of the other $CD4^+$ T cell subset, IFNγ, was involved. This may seem unlikely, since I have shown in Chapter 3 that OVA-specific IFNγ production *in vitro* after immunisation *in vivo* is particularly susceptible to inhibition by oral tolerance. However, chapter 6 describes the preferential release of OVA-specific IFNγ in the first few days after feeding OVA. This cytokine has well known cytostatic properties [293, 319] and its production is relatively preserved in mice tolerized by i.v. injection of staphylococcal enterotoxin B [228]. Furthermore, IFNγ is required for the tolerance induced in rats by intranasal administration of OVA [128]. Therefore, I considered it important to address the role of endogenous IFNγ in the induction of oral tolerance .

I first attempted to determine if oral tolerance could be induced in the absence of IFNγ by administering a depleting anti-IFNγ mab to animals around the time of feeding either saline or 25mg OVA. Although the OVA-specific DTH responses generated in OVA/CFA immunised control animals were significantly reduced by anti-IFNγ antibody, this response was tolerised normally in mice fed 25mg OVA before immunisation (Fig 4.5a). The levels of OVA-specific serum IgG were also significantly reduced in control animals treated with anti-IFNγ and again this response was tolerised normally in mice fed 25mg OVA before immunisation (Fig 4.5b).

Serum levels of OVA-specific IgG1 in saline fed immunised control mice were similar, irrespective of whether animals had received anti-IFNγ or isotype-matched antibody and these responses were significantly reduced to a similar extent by prior feeding of 25mg OVA (Fig 4.6a). In marked contrast, the levels of IgG2a were significantly reduced in control mice treated with anti-IFNγ compared with isotype-matched antibody (Fig 4.8b), highlighting the importance of IFNγ for this isotype and perhaps explaining the low levels of total OVA-specific IgG responses in these mice. However, IgG2a responses were tolerised normally both in anti-IFNγ and isotype antibody treated mice fed 25mg OVA before

immunisation (Fig 4.6b). Together, these findings do not support a role for IFNγ in the induction of oral tolerance by feeding a high dose of antigen.

I had intended to extend this study to address the role of IFNγ in tolerance induced by a low dose of fed OVA. However, insufficient antibody prevented this work from being carried out in normal animals. Instead, I used IFNγR$^{-/-}$ mice, which are genetically engineered to lack the receptor for IFNγ and hence are unresponsive to the cytokine [307]. This system also allowed me to examine the role of IFNγ in both the induction and expression of oral tolerance induced by either 2 or 25mg fed OVA without the need for chronic administration of antibody.

In vivo Responses

The DTH responses of WT control animals were significantly higher than those of IFNγR$^{-/-}$ control mice after immunisation with OVA/CFA (Fig 4.7a). Despite this difference, DTH responses were significantly reduced in both WT and IFNγR$^{-/-}$ mice by feeding 25mg OVA before immunisation (Fig 4.7a). Individual WT mice fed 2mg OVA showed considerable variability in DTH responses and were therefore not significantly different from their WT controls (Fig 4.7a). However, when IFNγR$^{-/-}$ mice were fed this low antigen dose prior to immunisation, their subsequent DTH responses were significantly reduced in comparison to controls (Fig 4.7a), indicating that endogenous IFNγ was not required for the inhibition of cell mediated responses *in vivo* by feeding either a high or low dose of OVA.

OVA-specific IgG responses were comparable in WT and IFNγR$^{-/-}$ control mice after immunisation (Fig 4.7b). As with DTH responses, feeding 25mg OVA to either WT or IFNγR$^{-/-}$ mice before immunisation significantly inhibited IgG responses (Fig 4.7b). Furthermore, a 2mg dose of fed OVA produced significant IgG tolerance in IFNγR$^{-/-}$, but not WT, mice (Fig 4.9b), indicating that endogenous IFNγ was not required for the inhibition of humoral immune responses *in vivo* by feeding OVA.

Although OVA-specific IgG1 responses were significantly lower in saline fed IFNγR$^{-/-}$ mice compared with WT controls after immunisation, the responses of both groups were significantly reduced by feeding either 2 or 25mg OVA before immunisation (Fig 4.8a). OVA-specific levels of IgG2a were comparable in saline fed IFNγR$^{-/-}$ mice and WT controls and again were significantly reduced by feeding OVA before immunisation (Fig 4.8b). Therefore, a lack of responsiveness to IFNγ does not preclude the normal induction of oral tolerance *in vivo* .

In vitro Responses

In contrast to CMI *in vivo,* the OVA-specific proliferative responses of control IFNγR$^{-/-}$ cells *in vitro* were significantly higher than those of WT controls (Fig 4.9), suggesting that the cytostatic effects of IFNγ may normally inhibit this response. However, feeding either 2 or 25mg OVA prior to immunisation of both WT and IFNγR$^{-/-}$ mice significantly reduced their specific proliferative responses (Fig 4.9), implying that IFNγ was not required for tolerance of proliferation *in vitro.*

OVA-specific production of IFNγ was virtually negligible in cultures of control IFNγR$^{-/-}$ cells, whereas OVA-specific IL5 was produced in levels comparable with WT controls and OVA-specific IL3 and IL10 were significantly enhanced beyond control levels (Fig 4.12). Feeding either 2 or 25mg OVA prior to immunisation of both WT and IFNγR$^{-/-}$ mice significantly reduced every specific cytokine response examined, indicating that IFNγ was not required for this tolerance *in vitro.*

Taken together with the normal oral tolerance induced in mice depleted of IFNγ, my findings show that oral tolerance is induced normally both *in vivo* and *in vitro* in the absence of endogenous IFNγ.

(C) The Role of TGFβ in Oral Tolerance

As the experiments described so far in this chapter appeared to exclude a role for conventional cytokine mediators produced by either Th1 or Th2 cells, I went on to examine the possibility that an alternative cytokine,TGFβ, might be important. Abundant in the normal intestine [299, 320], TGFβ is immunosuppressive in many situations [299] and has recently been implicated as a mediator of oral tolerance in rodents [282, 285]. Moreover, others in this lab have detected TGFβ *in vitro* early in the response to fed OVA [321].

(i) Effects of Neutralising TGFβ *in vivo*

I first examined the role of endogenous TGFβ in the induction of oral tolerance by administering anti-TGFβ antibody to animals around the time of feeding saline, 2 or 25mg OVA. Although I did not address the effectiveness of this treatment in removing endogenous TGFβ, the antibody used and the dose chosen were identical to other published work where TGFβ was neutralised *in vivo* [322].

In vivo Responses

When saline fed control animals were treated with anti-TGFβ, their subsequent DTH responses after immunisation were raised significantly above those of controls given isotype-matched antibody (Fig 4.11a), confirming that anti-TGFβ was having some biological effect *in vivo*. However, anti-TGFβ could not prevent the inhibition of DTH responses which occurred in mice fed either 2 or 25mg OVA before immunisation (Fig 4.11a), indicating that depletion of TGFβ did not prevent tolerance of CMI *in vivo*.

The serum levels of OVA-specific total IgG were comparable in control animals treated with either isotype-matched control antibody or anti-TGFβ (Fig 4.11b). However, only those mice given the isotype-matched control antibody and fed either 2 or 25mg OVA showed a significant reduction in specific IgG antibodies compared with their controls (Fig 4.11b). Although the IgG levels in OVA fed mice given anti-TGFβ were generally slightly

lower than those of anti-TGFβ treated controls, this difference was not significant, perhaps due to the small numbers of mice which had to be used because of the limited supply of anti-TGFβ. Alternatively, the possibility remains that TGFβ is required for oral tolerance of humoral, but not CMI responses.

In vitro Responses

The OVA-specific proliferative responses of PLN cells from mice given isotype-matched antibody showed the expected reductions when animals were fed either 2 or 25mg OVA before immunisation (Fig 4.12). The OVA-specific proliferative responses of all groups treated with anti-TGFβ were very low in comparison (Fig 4.12), but still significantly higher than observed in medium alone, where proliferation was virtually negligible (results not shown). In addition, the OVA-specific responses of anti-TGFβ-treated mice fed 25mg OVA were significantly higher than those of animals fed the same dose of antigen and given isotype antibody (Fig 4.12), suggesting that this aspect of oral tolerance may be modulated by depletion of TGFβ. However, low levels of response make it difficult to interpret.

In contrast, the production of OVA-specific IL5, IL10 and IFNγ by cells from saline fed mice given anti-TGFβ was comparable with those of controls receiving the isotype-matched antibody (Fig 4.13). In addition, cytokine secretion by cells from mice fed either 2 or 25mg OVA was significantly inhibited compared with immunised controls, regardless of whether the animals had received anti-TGFβ or isotype antibody (Fig 4.13). Every cytokine response was negligible for all groups cultured in medium alone. Therefore, endogenous TGFβ was not required for tolerance of cytokine secretion *in vitro* .

Overall, my findings suggest that TGFβ is not required for the induction of most aspects of oral tolerance, although its role in tolerance of antibody and proliferative responses remain unclear. Unfortunately, I could not repeat this study *in vivo* due to the limited supply of antibody.

(ii) The Role of TGFβ in the Expression of Oral Tolerance *in vitro*

Most studies which have indicated a role for TGFβ in oral tolerance examined the effector phases when tolerance has been induced and recalled [274]. As it is therefore conceivable that TGFβ might only be required to maintain immune suppression, I explored the effect of neutralising TGFβ during culture of tolerised PLN cells *in vitro*. Again, I chose a dose of neutralising antibody above that which had been used in other published work to deplete TGFβ actively *in vitro* [323].

In this experiment, the OVA-specific proliferation of all groups of cells cultured in the presence of isotype control antibody was very low (Fig 4.14), preventing detection of tolerance in cells from OVA fed mice. Although the reasons for this remain unclear, OVA-specific proliferative responses were observed in the presence of anti-TGFβ antibody and these were significantly reduced in cells from mice fed either 2 or 25mg OVA (Fig 4.14). All groups made low proliferative responses when cultured in the absence of OVA, although this response was somewhat enhanced by the addition of anti-TGFβ (results not shown). Thus TGFβ appears not to be required for expression of this aspect of oral tolerance.

The production of OVA-specific IL3, IL5 and IL10 by cells from saline fed mice was observed in the presence of isotype antibody and augmented by addition of anti-TGFβ to the cultures (Fig 4.15). The secretion of most of these cytokines by cells from mice fed either 2 or 25mg OVA was significantly inhibited compared with immunised controls, regardless of whether or not anti-TGFβ was present in the cultures (Fig 4.15). One exception to this pattern was that very little IFNγ was produced by any cells cultured in the presence of isotype control antibody (Fig 4.15), paralleling the defective proliferative responses under the same conditions. In contrast, the addition of anti-TGFβ to cells from saline fed mice increased their IFNγ production above the level of detection by ELISA (Fig 4.15), making it difficult to discern the effects of feeding OVA. Only cells from mice fed 2mg OVA appeared to produce less IFNγ than controls in the presence of anti-TGFβ (Fig 4.15). Insufficient

supernatants remained to investigate if this was a problem with the ELISA or not. All cytokine responses were negligible in medium alone, even in the presence of anti-TGFβ.

Overall, the results above do not support a critical role for TGFβ in the expression of oral tolerance *in vitro*.

Summary and Conclusions

The results presented in this chapter show that mice fed 2 or 25mg OVA in the absence of IL4, IL10, TGFβ or IFNγ develop oral tolerance normally, indicating that these cytokines are not required for the induction of unresponsiveness. In addition, I found no evidence that they are involved in maintaining unresponsiveness in tolerised mice, as oral tolerance was expressed normally in $IL4^{-/-}$ mice, $IFN\gamma R^{-/-}$ mice and in tolerant cell cultures treated with anti-TGFβ.

$IL4^{-/-}$ mice displayed normal oral tolerance of specific DTH and IgG levels *in vivo* and of proliferative, IL3, IL5 and IFNγ responses *in vitro* despite their anticipated defects in IL4 and IL10 production [306]. These findings show that oral tolerance can be induced and maintained in the absence of IL4 or IL10, and that this applies to feeding either a high or low dose of antigen.

In addition, no role was found for endogenous IFNγ in either the induction or maintenance of oral tolerance, as normal immune suppression of effector responses occurred in animals depleted of IFNγ at the time of feeding as well as in $IFN\gamma R^{-/-}$ mice.

Mice given neutralising anti-TGFβ antibody also developed specific tolerance of DTH responses *in vivo* and IL5, IL10 and IFNγ responses *in vitro* after feeding 2 or 25mg OVA. Furthermore, neutralisation of TGFβ *in vitro* did not reverse the inhibition of proliferation and secretion of IL3, IL5 or IL10 seen in cells from orally tolerised animals. However, some exceptions to these findings occurred, with apparently no tolerance of IgG or proliferation in anti-TGFβ treated mice and with variable effects on the *in vivo* and *in*

vitro inhibition of IFNγ. Therefore, TGFβ remains a potential mediator of some aspects of oral tolerance and this possibility requires further investigation.

The results of this chapter do not support other reports that tolerance induced in mice by feeding low doses of antigen is mediated entirely by suppressive cytokines. However, it remains possible that oral tolerance does reflect active regulation by an effector function other than cytokine secretion. Equally, oral tolerance could reflect direct inactivation of antigen responsive cells. Both theories will be addressed in subsequent chapters.

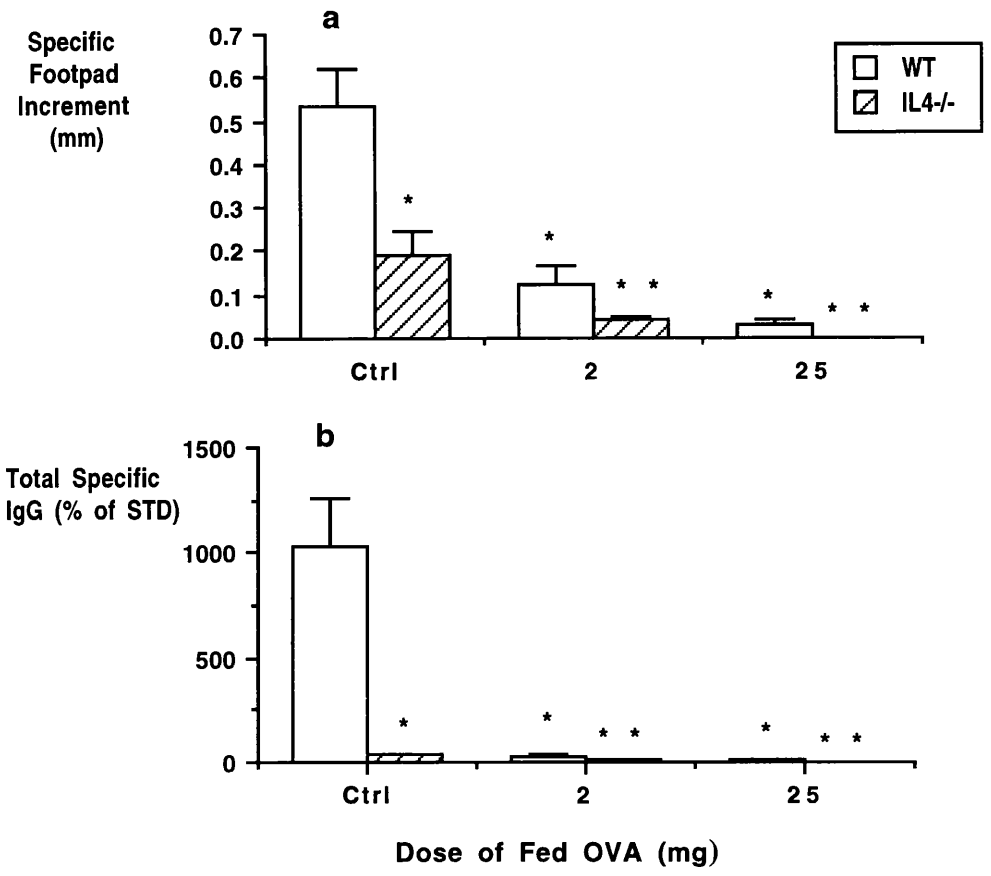

Figure 4.1 IL4$^{-/-}$ Mice Display Normal Oral Tolerance of Effector Responses *in vivo*.

a) OVA-specific DTH responses in C57Bl/6 (WT) and IL4$^{-/-}$ mice immunised 21 days before with OVA/CFA s.c. The results shown are mean specific increments in footpad thickness ± 1 SEM for 6 mice per group in animals fed saline (Ctrl), 2 or 25mg OVA 10 days prior to immunisation. (*$p<0.05$ versus WT Ctrl; **$p<0.05$ versus IL4$^{-/-}$ Ctrl). b) Total OVA-specific serum IgG responses of C57Bl/6 (WT) and IL4$^{-/-}$ mice 21 days after immunisation with OVA/CFA. Results shown are % hyperimmunised control serum and are the means ± 1 SEM for individual sera from 6 mice per group in mice fed saline (Ctrl), 2 or 25mg OVA 10 days prior to immunisation. (*$p<0.05$ versus WT Ctrl; **$p<0.05$ versus IL4$^{-/-}$ Ctrl). Similar results were obtained in 2 other experiments examining 2mg and 25mg fed OVA individually.

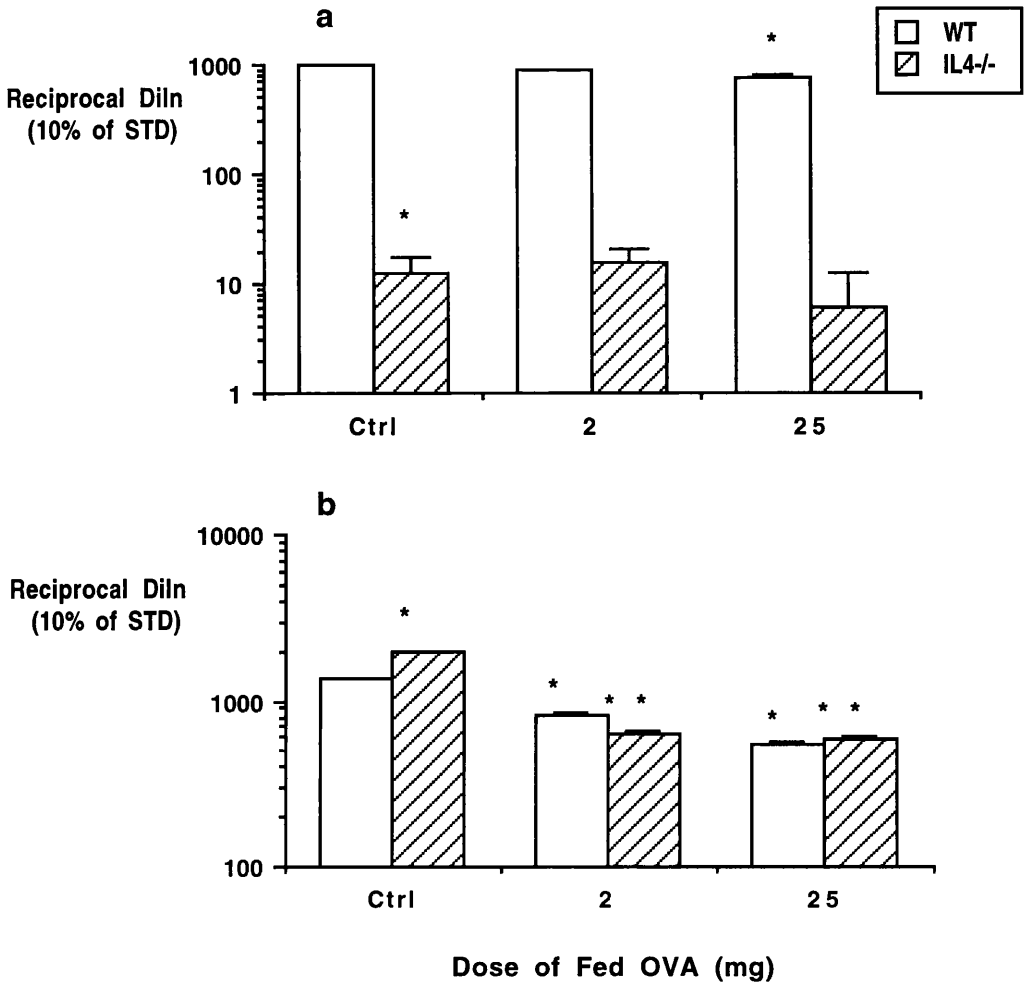

Figure 4.2 IL4$^{-/-}$ Mice Display Normal Oral Tolerance of Serum IgG Isotypes.

Primary OVA-specific IgG1 a) and IgG2a b) responses in C57Bl/6 (WT) or IL4$^{-/-}$ mice 21 days after immunisation with OVA/CFA. The results shown are reciprocal dilutions giving an OD value equivalent to 10% of hyperimmunised control serum and are the means ± 1 SEM for individual sera from 6 mice per group in animals fed saline (Ctrl), 2 or 25mg OVA 10 days prior to immunisation. (*$p<0.05$ versus WT Ctrl; **$p<0.05$ versus IL4$^{-/-}$ Ctrl). Similar results were obtained in 2 other experiments examining 2mg and 25mg fed OVA individually.

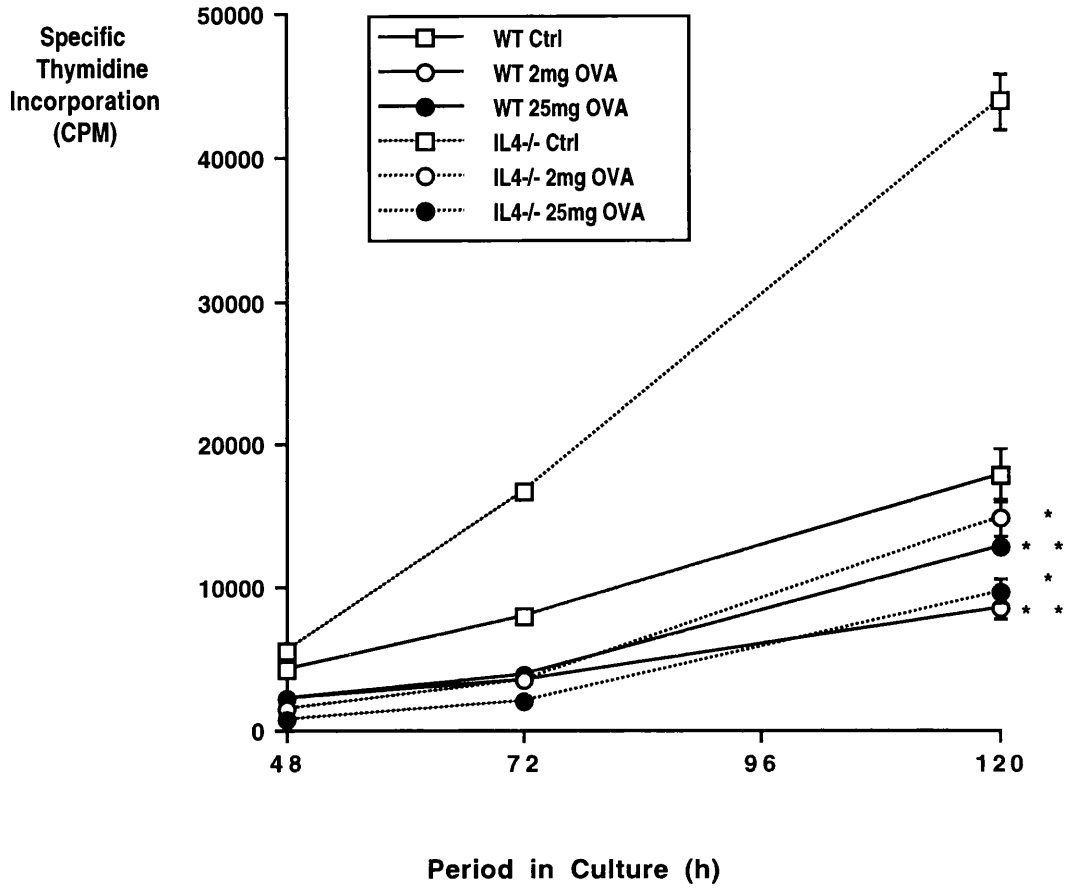

Figure 4.3 IL4$^{-/-}$ Mice Display Normal Oral Tolerance of Proliferative Responses *in vitro*.

Proliferative responses of PLN cells removed 14 days after s.c. immunisation of C57Bl/6 (WT) or IL4$^{-/-}$ mice with OVA/CFA and cultured for 120h with OVA (1mg/ml). Results shown are mean ^{3}H-TdR incorporation (CPM) ± 1 SEM in quadruplicate cultures of cells pooled from 5 animals per group in saline (Ctrl), 2 or 25mg OVA fed mice. (*$p<0.05$ versus WT Ctrl; **$p<0.05$ versus IL4$^{-/-}$ Ctrl). Similar results were obtained in 2 other experiments examining 2mg and 25mg fed OVA individually.

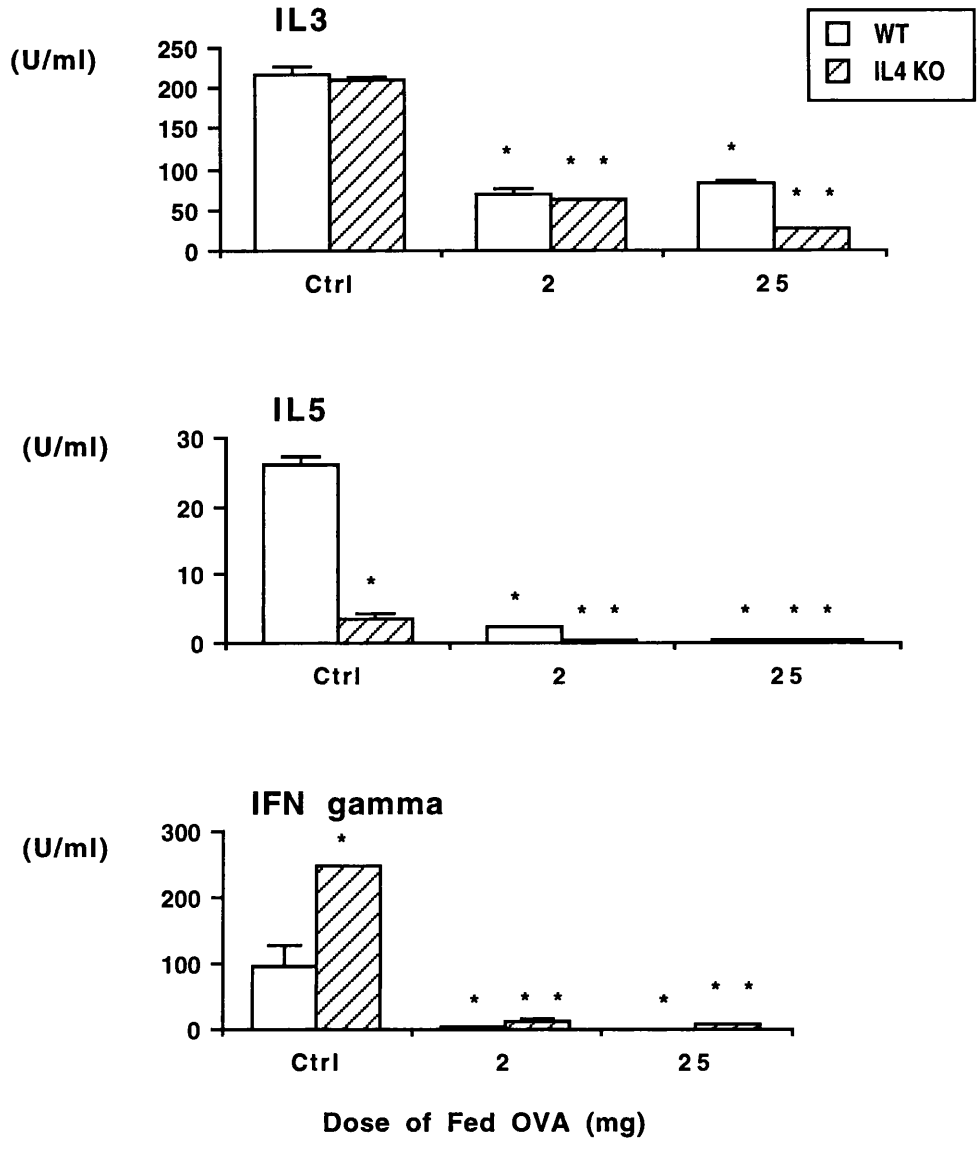

Figure 4.4 IL4$^{-/-}$ Mice Display Normal Oral Tolerance of Specific Cytokines *in vitro*.

OVA-specific production of IL3, IFNγ and IL5 by PLN cells removed 14 days after s.c. immunisation of C57Bl/6 (WT) or IL4$^{-/-}$ mice with OVA/CFA and restimulated for 72h with OVA (1mg/ml). Results shown are mean cytokine levels (U/ml) ± 1 SEM in cultures of cells pooled from 5 mice per group in saline (Ctrl), 2 or 25mg OVA fed mice. (*$p<0.05$ versus WT Ctrl; **$p<0.05$ versus IL4$^{-/-}$ Ctrl). Similar results were obtained in 2 other experiments examining 2mg and 25mg fed OVA individually.

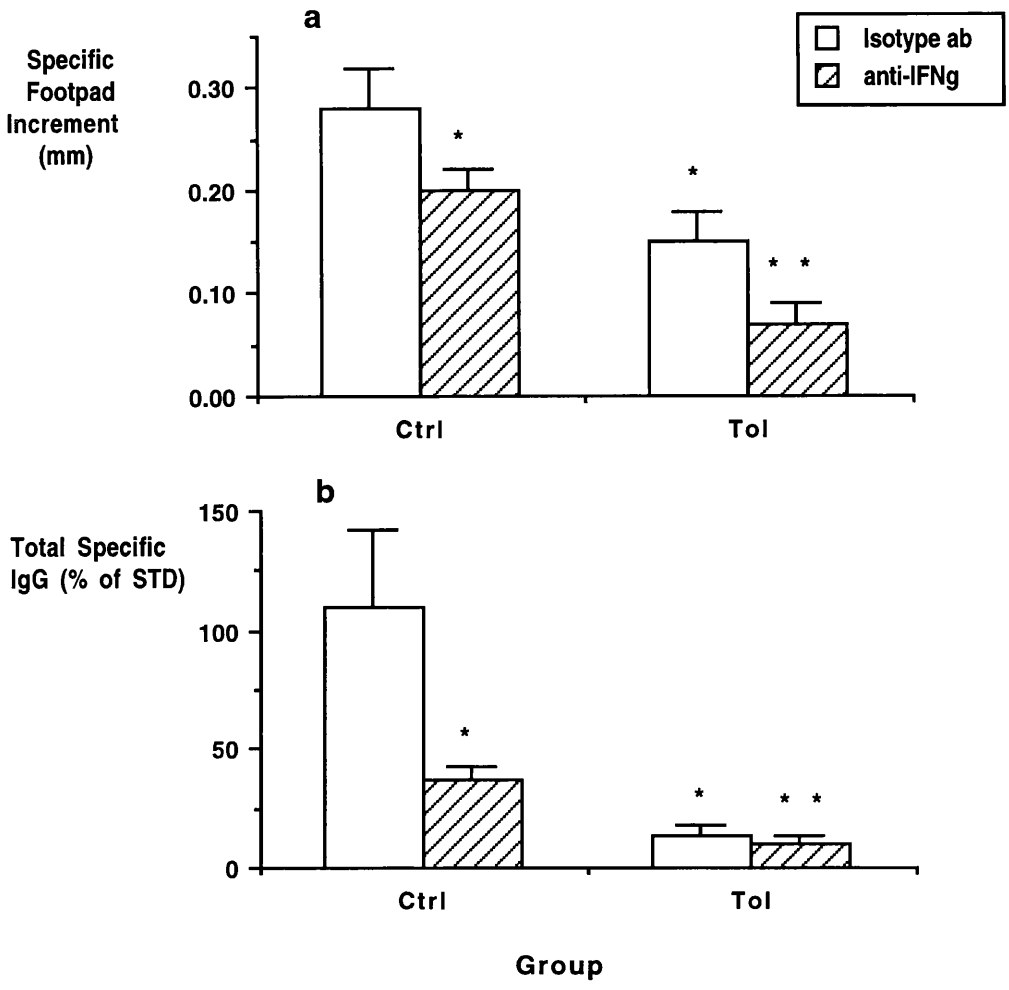

Figure 4.5 Effects of Neutralising IFNγ *in vivo* on the Induction of Oral Tolerance.

a) OVA-specific DTH responses in BALB/c mice immunised 21 days before with OVA/CFA s.c. Results shown are mean specific increments in footpad thickness ± 1 SEM for 6 mice per group in animals fed saline (Ctrl) or 25mg OVA (Tol) 10 days prior to immunisation and treated with 0.5mg either of anti-IFNγ or an isotype-matched control antibody 2 days before and 2 days after feeding. b) Total OVA-specific serum IgG responses 21 days after immunisation with OVA/CFA. Results shown are % hyperimmunised control serum and are the means ± 1 SEM for individual sera from 6 mice per group. (*$p<0.05$ versus Ctrl given isotype ab; **$p<0.05$ versus Ctrl given anti-IFNγ).

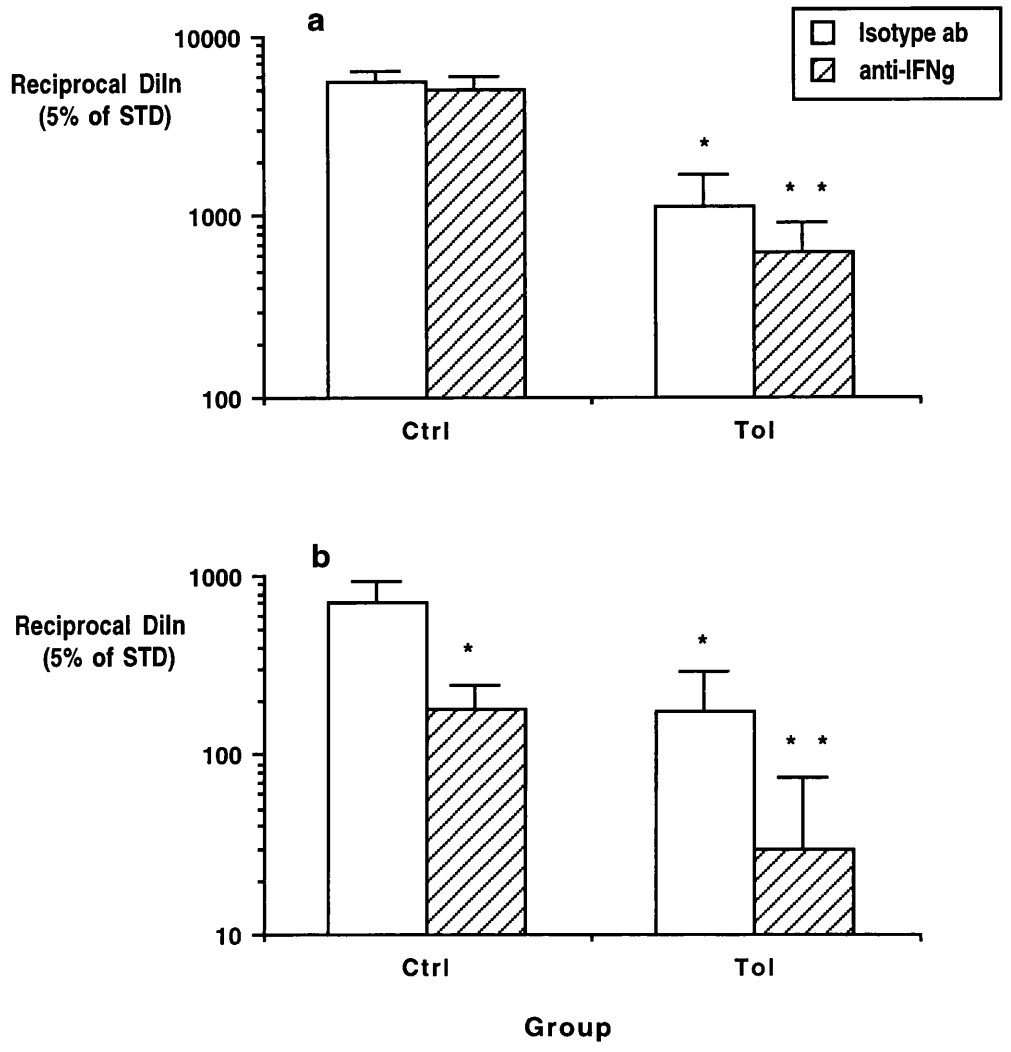

Figure 4.6 Effects of Neutralising IFNγ *in vivo* on Oral Tolerance of Serum IgG Isotypes.

Primary OVA-specific IgG1 a) and IgG2a b) responses 21 days after immunisation with OVA/CFA. The results shown are reciprocal dilutions giving an OD value equivalent to 5% of hyperimmunised control serum and are the means ± 1 SEM for individual sera from 6 mice per group in animals fed saline (Ctrl) or 25mg OVA (Tol) 10 days prior to immunisation and treated with 0.5mg either of anti-IFNγ or an isotype-matched control antibody 2 days before and 2 days after feeding. (*$p<0.05$ versus Ctrl given isotype ab; **$p<0.05$ versus Ctrl given anti-IFNγ).

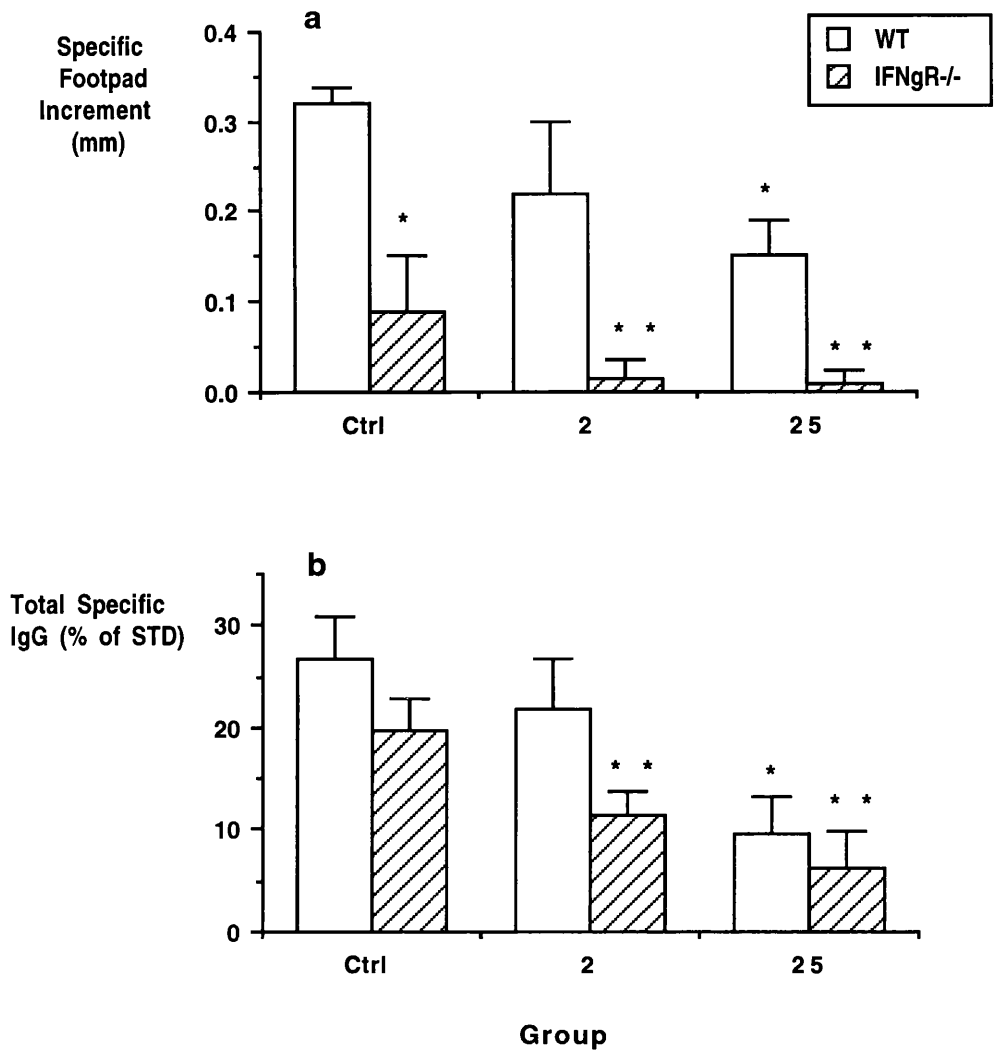

Figure 4.7 IFNγR$^{-/-}$ Mice Display Normal Oral Tolerance of Effector Responses *in vivo*.

a) OVA-specific DTH responses in IFNγR$^{-/-}$ and C57BL/6 (WT) mice immunised s.c. 21 days before with OVA/CFA. Results shown are mean specific increments in footpad thickness ± 1 SEM for 6 mice per group in animals fed saline (Ctrl), 2 or 25mg OVA 10 days prior to immunisation. (*$p<0.05$ versus WT Ctrl; **$p<0.05$ versus IFNγR$^{-/-}$ Ctrl). b) Total OVA-specific serum IgG responses 21 days after immunisation with OVA/CFA. Results shown are % hyperimmunised control serum and are the means ± 1 SEM for individual sera from 6 mice per group. (*$p<0.05$ versus WT Ctrl; **$p<0.05$ versus IFNγR$^{-/-}$ Ctrl). Similar results were obtained in a replicate experiment.

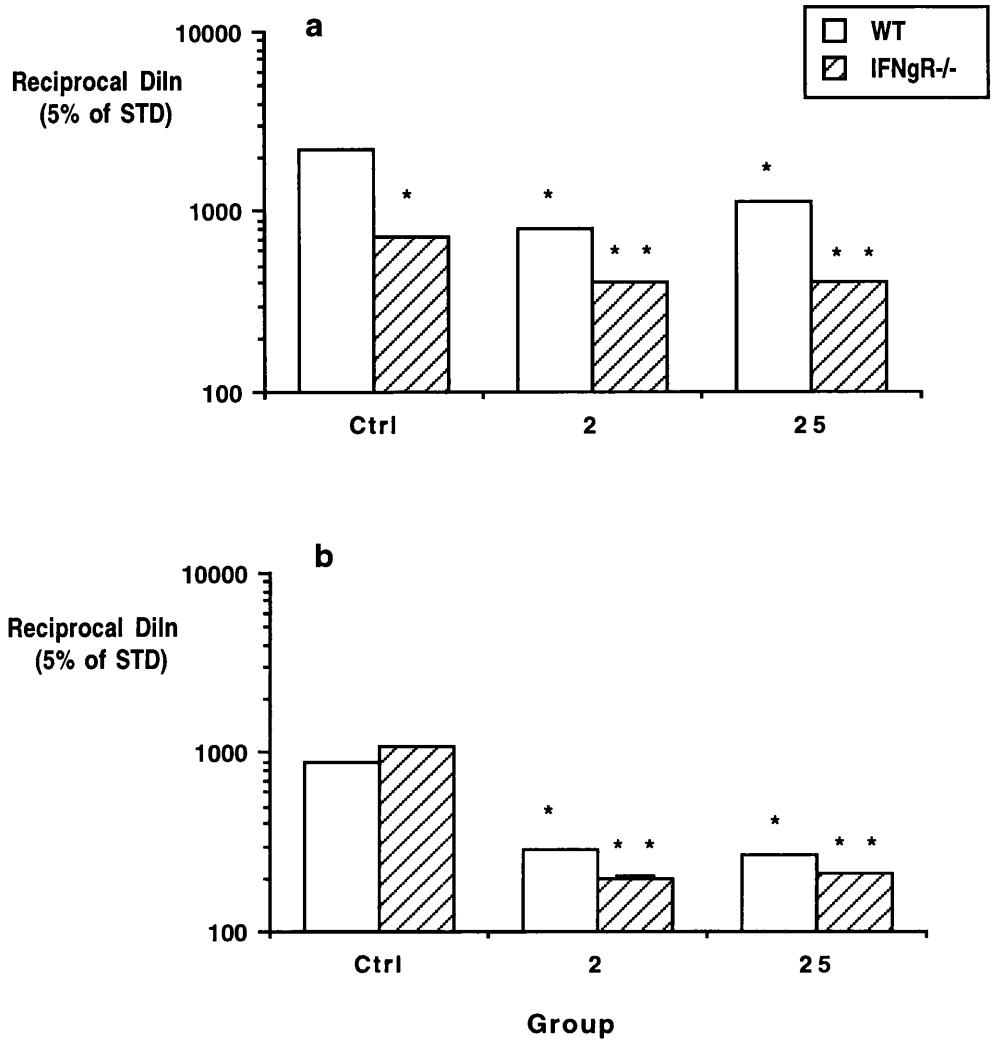

Figure 4.8 IFNγR$^{-/-}$ Mice Display Normal Oral Tolerance of Specific Serum IgG Isotypes.

Primary OVA-specific IgG1 a) and IgG2a b) responses in C57Bl/6 (WT) or IFNγR$^{-/-}$ mice 21 days after immunisation with OVA/CFA. The results shown are reciprocal dilutions giving an OD value equivalent to 5% of hyperimmunised control serum and are the means ± 1 SEM for individual sera from 6 mice per group in animals fed saline (Ctrl), 2 or 25mg OVA 10 days prior to immunisation. (*$p<0.05$ versus WT Ctrl; **$p<0.05$ versus IFNγR$^{-/-}$ Ctrl). Similar results were obtained in a replicate experiment.

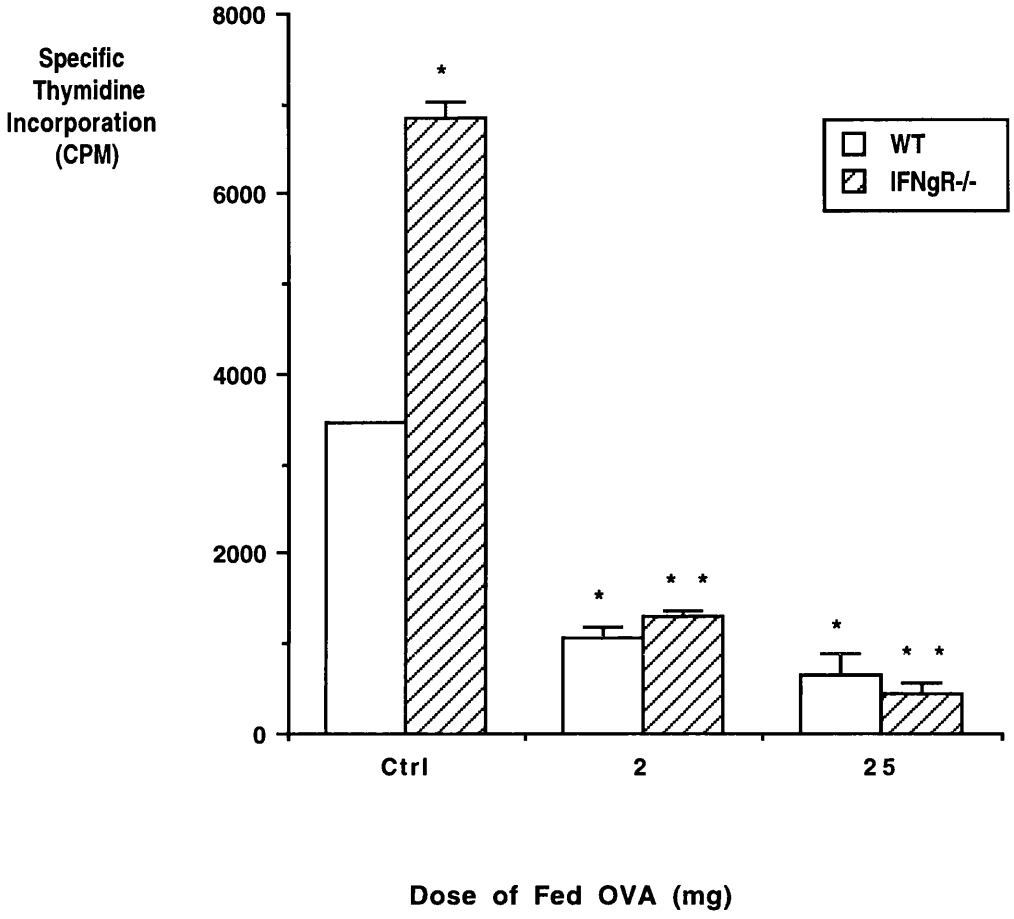

Figure 4.9 IFNγR$^{-/-}$ Mice Display Normal Oral Tolerance of Specific Proliferative Responses *in vitro*.

OVA-specific proliferative responses of PLN cells removed 14 days after s.c. immunisation of C57Bl/6 (WT) or IFNγR$^{-/-}$ mice with OVA/CFA and cultured over 120h with OVA (1mg/ml). Results shown are mean ^{3}H-TdR incorporation (CPM) ± 1 SEM in quadruplicate cultures of cells pooled from 5 animals per group in saline (Ctrl), 2 or 25mg OVA fed mice. (*$p<0.05$ versus WT Ctrl cells; **$p<0.05$ versus IFNγR$^{-/-}$ Ctrl cells). Similar results were obtained in a replicate experiment.

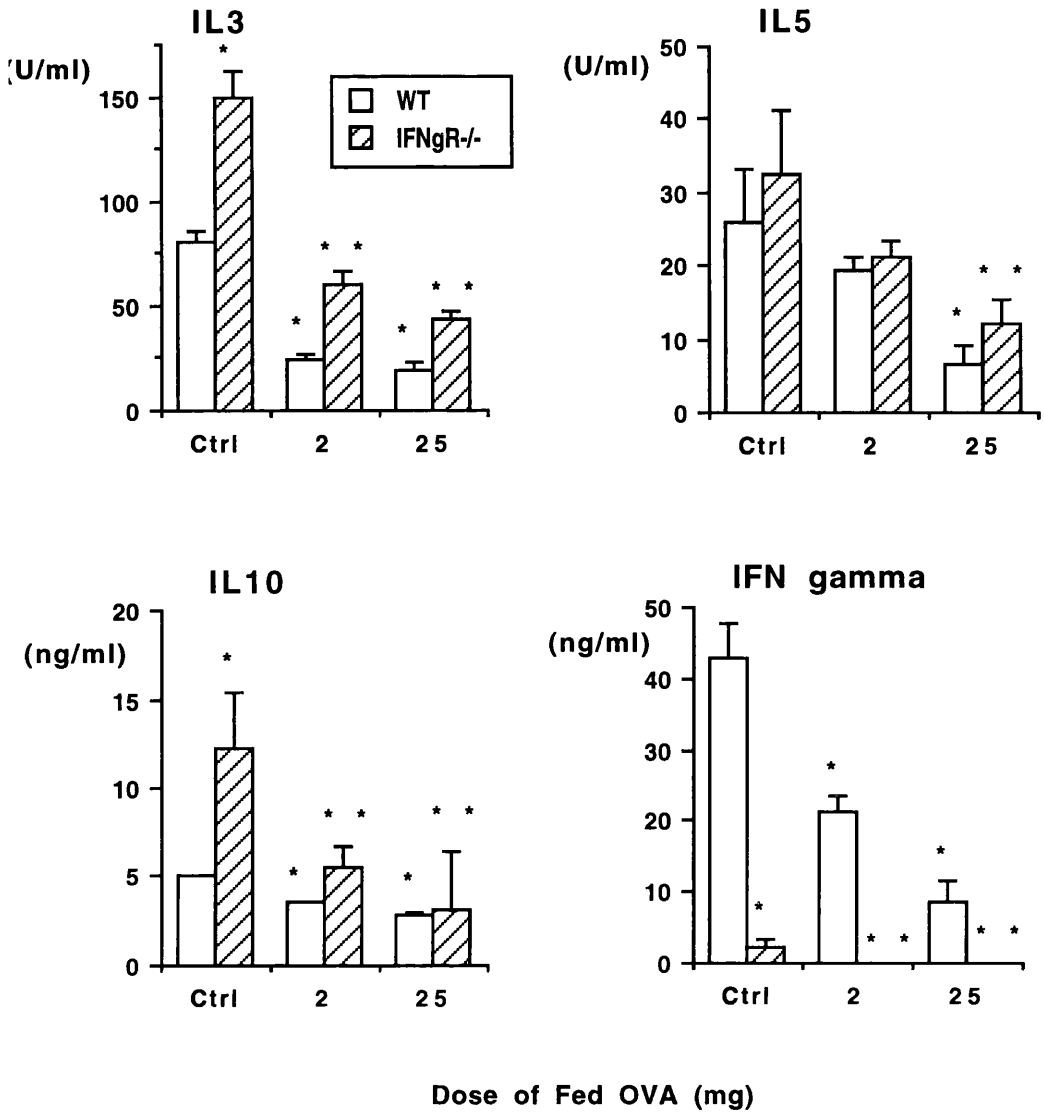

Figure 4.10 IFNγR$^{-/-}$ Mice Display Normal Oral Tolerance of Specific Cytokine Production *in vitro*.

OVA-specific production of IL3, IL5, IL10 and IFNγ by PLN cells removed 14 days after s.c. immunisation of C57BL/6 (WT) or IFNγ$^{-/-}$ mice with OVA/CFA and restimulated for 72h with 1mg/ml OVA. Results shown are mean cytokine levels (U/ml or ng/ml) ± 1 SEM in cultures of cells pooled from 5 mice per group in saline (Ctrl), 2 or 25mg OVA fed mice. (*$p<0.05$ versus WT Ctrl; **$p<0.05$ versus IFNγ$^{-/-}$ Ctrl). Similar results were obtained in a replicate experiment.

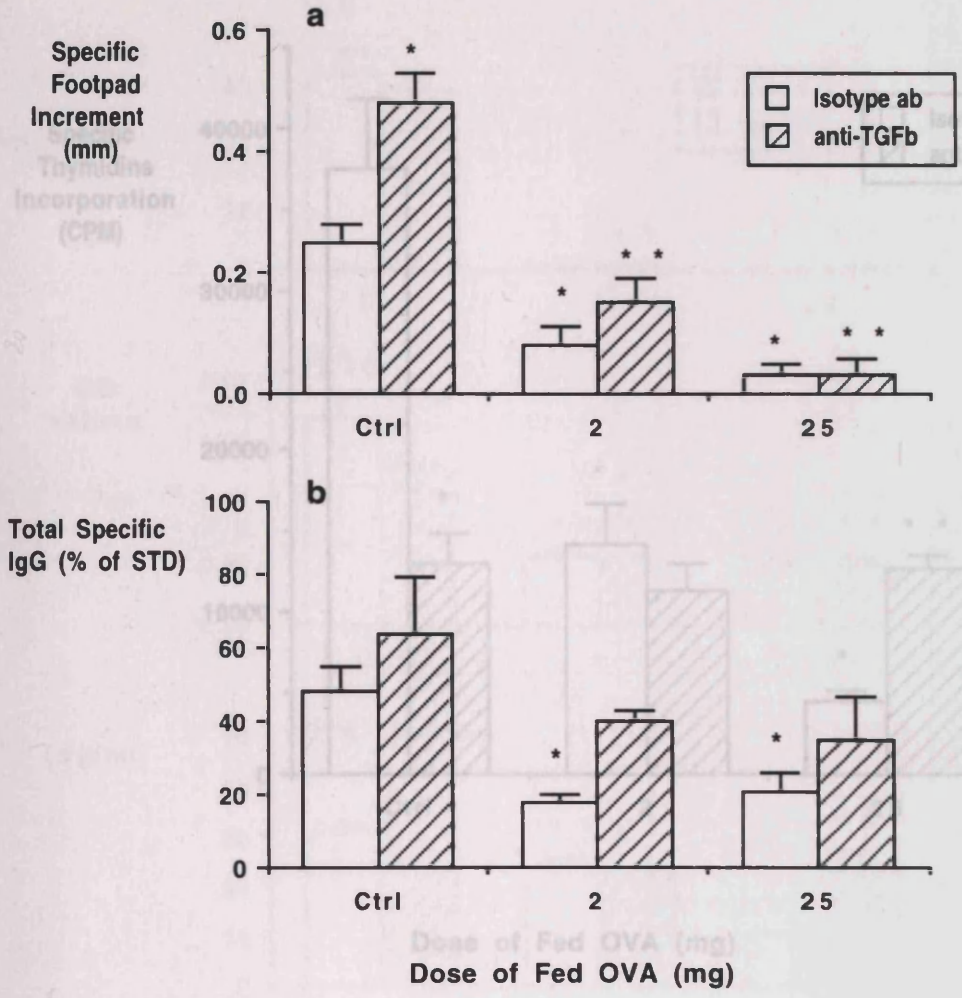

Figure 4.11 Neutralisation of TGFβ *in vivo* May Not Prevent the Induction of Oral Tolerance.

a) OVA-specific DTH responses in BALB/c mice immunised 21 days before with OVA/CFA s.c. Results shown are mean specific increments in footpad thickness ± 1 SEM for 5 mice per group in animals fed saline (Ctrl), 2 or 25mg OVA 10 days prior to immunisation and treated with 0.5mg of either anti-TGFβ or an isotype-matched control antibody 2 days before and 2 days after feeding. (*$p<0.05$ versus Ctrl given isotype ab; **$p<0.05$ versus Ctrl given anti-TGFβ). b) Total OVA-specific serum IgG responses 21 days after immunisation with OVA/CFA. Results shown are % hyperimmunised control serum and are the means ± 1 SEM for individual sera from 4 or 5 mice per group. (*$p<0.05$ versus Ctrl given isotype ab; **$p<0.05$ versus Ctrl given anti-TGFβ). This experiment was not repeated.

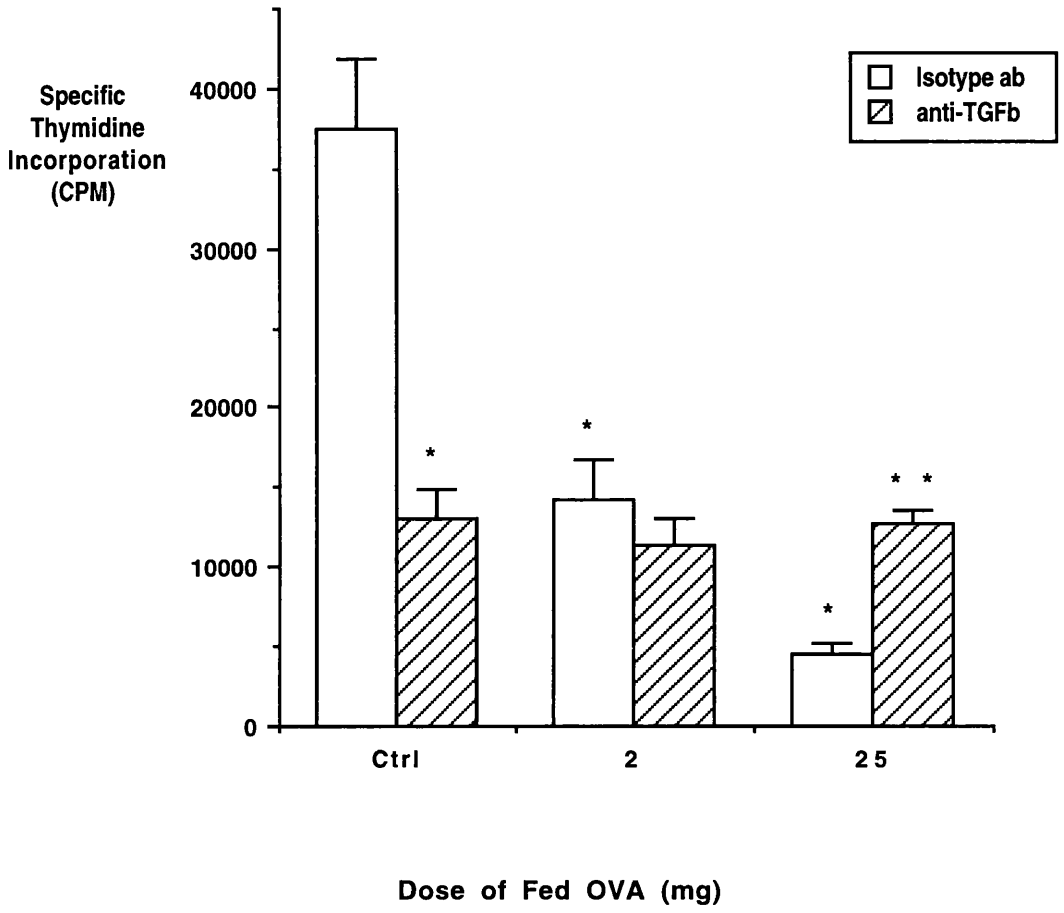

Figure 4.12 Neutralisation of TGFβ *in vivo* May not Prevent Oral Tolerance of Proliferative Responses *in vitro*.

OVA-specific proliferative responses of PLN cells removed 14 days after s.c. immunisation of mice with OVA/CFA and cultured for 96h with 1mg/ml OVA. Results shown are mean ^{3}H-TdR incorporation (CPM) ± 1 SEM in quadruplicate cultures of cells pooled from 5 animals per group in saline (Ctrl), 2 or 25mg OVA fed mice treated with either anti-TGFβ or an isotype-matched control antibody 2 days before and 2 days after feeding. (*$p<0.05$ versus Ctrl cells given isotype ab, **$p<0.05$ versus 25mg fed OVA+ isotype ab group).

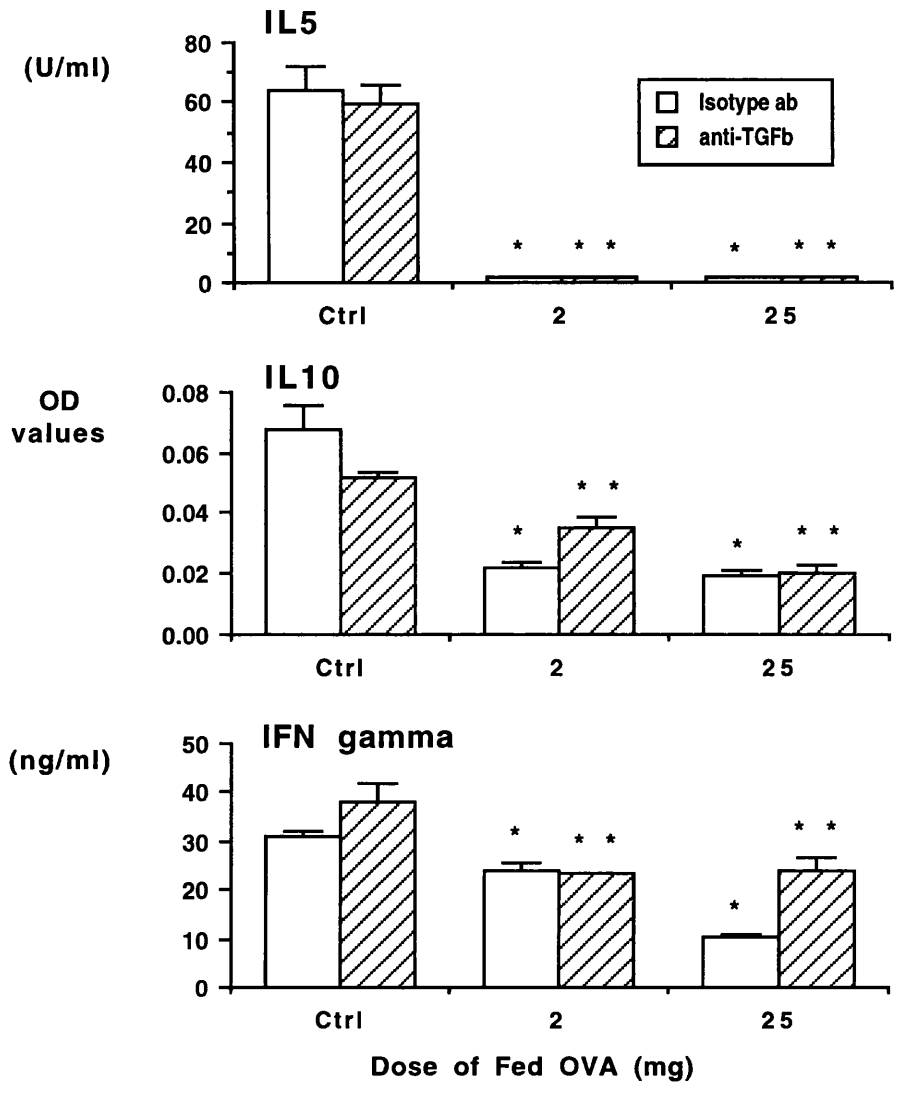

Figure 4.13 Neutralisation of TGFβ *in vivo* Does not Prevent Oral Tolerance of Cytokine Production *in vitro*.

OVA-specific production of IL5, IL10 and IFNγ by PLN cells removed 14 days after s.c. immunisation of mice with OVA/CFA and restimulated for 72h with 1mg/ml OVA. Results shown are mean cytokine levels (U/ml, OD values or ng/ml) ± 1 SEM in cultures of cells pooled from 5 mice per group in saline (Ctrl), 2 or 25mg OVA fed mice treated with either anti-TGFβ or isotype-matched control antibody 2 days before and 2 days after feeding. (*$p<0.05$ versus Ctrl cells given isotype ab; **$p<0.05$ versus Ctrl cells given anti-TGFβ).

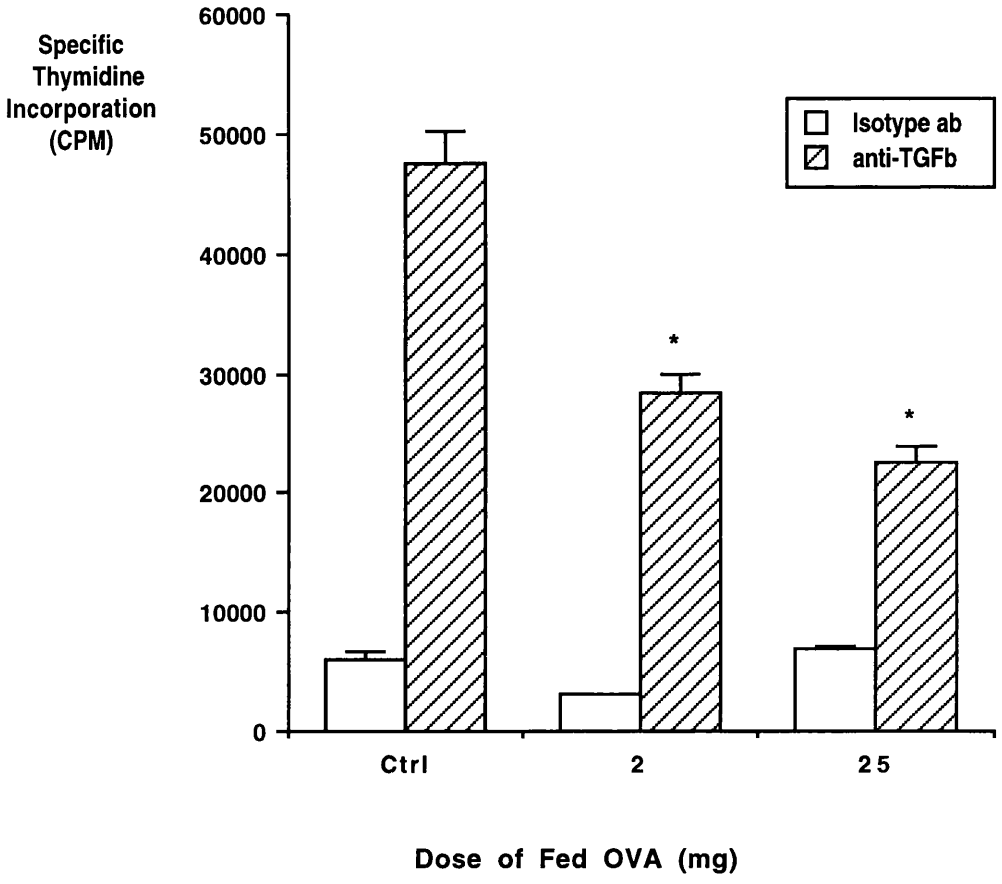

Figure 4.14 Effects of Neutralising TGFβ *in vitro* on Oral Tolerance of Proliferative Responses.

OVA-specific proliferative responses of PLN cells removed 14 days after s.c. immunisation of mice with OVA/CFA and cultured for 96h with 1mg/ml OVA ± anti-TGFβ or isotype-matched control antibody (both at 50μg/ml). Results shown are mean ^{3}H-TdR incorporation (CPM) ± 1 SEM in quadruplicate cultures of cells pooled from 5 animals per group in saline (Ctrl), 2 or 25mg OVA fed mice. (*$p<0.05$ versus Ctrl cells given anti-TGFβ).

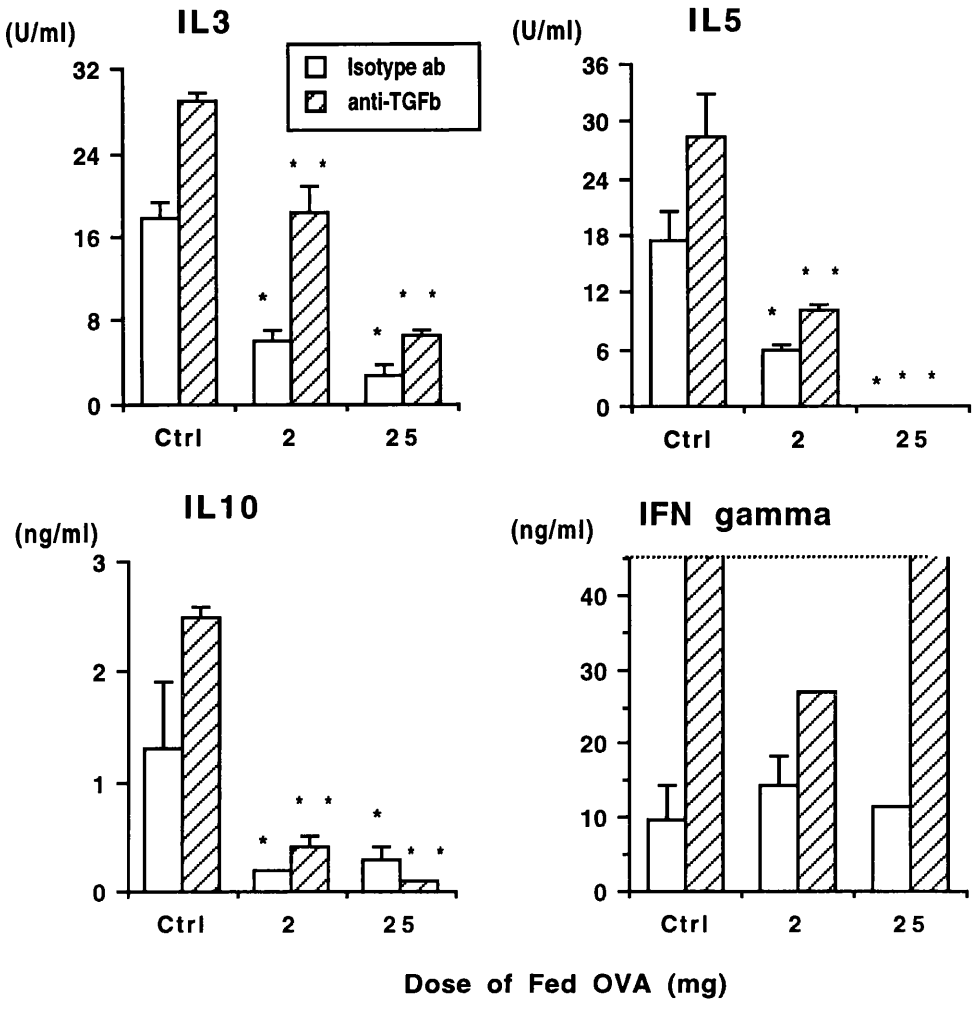

Figure 4.15 Effects of Neutralising TGFβ *in vitro* on Oral Tolerance of Cytokine Production.

OVA-specific production of IL3, IL5, IL10 and IFNγ by PLN cells removed 14 days after s.c. immunisation of mice with OVA/CFA and restimulated for 72h with 1mg/ml OVA ± anti-TGFβ or isotype-matched control antibody (both at 50μg/ml). Results shown are mean cytokine levels (U/ml or ng/ml) ± 1 SEM in cultures of cells pooled from 5 mice per group in saline (Ctrl), 2 or 25mg OVA fed mice. The dotted line respresents upper detection limit for ELISA. (*$p<0.05$ versus Ctrl cells given isotype ab; **$p<0.05$ versus Ctrl cells given anti-TGFβ).

Chapter 5: The Role of CD8$^+$ T Cells in Oral Tolerance

Introduction

The findings presented in the previous chapters did not support a role for regulatory CD4$^+$ T cell subsets in the peripheral tolerance induced by feeding a high or low dose of antigen. However, this work does not preclude the possibility that oral antigen could induce active suppression mediated by other regulatory T cells, such as those expressing CD8 coreceptor. This idea would be compatible with early studies of oral tolerance, which demonstrated a suppressive mechanism transferrable to naive recipients with CD8$^+$ T cells [93, 97, 109, 324]. Although the presence of functional CD8$^+$ suppressor T (T_S) cells remains controversial, it is now apparent that CD8$^+$ T cells can play a regulatory role in a number of immune responses, either through classical cytotoxic effects on APC [275] or via the production of suppressive cytokines such as IFNγ [128] and TGFβ [274]. Furthermore, recent studies of oral tolerance have also suggested that CD8$^+$ T cells can transfer suppression [117].

In an attempt to clarify this issue, I examined the regulatory function of CD8$^+$ T cells in our model of oral tolerance to OVA by measuring class I MHC-restricted T cell activity *in vitro* and assessing the induction of oral tolerance in CD4- or CD8-depleted mice. I also determined how this was influenced by the dose of fed antigen, as it has been suggested that only low dose regimes stimulate active suppressor mechanisms.

Experimental Protocol

Tolerance was induced by feeding mice 2 or 25mg OVA and systemic immune responses were assessed after parenteral challenge with 100μg of either OVA ISCOMS i.p or OVA/CFA s.c.. To detect class I MHC-restricted CTL activity, spleen cells from immunised C57Bl/6 mice were restimulated for 5 days *in vitro* with EG7.OVA cells before analysis of cytotoxicity using ^{51}Cr labelled EG7.OVA or EL4 cells.

To deplete $CD4^+$ and $CD8^+$ T cells, BALB/c mice were given 0.5mg anti-CD4/8 monoclonal antibody 4 days prior to and on the day of feeding OVA. Controls received rat IgG or serum.

Results

(A) Effects of Fed OVA on Subsequent Immune Responses Generated by OVA ISCOMS

To determine if class I MHC-restricted cytotoxicity was important in regulating oral tolerance, I first examined how conventional $CD8^+$ CTL responses were influenced by feeding tolerogenic doses of OVA. To generate OVA-specific $CD8^+$ T cells *in vivo*, I took advantage of an immunisation model developed in the lab using OVA incorporated into ISCOMS containing the adjuvant Quil A [325].

DTH and Antibody Production *in vivo*

I first ensured that OVA-specific DTH and serum IgG responses generated in mice immunised with OVA ISCOMS could be reduced by prior feeding of OVA. This was found with both 2mg and 25mg fed OVA (Fig 5.1a&b), demonstrating normal oral tolerance under these immunisation conditions.

Systemic CTL Responses

Control mice immunised with OVA ISCOMS were also primed to develop high levels of splenic CTL activity after restimulation *in vitro* (Fig 5.2). This response was already known to be entirely due to $CD8^+$ T cells and mediated by classical class I MHC-restricted CTL [325]. In my hands, the cytotoxicity was OVA-specific since significant levels of killing were only found against EG7.OVA cells and not the parental EL4 cells, which do not express the OVA gene (Fig 5.2). Feeding either 2 or 25mg OVA prior to immunisation abolished the subsequent CTL response to OVA ISCOMS (Fig 5.2), indicating that this function was also suppressed by oral tolerance and that classical CTL were unlikely to mediate oral tolerance *in vivo*.

To deplete $CD4^+$ and $CD8^+$ T cells, BALB/c mice were given 0.5mg anti-CD4/8 monoclonal antibody 4 days prior to and on the day of feeding OVA. Controls received rat IgG or serum.

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(B) CD4-dependence of Orally Tolerised CTL Responses Generated by OVA ISCOMS

Although these results showed that feeding OVA could directly suppress $CD8^+$ T cell function, the CTL responses primed by ÓVA ISCOMS immunisation have been found to be dependent on the helper activity of $CD4^+$ T cells [326]. Thus the suppression of the CTL responses in OVA fed mice could reflect the profound inhibition of $CD4^+$ T cell responses documented in the previous chapters. In order to clarify this, I first examined whether the defective ability of tolerised spleen cells to generate CTL when restimulated *in vitro* could be overcome by addition of functional OVA-specific $CD4^+$ T cells. In the restimulation assay I therefore depleted $CD4^+$ or $CD8^+$ T cells from the immunised control or tolerant mice and recombined them before restimulation with EG7.OVA cells (see Table 5.1). FACS analysis confirmed the depletion of >95% of the appropriate cell population (Fig 5.3). However, on the two occasions when this experiment was performed, no OVA-specific cytotoxic response could be detected in any of the recombined cell populations or in the unseparated spleen cells from control mice (results not shown), suggesting that the batches of OVA ISCOMS used for immunisation had been ineffective in priming a CTL response *in vivo*. Consistent with this explanation, I was also unable to detect priming of OVA-specific DTH responses in animals immunised with the same batches of OVA ISCOMS (results not shown). Unfortunately, time restraints prevented me from repeating these experiments.

(C) Effects of Fed OVA on $CD8^+$ T Cell Responses Generated by OVA/CFA

As an alternative means of addressing the CD4-dependency of CTL tolerance, I attempted to induce OVA-specific CTL responses which did not require $CD4^+$ T cells *in vivo,* by exploiting a recent report that s.c. immunisation of mice with OVA/CFA generated CD4-independent $CD8^+$ CTL [327]. Consistent with these results, I found that C57Bl/6 mice immunised with OVA/CFA displayed high levels of OVA-specific CTL activity after restimulation with EG7.OVA *in vitro*. These CTL responses did not require $CD4^+$ T cells *in vivo*, as depletion of $CD8^+$, but not $CD4^+$ T cells around the time of immunisation abolished

subsequent CTL activity (Fig 5.4). However, previous work showed that the CTL effector cells themselves were $CD8^+$, class I MHC-restricted T cells that recognise $OVA_{257-264}$ and K^b [327].

Feeding either 2 or 25mg OVA had no efffect on the CTL responses primed by OVA/CFA (Fig 5.5a), despite the fact that the presence of oral tolerance was confirmed in the OVA fed animals by a lowered OVA-specific serum IgG response compared with immunised control mice (Fig 5.5b). Thus, neither a low nor a high dose of fed OVA could reduce CD4-independent CTL responses in mice immunised with OVA/CFA.

(D) Effects of Depleting $CD4^+$ or $CD8^+$ T Cells on the Induction of Oral Tolerance

As the above results indicated that fed OVA could not tolerise CD4-independent CTL responses, I next examined if the presence of $CD8^+$ T cells was required for oral tolerance induciton, by depleting them *in vivo* around the time of feeding. Additional mice were depleted of $CD4^+$ T cells to explore their requirement in the phenomenon.

These regimes depleted ≥95% $CD4^+$ or $CD8^+$ cells (Fig 5.6), but depleted mice were able to mount systemic DTH and IgG responses which were comparable to those of undepleted control mice (Fig 5.7a&b), indicating that functional levels of $CD4^+$ and $CD8^+$ T cells had recovered by the time systemic immune responses were assessed. As expected, these responses were significantly reduced in mice fed 25mg OVA prior to immunisation and equivalent suppression was observed in OVA fed mice depleted of $CD8^+$ cells (Fig 5.7a&b). In contrast, OVA fed mice depleted of $CD4^+$ cells showed no suppression of DTH and antibody responses, which were comparable to those of immunised control animals (Fig 5.7a&b).

Similar results were obtained in mice tolerised by feeding 2mg OVA, where the tolerance of IgG and DTH responses was unaffected by depleting $CD8^+$ T cells at the time of feeding (Fig 5.8a&b). Although DTH tolerance was reversed in mice depleted of $CD4^+$ T cells (Fig 5.8a), CD4-depleted mice had no antibody responses in this experiment (Fig

5.8b), suggesting that helper T cell function had not recovered by the time of immunisation and making interpretation of the results in tolerant mice impossible.

Thus, $CD4^+$, but not $CD8^+$ T cells are required for the induction of oral tolerance to OVA, irrespective of the feeding dose.

Summary and Conclusions

The results from this chapter demonstrate that the peripheral immune responses suppressed by feeding either a high or low dose of OVA include $CD8^+$ CTL responses and require the presence of $CD4^+$, but not $CD8^+$ T cells during the induction phase.

Although CTL responses mediated by $CD8^+$ T cells were suppressed, only CD4-dependent CTL were inhibited by oral tolerance, suggesting that this effect was secondary to tolerance of IL2-producing $CD4^+$ T cells rather than direct suppression of specific $CD8^+$ T cells by the fed antigen. However, preliminary experiments performed to address this issue were unsuccessful and lack of time prevented further studies examining whether administering exogenous IL2 to restimulation cultures might have overcome CTL unresponsiveness. Nevertheless, even if $CD8^+$ T cells are not tolerised directly by feeding OVA, my other experiments show that this population probably does not mediate oral tolerance. Thus, the CD4-independent CTL induced by OVA/CFA immunisation were neither primed nor suppressed by feeding OVA, suggesting that these cells were ignorant of fed antigen. Furthermore, oral tolerance was induced normally by both low and high doses of OVA in mice depleted of $CD8^+$ cells.

Taken together, my findings indicate that $CD8^+$ T cells do not play a role in the induction of oral tolerance, irrespective of the dose of antigen used. In contrast, my results show that $CD4^+$ T cells are essential for the induction of oral tolerance and the mechanisms underlying this effect will be explored in more detail in the following chapter.

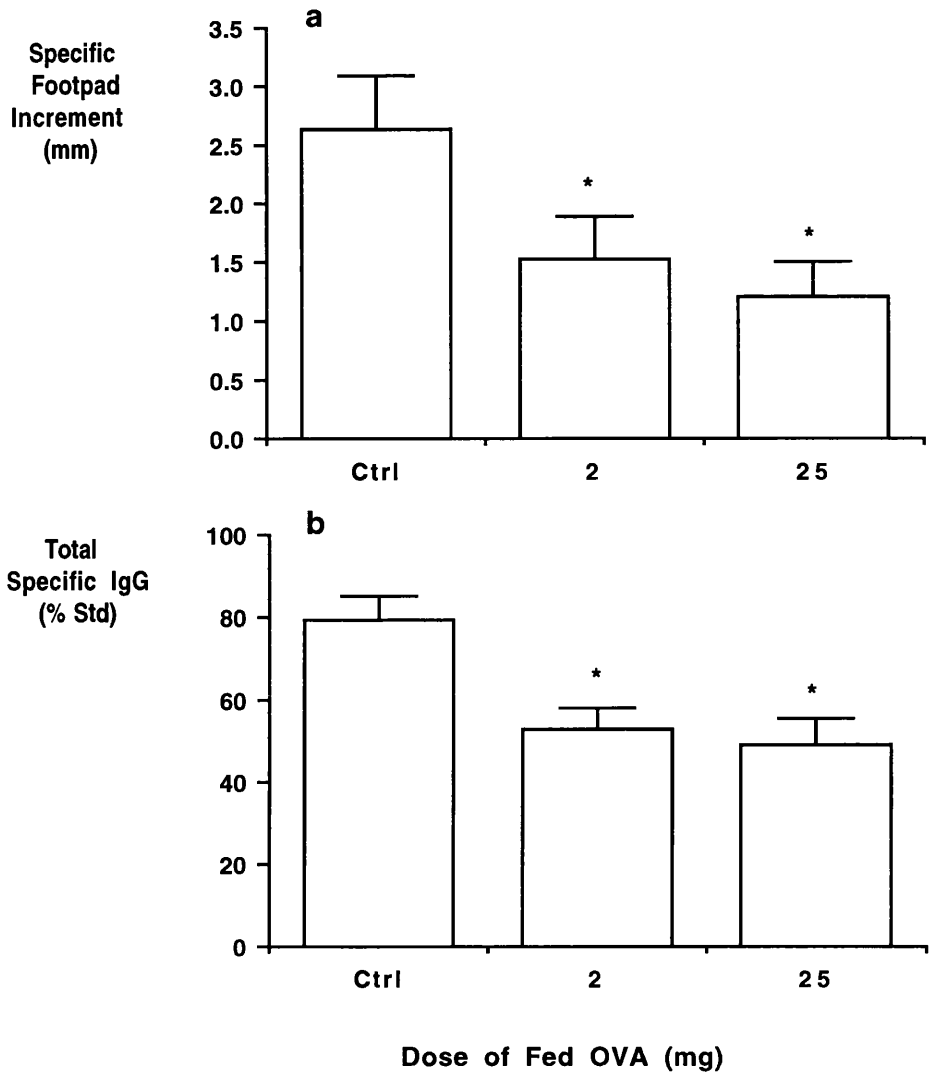


Figure 5.1 Suppression of OVA ISCOMS-induced Systemic DTH and Antibody Production by Feeding OVA.

a) OVA-specific DTH responses in mice 21 days after i.p. immunisation with 3μg OVA ISCOMS. The results shown are mean specific increments in footpad thickness ± 1 SEM for 6 mice per group in C57Bl/6 mice fed saline (Ctrl), 2 or 25mg OVA 10 days prior to immunisation. (* $p<0.05$ versus Ctrl). b) Total OVA-specific serum IgG responses 21 days after i.p. immunisation with 3μg OVA ISCOMS. The results shown are % hyperimmunised control serum and are the means ± 1 SEM for individual sera from 6 mice per group. (* $p<0.05$ versus Ctrl). Similar results were obtained in a replicate experiment.

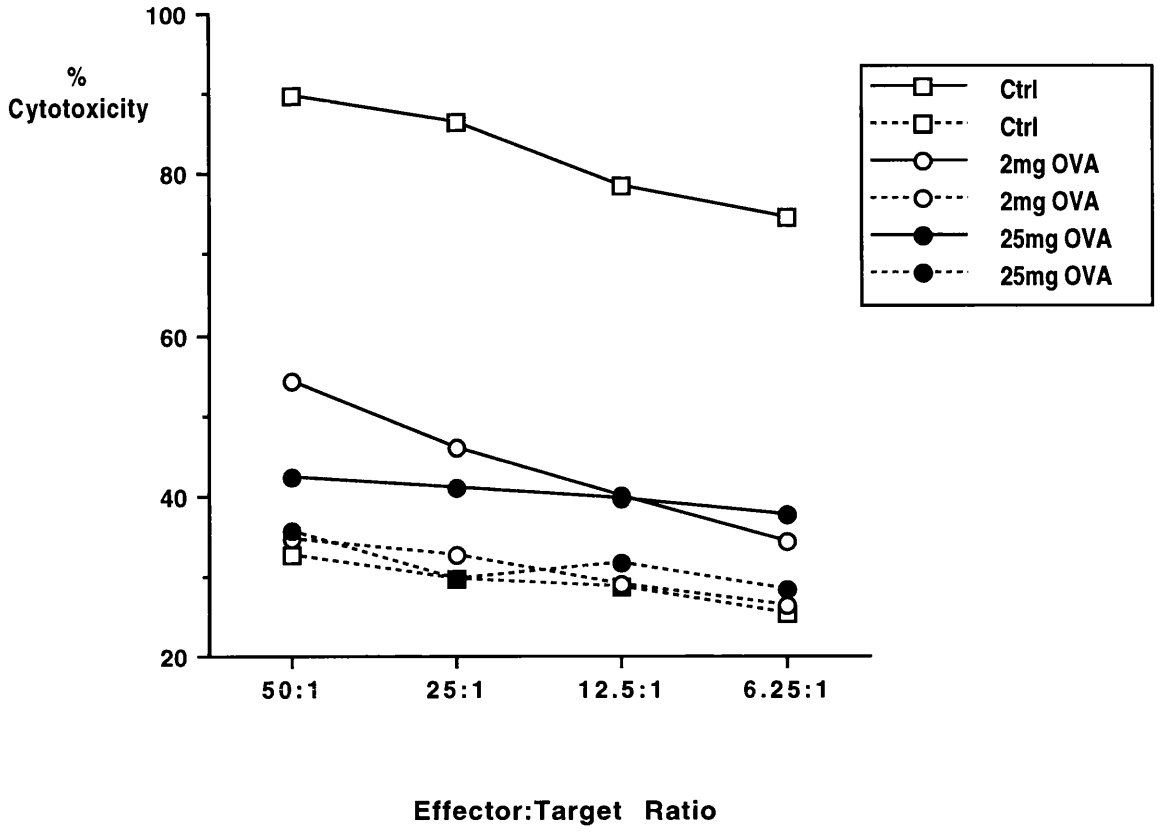


Figure 5.2 Effects of Feeding OVA on Systemic CTL Responses Induced by OVA ISCOMS

Systemic CTL responses in mice 14 days after i.p. immunisation with 3μg OVA ISCOMS. Spleen cells were removed and restimulated for 5 days *in vitro* with OVA-transfected EG7.OVA cells, before being assayed for OVA-specific CTL activity in a ^{51}Cr-release assay using EG7.OVA (bold line) or non-OVA-expressing EL4 (dotted line) target cells at the indicated effector:target cell ratios. The results shown are from quadruplicate assays using lymphocytes pooled from 6 mice per group in C57BL/6 mice fed saline (Ctrl), 2 or 25mg OVA 10 days before immunisation. Similar results were obtained in a replicate experiment.

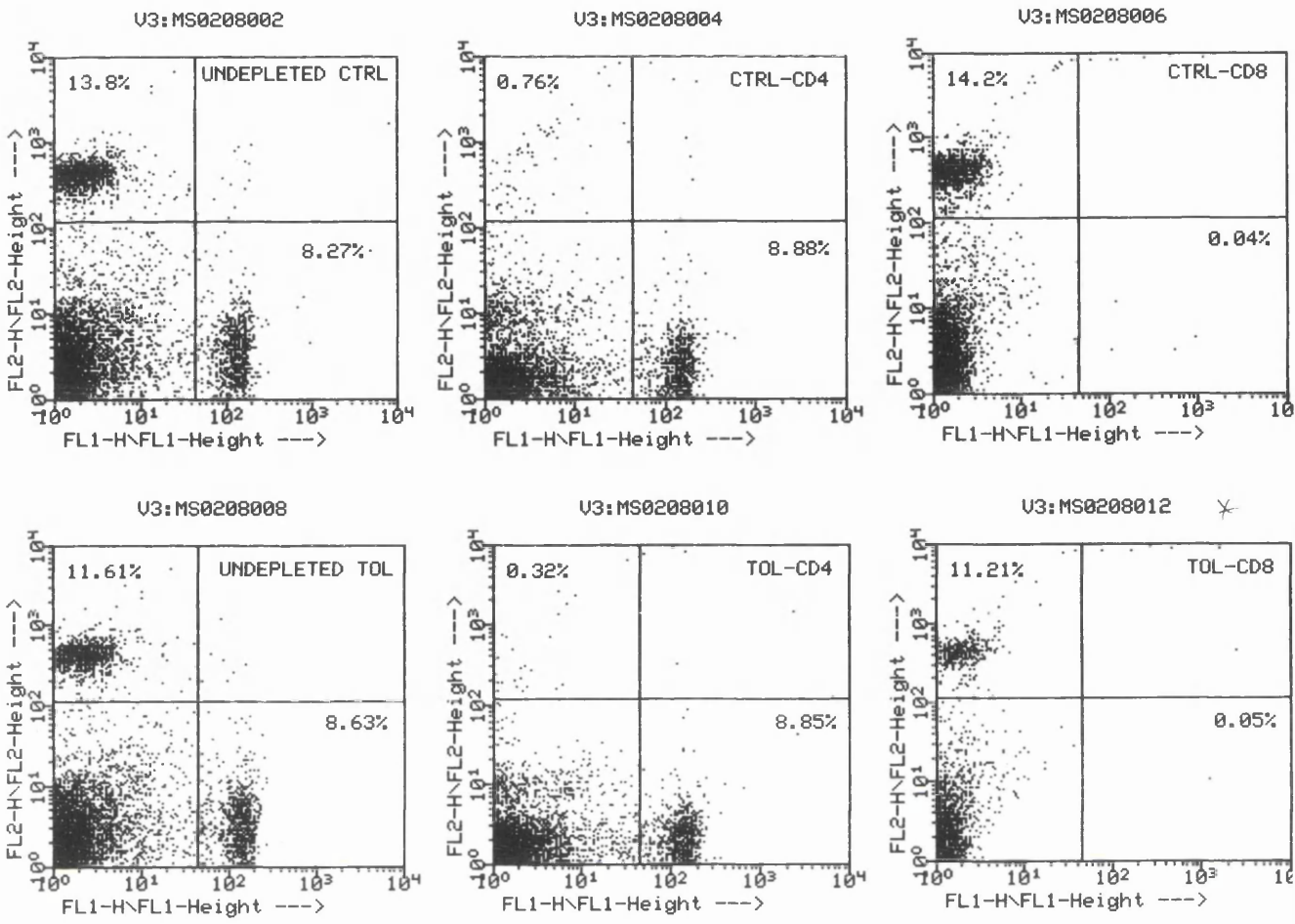


Figure 5.3 Efficacy of *in vitro* Purification CD4⁺ and CD8⁺ Spleen Cells by Antibody-mediated Lysis.

Spleen cells removed from mice 14 days after i.p. immunisation with 100µg OVA ISCOMS were depleted by incubation with 100µg/ml of anti-CD4 (YTS-191) or anti-CD8 (YTS-169) antibody and complement before staining with anti-CD4-PE and anti-CD8-FITC antibodies. The results shown are FACS plots of undepleted, CD4- or CD8-depleted lymphocytes from 3 mice per group in animals fed saline (Ctrl) or 25mg OVA (Tol) 10 days before immunisation.

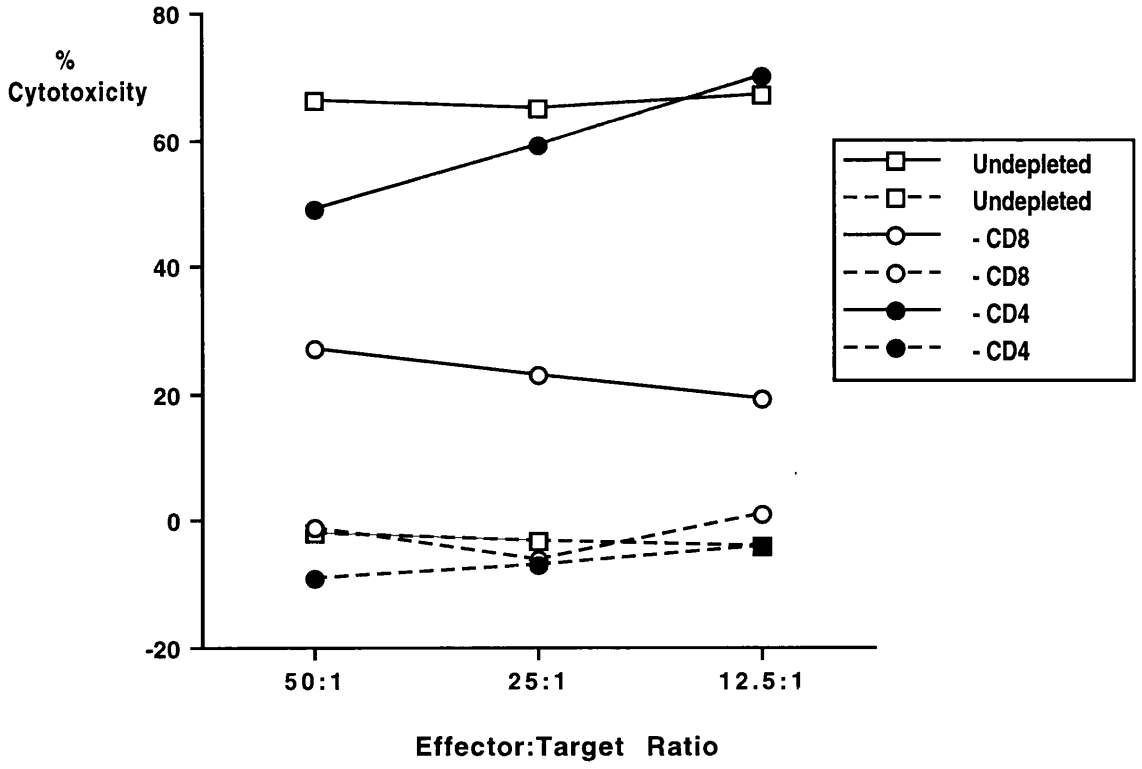


Figure 5.4 Effects of Depleting CD4+ or CD8+ Cells *in vivo* on the Generation of Systemic CTL by OVA/CFA.

Systemic CTL responses in mice 14 days after s.c. immunisation with 100μg OVA/CFA. Spleen cells were removed and restimulated for 5 days *in vitro* with OVA-transfected EG7.OVA cells, before being assayed for OVA-specific CTL activity in a ^{51}Cr-release assay using EG7.OVA (bold line) or non-OVA-expressing EL4 (dotted line) target cells at the indicated effector:target cell ratios. The results shown are from quadruplicate assays using lymphocytes pooled from 6 mice per group in C57BL/6 mice injected i.p. with 0.2ml saline (Ctrl), 0.5mg of anti-CD4 (YTS-191) or anti-CD8 (YTS-169) antibody 4 days before and on the day of immunisation. Similar results were obtained in a replicate experiment.

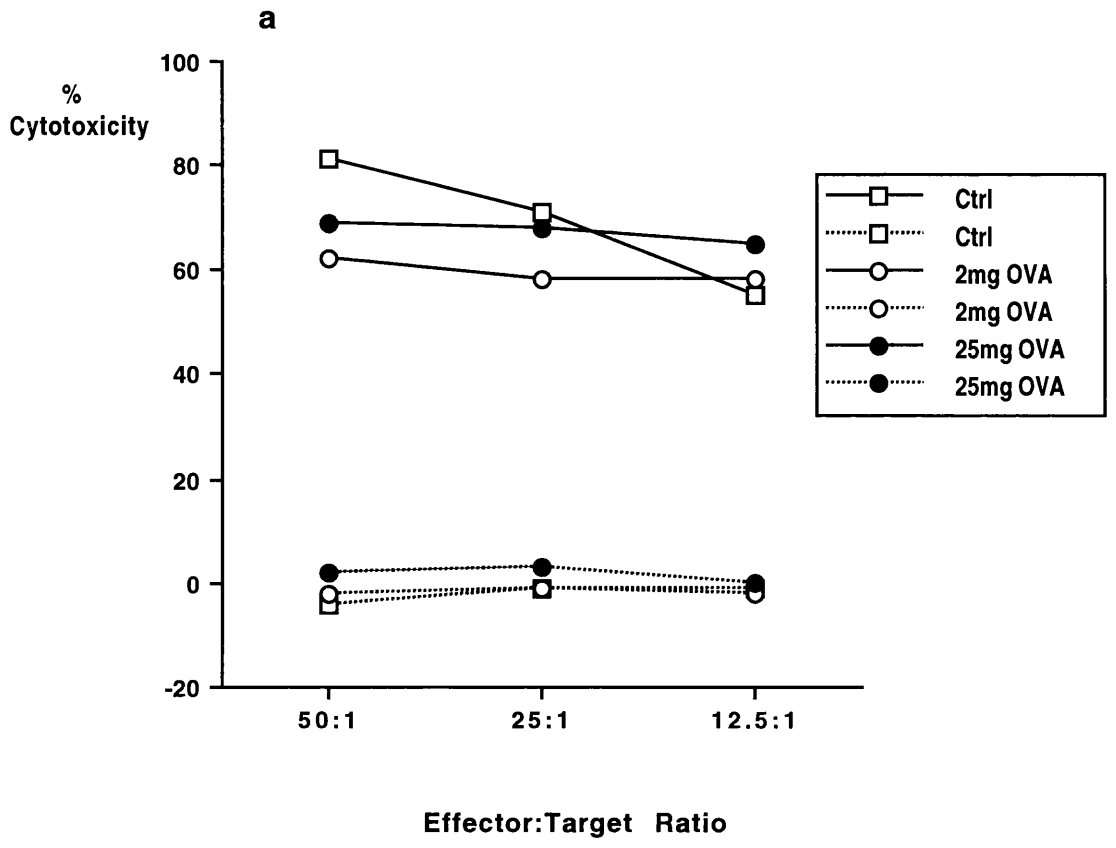


Figure 5.5a Effects of Feeding OVA on the CD4-independent CTL Responses Induced by OVA/CFA.

Systemic CTL responses in mice 14 days after s.c. immunisation with 100μg OVA/CFA. Spleen cells were removed and restimulated for 5 days *in vitro* with OVA-transfected EG7.OVA cells, before being assayed for OVA-specific CTL activity in a ^{51}Cr-release assay using EG7.OVA (bold line) or non-OVA-expressing EL4 (dotted line) target cells at the indicated effector:target cell ratios. The results shown are from quadruplicate assays using lymphocytes pooled from 6 mice per group in C57BL/6 mice fed saline (Ctrl), 2 or 25mg OVA 10 days before immunisation.

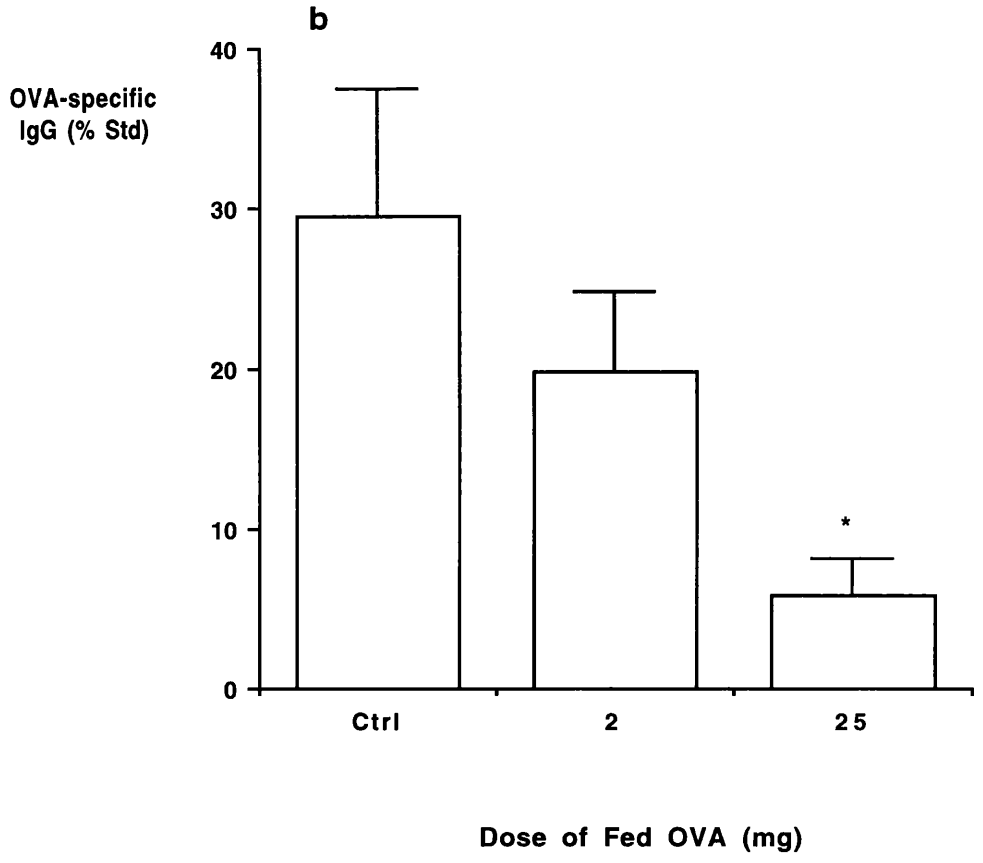


Figure 5.5b Effects of Feeding OVA on Systemic Antibody Responses Induced by OVA/CFA.

Total OVA-specific serum IgG antibody responses in mice 21 days after s.c. immunisation with 100μg OVA/CFA. The results shown are % hyperimmunised control serum and are the means ± 1 SEM for individual sera from 6 mice per group. The mean IgG levels of mice fed 2mg OVA were 33% lower than those of Ctrl mice. (* $p<0.05$ versus Ctrl). Similar results were obtained in a replicate experiment.

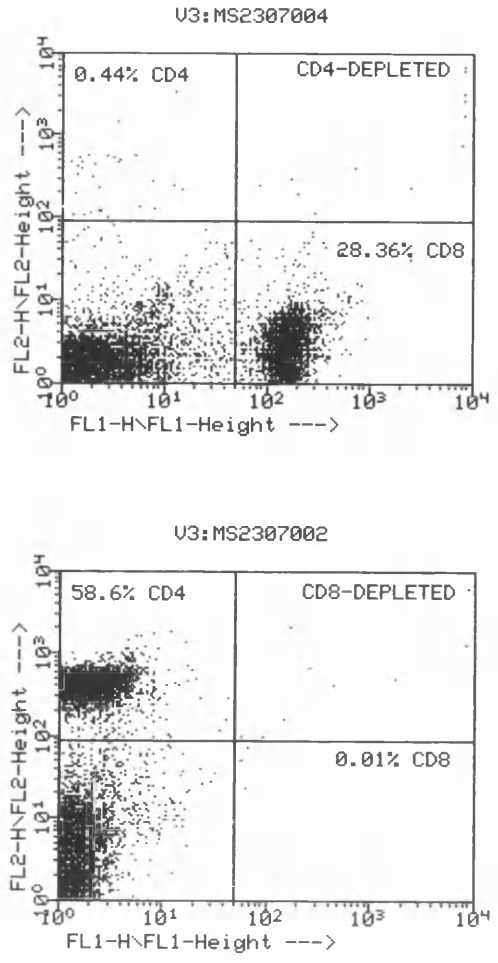


Figure 5.6 Efficacy of Depleting CD4⁺ or CD8⁺ Cells by Antibody Treatment *in vivo*.

To confirm depletion at the time of feeding, spleen cells were removed from a BALB/c mouse after i.p. injection with 0.5mg of anti-CD4 (YTS-191) or anti-CD8 (YTS-169) antibody 4 days before and on the day of sacrifice when the remaining animals in this experiment were treated as described in Fig 5.8. The results shown are the proportion of CD4⁺ and CD8⁺ T cells after staining with anti-CD4-PE and anti-CD8-FITC antibodies, as analysed by flow cytometry.

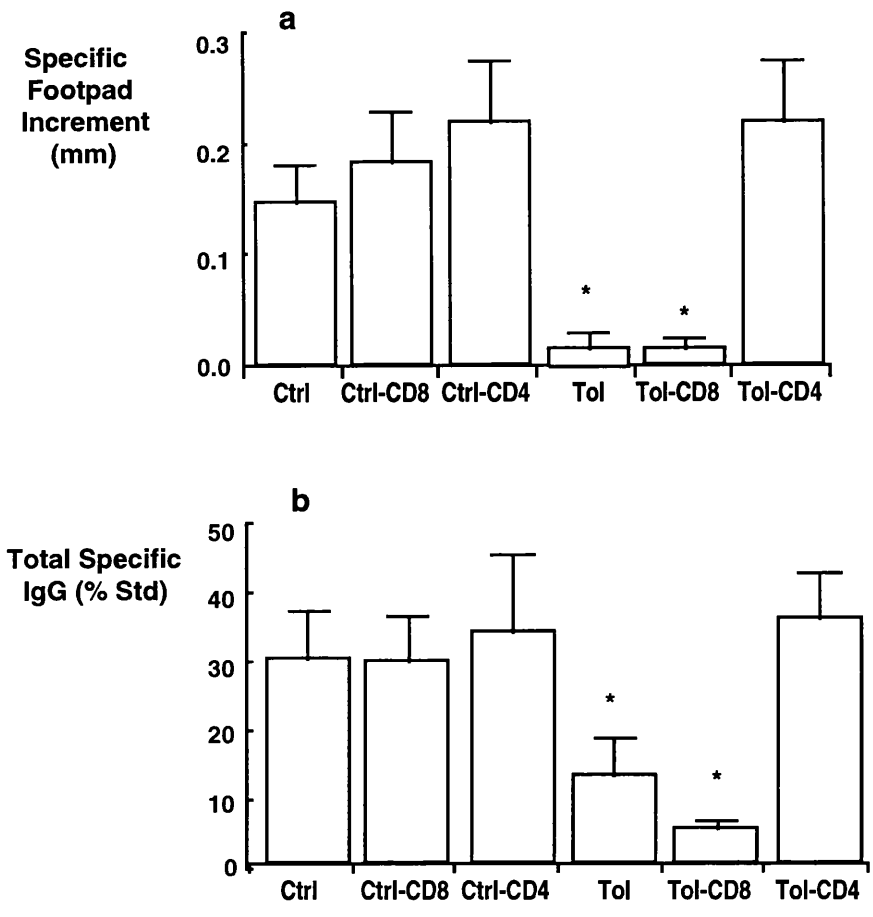


Figure 5.7 Effects of Depleting CD4$^+$ or CD8$^+$ Cells *in vivo* on Oral Tolerance Induced by Feeding a 25mg Dose of OVA.

a) OVA-specific DTH responses in BALB/c mice 21 days after s.c. immunisation with 100μg OVA/CFA. The results shown are mean specific increments in footpad thickness ± 1 SEM for 5 mice per group in animals fed saline (Ctrl) or 25mg OVA (Tol) 10 days before immunisation and injected i.p. with 0.2ml saline or 0.5mg either of anti-CD4 (YTS-191) or anti-CD8 (YTS-169) antibody 4 days before and on the day of feeding. (*$p<0.05$ versus Ctrl). b) Total OVA-specific serum IgG responses in mice 21 days after s.c. immunisation with 100μg OVA/CFA. The results shown are % hyperimmunised control serum and are the means ± 1 SEM for individual sera from 5 mice per group. (* $p<0.05$ versus Ctrl). Similar results were obtained in a replicate experiment.

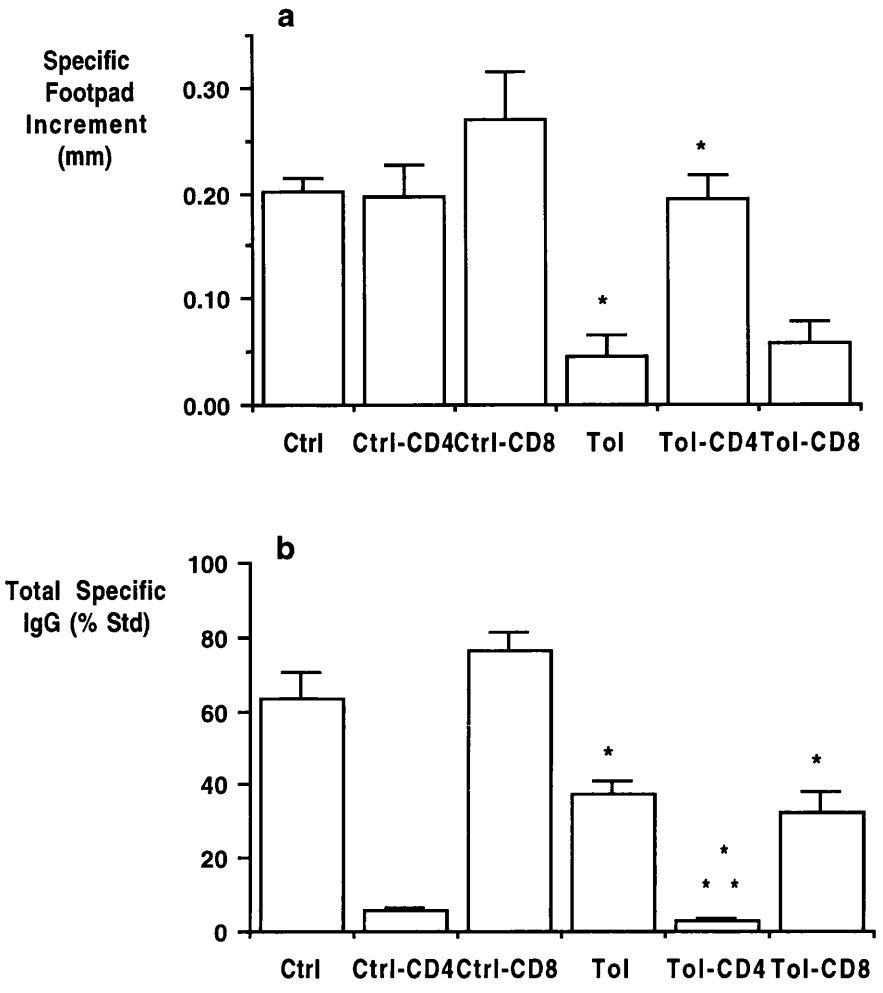


Figure 5.8 Effects of Depleting CD4$^+$ or CD8$^+$ Cells *in vivo* on Oral Tolerance Induced by Feeding a 2mg Dose of OVA.

a) OVA-specific DTH responses in BALB/c mice 21 days after s.c. immunisation with 100μg OVA/CFA. The results shown are mean specific increments in footpad thickness ± 1 SEM for 6 mice per group in animals fed saline (Ctrl) or 2mg OVA (Tol) 10 days before immunisation and injected i.p. with 0.2ml saline or 0.5mg either of anti-CD4 (YTS-191) or anti-CD8 (YTS-169) antibody 4 days before and on the day of feeding. (*$p<0.05$ versus Ctrl). b) Total OVA-specific serum IgG responses in mice 21 days after s.c. immunisation with 100μg OVA/CFA. The results shown are % hyperimmunised control serum and are the means ± 1 SEM for individual sera from 6 mice per group. (* $p<0.05$ versus Ctrl). Similar results were obtained in a replicate experiment.

Table 5.1 Spleen Cell Restimulation Cultures.

Spleen cells were removed from C57Bl/6 mice 14 days after s.c. immunisation with 100μg OVA/ISCOMS and used in the following combinations after depletion with 100μg/ml of anti-CD4 (YTS-191) or anti-CD8 (YTS-169) antibody and complement:

Group	Unfractionated	CD4-depleted	CD8-depleted
1	Unimmunised		
2	Control		
3	Tolerant		
4		Control	Control
5		Tolerant	Tolerant
6		Control	Tolerant
7		Tolerant	Control

Table shows recombination groups of spleen cells pooled from 10 mice per group in naive animals (Unimmunised) or mice fed either saline (Control) or 25mg OVA (Tolerant) 10 days prior to immunisation.

Chapter 6: The Role of Cell Death and Anergy in Oral Tolerance

Introduction

In the previous chapters, I have shown that feeding mice a single dose of OVA can inhibit all CD4-dependent effector T cell functions normally induced by subsequent systemic immunisation with OVA. Furthermore, my findings indicated that the oral tolerance induced by a wide range of doses is unlikely to arise through active suppression mediated by preferential induction of $CD4^+$ Th2 lymphocytes or $CD8^+$ T cells, but instead may reflect direct inactivation of specific T cells either by clonal anergy or deletion. Although direct inactivation has been documented in many models of peripheral tolerance induced parenterally and orally with exogenous superantigens [328, 329] or by administering conventional antigen to TcR transgenic animals [300, 330], neither anergy or deletion has been identified conclusively in physiologically relevant models of peripheral tolerance to nominal antigens in normal animals.

In this chapter I assessed the role of direct inactivation of T cells in oral tolerance mainly by examining for deletion via apoptosis *in vitro* and *in vivo* using both normal mice and lpr mice, which have a genetic defect in fas-mediated apoptosis. In preliminary studies, I also investigated a role for anergy in oral tolerance by attempting to reverse T cell unresponsiveness *in vitro* in the presence of IL2, a procedure well documented for this purpose [258]. Furthermore, as T cell anergy or deletion are often preceded by a period of partial activation [328, 331, 332], I assessed whether tolerogenic doses of fed OVA induced antigen specific immune activation before unresponsiveness.

Experimental Protocol

Oral tolerance was induced by feeding mice 2 or 25mg OVA prior to s.c. immunisation with OVA/CFA. PLN cells were subsequently removed to be assessed *in*

vitro for evidence of cell death using light and electron microscopy, flow cytometric analysis and nuclear DNA analysis.

To investigate the molecular mechanism(s) of apoptosis in oral tolerance, I examined fas-fasL interactions, as these have been implicated in cell death [235]. Fas-Fc fusion protein was employed in an attempt to block apoptosis of tolerant lymphocytes *in vitro*, while fas-mediated apoptosis *in vivo* was studied using OVA fed fas-defective MRL lpr mice.

To determine the potential contribution of anergy to oral tolerance, I restimulated PLN cells from orally tolerised animals for 5 days ± rIL2 (50U/ml) before assessing their proliferative responses to OVA (1mg/ml).

I also determined if T cells were activated prior to becoming unresponsive to oral antigen by using animals which had been fed OVA up to 10 days previously but were not parenterally immunised. Spleen, MLN and Peyer's patches from these mice were assessed for antigen-specific proliferation, cytokine production and entry into cell cycle *in vitro*.

Results

(A) Cells From Orally Tolerised Mice are Predisposed to Die by Apoptosis *in vitro*

(i) Compromised Viability of Cells From Orally Tolerised Animals in Culture

I first suspected that cells from tolerised mice were unusually susceptible to die *in vitro* during casual inspection of the PLN cells cultured for collection of supernatants for cytokine analysis. As these tolerant cultures displayed marked cell loss, I decided to explore this phenomenon in more detail. PLN cells from mice fed 2 or 25mg OVA prior to immunisation were cultured under different conditions and their viability assessed by phase contrast microscopy. When lymphocytes were cultured in medium without antigen, cell loss occurred in all groups over 120h (Fig 6.1a). However, at each time point assessed, cultures from mice fed OVA prior to immunisation showed more cell loss than those from immunised

control animals. By the end of the culture, 45.3% of control cells remained viable, compared with 10.3 or 19.3% of cells from animals fed 2 or 25mg OVA (Fig 6.1a). Although addition of OVA enhanced the cell survival in every culture, the number of viable cells from OVA fed mice was consistently lower than control cell numbers at each time point assessed (Fig 6.1b). By the end of the culture, 61.5% of control cells remained viable compared with 34.5 or 29.0% of cells from animals fed 2 or 25mg OVA, respectively (Fig 6.1b). In contrast, the number of viable cells in both control and tolerant cultures containing PPD were more comparable at each time point examined (Fig 6.1c). The cell death in these cultures was less marked than that observed upon restimulation with OVA and, by the end of culture, 78.3% control cells remained compared with 69.5 or 73.0% of cells from animals fed 2 or 25mg OVA, respectively (Fig 6.1c), indicating that tolerant cultures could sustain growth of viable lymphocytes, providing an antigen recognised by functionally active cells was present.

To investigate further the apparent predisposition of tolerant cells to die *in vitro*, I compared their viability to that of naive cells. When cultured in medium alone, the number of viable cells in both groups was strikingly similar, but far lower than that found in immunised control cultures at each time point examined (Fig 6.2a), suggesting that tolerant and naive cells display a similar susceptibility to cell death *in vitro*. However, differences between these groups became apparent upon the addition of OVA, which enhanced cell survival in tolerant and immunised control cultures with no appreciable effect on naive cell viability (Fig 6.2b). As observed previously under these conditions, the number of viable cells in tolerant cultures remained lower than control cell numbers throughout the culture period (Fig 6.2b).

(ii) Morphological Analysis of Tolerised Lymph Node Cells

I next examined if the loss of cell viability in tolerant cultures correlated with altered morphology *in vitro*. Phase contrast light microscopy showed that PLN cells from immunised control animals remained small and phase bright during a 120h culture in the absence of OVA (Fig 6.3a). In contrast, cultures of cells from mice fed 25mg OVA prior to

immunisation showed a rapidly increasing number of small, phase dark cells after 48h with few intact cells and lots of cellular fragments with the appearance of apoptotic bodies present by 120h (Fig 6.3b). In parallel, increasing numbers of large, granulated cells became apparent, many seeming to contain ingested cellular debris (Figs 6.3b) and having the appearance of activated macrophages. These features are consistent with apoptosis of some cells, followed by their ingestion by macrophages. Addition of OVA to the cultures induced immunised control cells to become activated as evidenced by their increased size (Fig 6.3c), polarisation (not shown) and blastogenesis (Fig 6.3d). These features were first apparent by 48h and increased progressively thereafter. In contrast, OVA restimulated cells from tolerant cultures showed greatly reduced polarisation, with little blastogenesis (Fig 6.3e). Rather, these cells had a morphology similar to control cells cultured in the absence of specific antigen, with more small and phase bright cells than was observed in parallel cultures in the absence of OVA.

To analyse these changes in more detail, the cultures were examined by electron microscopy. In the absence of antigen, immunised control cultures were comprised mainly of small cells with the appearance of resting lymphocytes (Fig 6.4a). These were present to a far lesser extent in tolerant cultures, which contained large numbers of cells with the pyknotic nuclei and membrane blebbing characteristic of apoptosis (Fig 6.4b). Upon addition of OVA, the vast majority of cells in the control cultures were enlarged with the appearance of activated lymphocytes (Fig 6.4c). In marked contrast, tolerant cells cultured with OVA had an appearance similar to control cells cultured without antigen, showing little evidence of apoptosis (Fig 6.4d).

(iii) Flow Cytometric Analysis of Lymph Node Cells from Tolerant Cultures

To determine whether these morphological changes affected all populations of tolerant T cells, I analysed the size, granularity and phenotype of lymphocytes gated for expression of CD4 or CD8 by flow cytometry. After 120h in the absence of OVA, cultures from immunised control mice comprised cells mainly with the size and granularity of resting lymphocytes, with a few other cells of larger size and slightly increased granularity,

presumed to be lymphoblasts (Fig 6.5a). In marked contrast, there were far fewer resting lymphocytes detected in cultures from 25mg OVA fed mice and there were virtually no lymphoblasts, consistent with the microscopic findings above. The loss of conventional lymphocytes affected both $CD4^+$ and $CD8^+$ T cell subsets, which were reduced to only 5.4 and 5.9% total, respectively, whereas 42.8% $CD4^+$ and 35.8% $CD8^+$ T cells remained in control cultures after 120h (Figs 6.5a,c,e&g). The cells lying outside the regions defined for live lymphocytes are likely to be dead. This population was particularly enhanced in tolerant cultures gated for either $CD4^+$ or $CD8^+$ cells (Table 6.1), consistent with the previous findings.

After restimulation of immunised control cultures with OVA, both the $CD4^+$ and $CD8^+$ T cell subsets contained an expanded proportion of cells with increased forward light scatter indicative of lymphoblasts (Figs 6.5b&f). Restimulation of tolerant cells with OVA resulted in the appearance of $CD4^+$ and $CD8^+$ T cells with the size and granularity of small lymphocytes, but these were still less than found in restimulated control cultures (46.1% of control $CD4^+$ and 67.8% of control $CD8^+$ T cells) and there was little evidence of blastogenesis (Figs 6.5d&h). Again, the proportion of $CD4^+$ or $CD8^+$ cells presumed dead were increased for tolerant cultures (Table 6.1).

Similar evidence for cell loss was observed in cultures from mice fed 2mg OVA compared with controls (Table 6.2). In the absence of antigen, the numbers of both $CD4^+$ and $CD8^+$ cells from mice fed 2mg OVA were markedly lower than their respective controls, but not as low as observed in cultures from mice fed 25mg OVA (Table 6.2). When OVA was present in culture, the cell loss was less marked, but again lower numbers of both $CD4^+$ and $CD8^+$ cells remained in culture from mice fed 2mg OVA compared with controls (Table 6.2) and the cultures from mice fed 25mg OVA contained even fewer $CD4^+$ and $CD8^+$ cells (Table 6.2).

These findings are consistent with the cell viability studies and show that cells of both $CD4^+$ and $CD8^+$ subsets from orally tolerant mice exhibit an increased propensity to die *in vitro*.

(iv) Analysis of DNA Content of Tolerised Lymph Node Cells

The preceding studies show that lymphocytes from orally tolerant mice are less viable than immunised control cells in culture and have a morphology consistent with apoptosis. To confirm and quantify these findings, I next examined nuclear DNA content using PI staining and flow cytometry. Small, resting lymphocytes have normal diploid DNA content and are in the G0/G1 phase of cell cycle. Activated lymphocytes contain more DNA and are in either the S or G2/M phase, while cells dying by apoptosis show a hypodiploid DNA content due to fragmented nuclear DNA leaching from the cell. These features are readily distinguished using PI staining (Fig 6.6).

After 72h culture in the absence of OVA, 56.1% lymphocytes from immunised control animals were in the G0/G1 phase of cell cycle and 32.5% cells displayed a hypodipoid DNA content (Fig 6.7a&b). Under the same conditions, cultures from animals fed 2 or 25mg OVA contained only 27.8 or 34.9% cells in G0/G1 phase, respectively, with the proportion of hypodiploid cells increasing to 61 and 50.5%, respectively (Fig 6.7a&b). The frequency of cells in the S and G2/M phases of cycle was similar for all cultures (Fig 6.7c). These findings are consistent with the previous morphological evidence that, in the absence of antigen, control cultures contained mainly quiescent lymphocytes, whereas tolerant cultures contained more cells with an apoptotic phenotype.

In the presence of OVA, more cells from every group were found in G0/G1 phase (Fig 6.7a) and the proportion of control cells with hypodiploid DNA content was reduced to 17.5% (Fig 6.7b). OVA-restimulated cultures from tolerant animals fed 2 or 25mg OVA also displayed a lower proportion of hypodiploid cells (32.5 or 26.4%, respectively) compared with values detected in the absence of antigen (Fig 6.7b). However, the numbers of hypodiploid cells in tolerant cultures never decreased to the same extent as that observed in control cultures under the same conditions (Fig 6.7b). In addition, the proportion of cells in S and G2/M phases of cycle was always lower for tolerant cells than for control cells after restimulation with OVA (Fig 6.7c), suggesting that antigen-specific activation of the tolerant cells was impaired. These results agree with the previous morphological study of OVA-

restimulated cultures, where both control and tolerant cells showed little evidence of apoptosis and control cultures contained more large blasting lymphocytes.

After restimulation of cultures with PPD, all groups contained a similarly decreased proportion of cells with hypodiploid DNA (Fig 6.7b) and more cells in the S and G2/M phases of cell cycle than observed in cultures containing OVA (Fig 6.7c). These findings are consistent with the cellular proliferation observed in the same cultures, where the responses of every group to PPD were always of greater magnitude than those directed against OVA.

(B) Molecular Mechanism of Apoptosis in Oral Tolerance

As my previous results suggested that lymphocytes from orally tolerised animals were more susceptible than control cells to die by apoptosis when cultured in the absence of antigen, it became important to determine the underlying molecular mechanism of this cell death. Given the evidence that a failure of fas-mediated apoptosis is associated with the breakdown of self-tolerance in some autoimmune disorders, such as systemic lupus erythematosus (SLE) [245, 333], and has been implicated in peripheral tolerance to superantigens [334], it seemed possible that this mechanism might also be important in oral tolerance.

Role of Fas-dependent Apoptosis in the *in vitro* Manifestations of Oral Tolerance

I first used a recently described fas-Fc fusion protein [310] to examine whether the apoptosis of cells from tolerant mice *in vitro* occurred by a fas-dependent mechanism. In parallel, I investigated the effect of this treatment on the immune effector responses generated by control and tolerant cells *in vitro*. Due to limited supplies of the reagent, it was not possible to carry out detailed dose response studies and therefore the fusion protein was used only at the concentration reported to be maximally effective in blocking fas-dependent apoptosis [335]. A TNFR-Fc fusion protein was used as a control since this molecule is

also effective in preventing apoptosis but blocks a fas-independent signalling pathway induced by TNFα [335].

(i) OVA-specific Proliferation

As expected, the proliferative response of PLN cells from mice fed 2 or 25mg OVA prior to immunisation was significantly reduced compared with that of control cells after restimulation with OVA (Fig 6.8). The addition of either fas-Fc fusion protein or control TNFR-Fc fusion protein to the cultures did not significantly alter the OVA-specific proliferative response of any group and did not restore the proliferative capacity of tolerant lymphocytes to control levels (Fig 6.8). The proliferative response of every group was negligible when cultured in the absence of antigen and was not influenced by fas-Fc fusion protein (results not shown).

(ii) Cytokine Production

OVA-specific IL3 and IFNγ secretion was also significantly reduced in cells from mice fed 2 or 25mg OVA prior to immunisation when compared with control cells (Fig 6.9) and this was not altered by the addition of fas-Fc fusion protein (Fig 6.9). Similarly, the fas-Fc fusion protein had no effect on control cytokine production. In the experiment shown, there was little or no production of OVA-specific IL5 and IL10 by either control or tolerant cells and these levels were not affected by the presence of fas-Fc fusion protein (data not shown). Due to a limited supply of reagents, this experiment did not include the TNFR-Fc fusion protein as a control and further studies were not possible.

(iii) Apoptosis

The hypodiploid cell content of cultures was assessed after 72h by PI staining. However, at this time point, the tolerant cultures contained no more hypodiploid cells than were observed in control cultures in the presence or absence of OVA (Fig 6.10) and the addition of fas-Fc fusion protein to tolerant cultures did not lower the proportion of hypodiploid cells to any greater extent than observed with control cultures (Fig 6.10). These

findings were unexpected since I normally find that tolerant cultures contain a greater proportion of hypodiploid cells than controls. However, it is possible that different effects might have been observed if a full kinetic study had been possible. Therefore, it remains to be determined conclusively if apoptosis of control or tolerised cells is differentially influenced by the addition of fas-Fc fusion protein.

As the reduced *in vitro* responsiveness of OVA-specific cells from orally tolerant animals was not altered by addition of fas-Fc fusion protein, these findings suggest that fas-mediated apoptosis is not required to maintain oral tolerance. However, it also remains possible that the fas-Fc fusion protein did not work in these experiments.

Role of Fas-dependent Apoptosis in Oral Tolerance *in vivo*

The apparent lack of effect of fas *in vitro* did not precude a role in the induction or maintenance of oral tolerance *in vivo* and to examine this directly, I performed a series of experiments feeding OVA to MRL lpr mice, which have a mutation in the fas gene, rendering it non-functional.

In vivo Responses

As observed in other normal mouse strains, the DTH responses of control MRL $lpr^{+/+}$ mice were significantly reduced in animals fed 2 or 25mg OVA before immunisation with OVA/CFA (Fig 6.11a). Unfed MRL lpr mice made significantly higher DTH responses than their littermate controls, but despite this, feeding either 2 or 25mg OVA induced significant tolerance, equivalent to or greater than that in the $MRL^{+/+}$ mice (Fig 6.11a).

Control MRL lpr mice also displayed higher total OVA-specific IgG and IgG2a serum antibody responses compared with control $MRL^{+/+}$ mice (Fig 6.11b & 6.12b), while IgG1 levels were similar in both groups (Fig 6.12a). All of these responses were significantly reduced in MRL lpr mice fed 25mg OVA prior to immunisation compared with control MRL lpr mice (Fig 6.11b & 6.12). In addition, the total IgG and IgG2a antibody levels were significantly reduced in immunised MRL lpr mice by a prior feed of 2mg OVA (Fig 6.11b &

6.12b). In this experiment, none of the antibody responses were reduced by feeding either dose of OVA to normal $MRL^{+/+}$ mice (Fig 6.11 & 6.12).

Thus, fas-mediated cell death is not required for orally induced tolerance of DTH or antibody responses *in vivo*.

In vitro Responses

The OVA-specific proliferation of PLN cells removed from $MRL^{+/+}$ mice after immunisation was significantly higher than that observed with MRL lpr control cells (Fig 6.13). Despite this difference, cells from both $MRL^{+/+}$ and MRL lpr mice fed 2 or 25mg OVA prior to immunisation were significantly reduced by equivalent extents compared with their respective controls (Fig 6.13).

The OVA-specific production of IL3 and IFNγ cytokines by PLN cells from MRL lpr mice was higher than that of $MRL^{+/+}$ animals, whereas IL5 production was reduced (Fig 6.14), indicating that the genetic defect had skewed immune responses to a Th1-like profile.

The OVA-specific production of IL3, IL10 and IFNγ was significantly reduced by feeding MRL lpr mice 2 or 25mg OVA prior to immunisation (Fig 6.14). Secretion of antigen-specific IL5 was also significantly reduced in MRL lpr mice by prior feeding of 25mg OVA (Fig 6.14), while 2mg fed OVA delayed the release of this cytokine (results not shown) but could not prevent it reaching levels comparable with controls by 120h culture (Fig 6.14). The reduction in cytokine production by cells from $MRL^{+/+}$ animals fed OVA was less consistent and was only observed with IL3 and IL5, although secretion of IL10 and IFNγ was also delayed by feeding 2 or 25mg OVA (results not shown). Therefore, normal functional tolerance was induced by feeding OVA to MRL lpr mice.

As OVA fed MRL lpr mice could be tolerised normally, I next examined whether the lpr mutation influenced the predisposition of tolerised cells to die by apoptosis when challenged *in vivo* and cultured *in vitro*. In this experiment, small, but dose-dependent, increases in the proportion of hypodiploid cells were detected by PI staining of cells from $MRL^{+/+}$ mice fed OVA before immunisation (Fig 6.15). As usual, the hypodiploid cell content of cultures was markedly reduced in the presence of OVA (Fig 6.15). A similar

increase in hypodiploid content was observed when MRL lpr cells from OVA fed mice were cultured in the absence of antigen and again this was rescued by restimulation with OVA (Fig 6.15). Therefore, my findings do not support a role for fas in the apoptosis of orally tolerant cells when cultured in the absence of antigen.

Together with the results obtained using the fas-Fc fusion protein, my findings are not consistent with a role for fas-mediated apoptosis in either the cell death occurring in tolerant cultures *in vitro,* or in the expression of oral tolerance *in vivo.*

(C) Anergy in Oral Tolerance

Although the results of this chapter suggest that T cells from orally tolerant animals are predisposed to die by apoptosis *in vitro*, the addition of OVA to tolerant cultures reduced cell death without altering immune unresponsiveness. This could be interpreted as evidence that OVA-specific T cells remained present in the tolerant cultures but were functionally impaired. This would suggest a role for anergy as well as deletion in oral tolerance. To examine this in more detail, I attempted to reverse the presumed anergy by culturing PLN with rIL2, a procedure which has been reported to allow anergised T cells to regain responsiveness to antigen upon subsequent restimulation (DeSilva, D.R. et.al. 1991. J. Immunol. 147:3261). PLN cells from mice fed 2 or 25mg OVA prior to OVA/CFA immunisation had decreased OVA-specific proliferation if cultured for 5 days in medium before restimulation with OVA (Fig 6.16). However, this tolerance was no longer observed when cells from mice fed 2mg OVA were cultured for 5 days in the presence of 50U/ml rIL2, as these cells now had OVA-specific proliferation comparable to OVA/CFA immunised controls (Fig 6.16). Similar findings were also made with cells from immunised mice fed 25mg OVA, where OVA-specific proliferation was significantly enhanced by prior exposure to rIL2 (Fig 6.16). However, these responses remained significantly lower than those of OVA/CFA immunised controls (Fig 6.16), indicating that the tolerance induced by a 25mg dose of OVA was only partly reversed. The subsequent OVA-specific proliferation of cells from control mice fed saline before parenteral immunisation with CFA $\pm$ OVA was not altered by prior exposure to rIL2 (Fig 6.16), indicating that this treatment did not induce a

non-specific proliferative response. The proliferative responses of all groups in medium alone were very low or negligible (results not shown).

Interestingly, preculture with rIL2 also decreased the cell loss found when tolerant PLN cells were subsequently cultured in the absence of antigen, restoring the cell recovery to levels similar to those of immunised controls (Table 6.3). The viability of control cells was not influenced by the presence of rIL2 (Table 6.3). Therefore, these findings are consistent with the presence of anergic cells in the tolerant cultures.

(D) Feeding Tolerogenic Doses of OVA Primes T Lymphocytes *in vivo*

The results of this and previous chapters indicate that oral tolerance may be associated with both anergy and deletion and, in other models of tolerance, these cells have been associated with a transient period of T cell activation [328, 331, 332]. To determine if a similar situation arose in oral tolerance, I examined for antigen-specific T cell priming immediately after feeding mice 25mg OVA, the dose which provokes the most profound tolerance.

(i) Proliferative Activity

Spleen cells taken from individual mice fed OVA 24h before showed consistent OVA-specific proliferation when restimulated over 96h culture (Fig 6.17a). Although these levels of proliferation varied between individual cultures, it was never observed with spleen cells from any unfed control mouse (Fig 6.17a), confirming the ability of feeding antigen to prime T cells. OVA-specific proliferative responses were also frequently observed using cells removed from individual mice examined 2 and 4 days after feeding OVA, but overall these responses were not significantly different from controls due to more variablity at these times (Fig 6.17b&c). This variability may reflect the low proportion of antigen-specific cells in normal mice fed OVA. The proliferative responses of all cells in medium alone were neglible (results not shown).

(ii) Entry into Cell Cycle

Similar variability was observed in repeat experiments and to try and obtain further evidence of oral priming, I examined these cells using the more sensitive technique of PI staining and flow cytometric analysis of cell cycle. This confirmed the presence of antigen-specific priming in spleen cells from OVA fed mice, as defined by their entry into the S and G2/M phases of cell cycle after 72h culture in the presence of OVA. Spleen cells from mice fed OVA 1, 2 or 3 days previously entered cell cycle when restimulated with OVA (Fig 6.18), with most activity being observed on day 1 and declining thereafter. Spleen cells from mice 4 or 10 days after feeding OVA or control animals fed saline, did not show increased numbers of cells in S or G2/M phases when cultured in the presence or absence of OVA (Fig 6.18a). However, when Con A was used to restimulate splenocytes, all groups showed a similar capacity to enter cell cycle (Fig 6.18b), demonstrating the viability and responsiveness of these cultures. Interestingly, there was no evidence of increased apoptosis in any cultures of OVA fed cells at any time of culture (data not shown), indicating that this phenomenon required *in vivo* challenge with OVA in adjuvant.

It is important to note that MLN or PP cells from either control or OVA fed mice could never be induced to enter cell cycle by OVA *in vitro* when examined at any time point after feeding (results not shown).

(iii) Cytokine Production

Further aliquots of cells taken from mice up to 10 days after feeding OVA were then assessed for the ability to produce cytokines when restimulated with OVA for up to 120h. Spleen cells from mice fed OVA up to 3 days previously, but not thereafter, produced detectable levels of OVA-specific IL3 and IFNγ (Fig 6.19a), while no OVA-specific IL5 and IL10 could be detected in any culture supernatant. No OVA-specific cytokine production occurred in cultures of MLN or PP cells from either control or OVA fed mice at any time point analysed. However, when Con A was used to restimulate these cultures, all groups showed a similar capacity to secrete each cytokine (Fig 6.19b), confirming the viability of the cultures.

These findings indicate that feeding OVA primes T cells *in vivo* to generate effector responses upon antigen-specific restimulation *in vitro*. However, this period of activation is transient, lasting for only a few days, and is followed by a state of immune unresponsiveness to identical restimulation *in vitro*.

To determine whether mice were tolerant to *in vivo* challenge during this period of immune priming, separate mice from these groups were immunised with OVA/CFA 1, 2, 3, 4 or 10 days after feeding OVA. When PLN cells were removed 14 days after challenging OVA fed mice with OVA/CFA they produced significantly lower OVA-specific IL3, IL10 and IFNγ compared with control cells from mice fed saline prior to immunisation (Fig 6.20). Thus OVA fed mice were tolerant to challenge despite the presence of primed cells.

Summary and Conclusions

The results presented in this chapter show that lymphocytes from orally tolerant mice display an increased propensity to die following restimulation with antigen *in vivo* and subsequent culture in the absence of antigen *in vitro*. This was comparable with naive cell death, occurred in mice fed either 2 or 25mg doses of OVA and affected both $CD4^+$ and $CD8^+$ T cells. Dying lymphocytes from unstimulated cultures of tolerised cells developed morphological features indicative of apoptosis, including nuclear fragmentation and membrane blebbing, and these changes were accompanied by an increased proportion of cells with hypodiploid DNA content. The cell death was probably more extensive than could be reasonably accounted for by deletion of specific clones alone, but the addition of OVA to the tolerant cultures reduced levels of apoptosis, indicating that the cell death was influenced by antigen and suggesting that some OVA-specific T cells remained present when lymph node cells were removed from tolerant animals. These cells may have been anergic as their unresponsiveness was found to be reversed, at least in part, by exposure to rIL2 before restimulation with OVA *in vitro*.

Although the results are compatible with direct inactivation of T cells in oral tolerance, this did not involve fas-dependent apoptosis either *in vivo* or *in vitro*. Entirely

normal oral tolerance of all immune functions was observed in fas-deficient lpr mice and the functional defects associated with oral tolerance were not reversed by a fas-Fc fusion protein, which blocks fas-dependent apoptosis in other systems.

My findings also indicated that induction of a tolerant phenotype was preceded by transient T cell activation after feeding OVA. This was evidenced by the capacity of spleen cells from OVA fed mice to enter cell cycle and secrete cytokines when restimulated with OVA during the first 3 days after feeding but not thereafter. However, these mice were tolerant to challenge during this time.

Together, the findings presented in this chapter support the possibility that a transient priming of specific T lymphocytes by fed antigen leads to functional inactivation and increased susceptibility to apoptosis in the absence of antigen *in vitro.* These results support those in previous chapters showing that all T cell functions are profoundly tolerised by feeding antigen and suggest that this phenomenon may be exploited to treat a wide range of immunological disorders. For this reason, I thought it important next to study the longevity of oral tolerance.

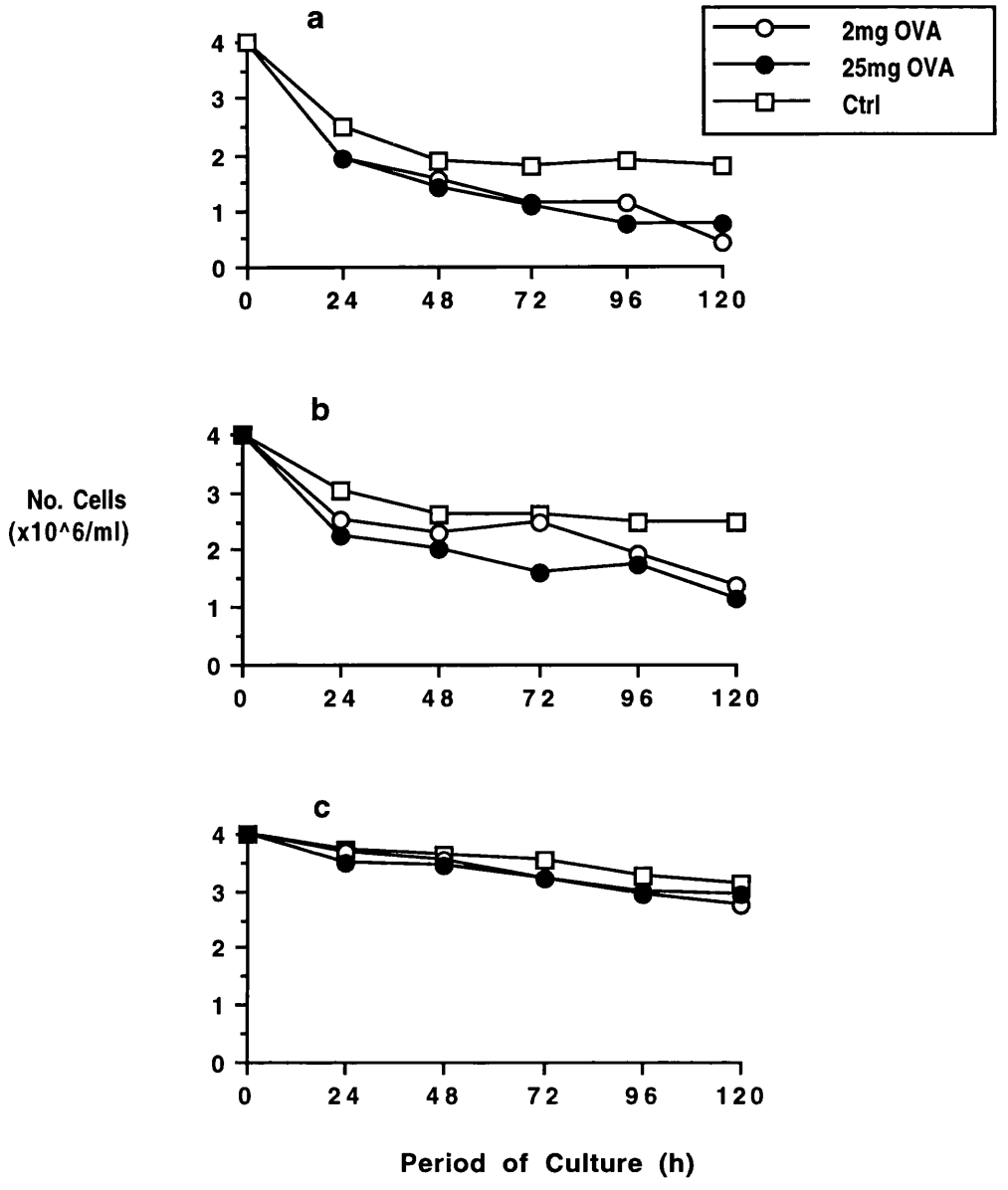


Figure 6.1 Viability of Lymphoid Cells from Orally Tolerised Animals is Compromised *in vitro*.

Viability of PLN cells removed 14d after immunisation of mice with OVA/CFA and cultured in (a) medium, (b) OVA (1mg/ml) or (c) PPD (50μg/ml) for a period of 120h. The results shown are the absolute numbers of viable cells, pooled from 5 mice per group from animals fed saline (Ctrl), 2 or 25mg OVA 10 days before immunisation. Similar results were obtained in 3 repeat experiments.

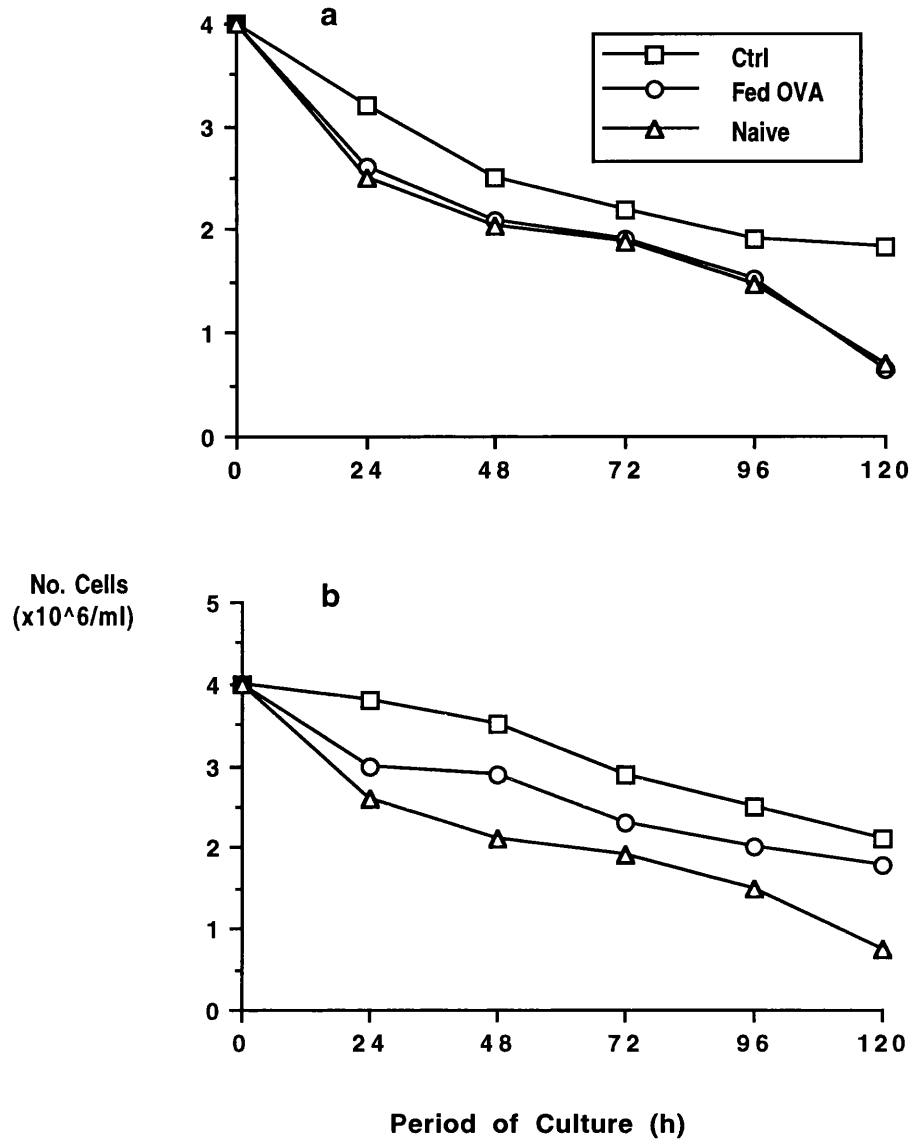
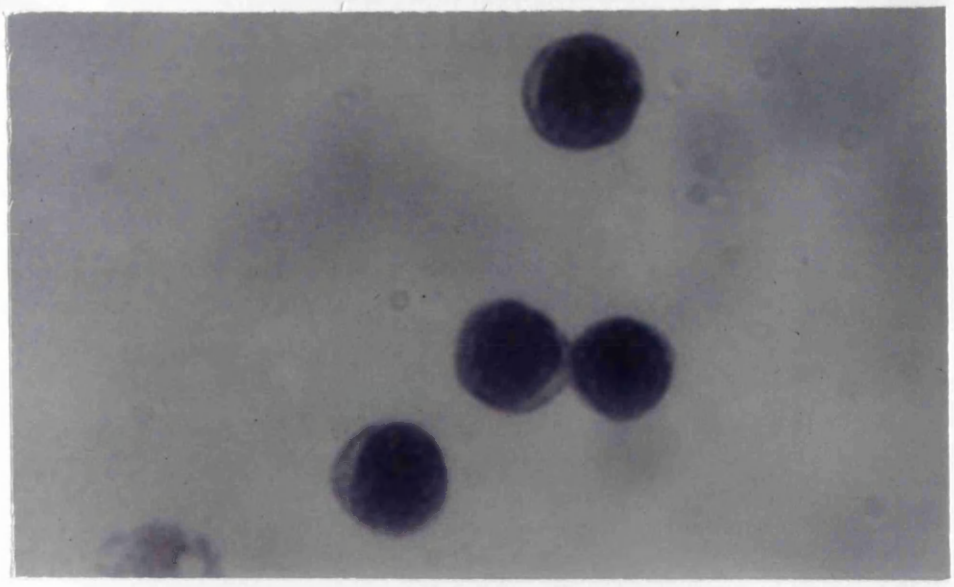


Figure 6.2 Viability of Lymphoid Cells from Orally Tolerant and Naive Animals is Similar

Viability of PLN cells removed 14d after immunisation of mice with OVA/CFA and cultured in (a) medium or (b) OVA (1mg/ml) for a period of 120h. The results shown are the absolute numbers of viable cells, pooled from 5 mice per group from naive mice or animals fed saline (Ctrl) or 25mg OVA 10 days before immunisation.

A



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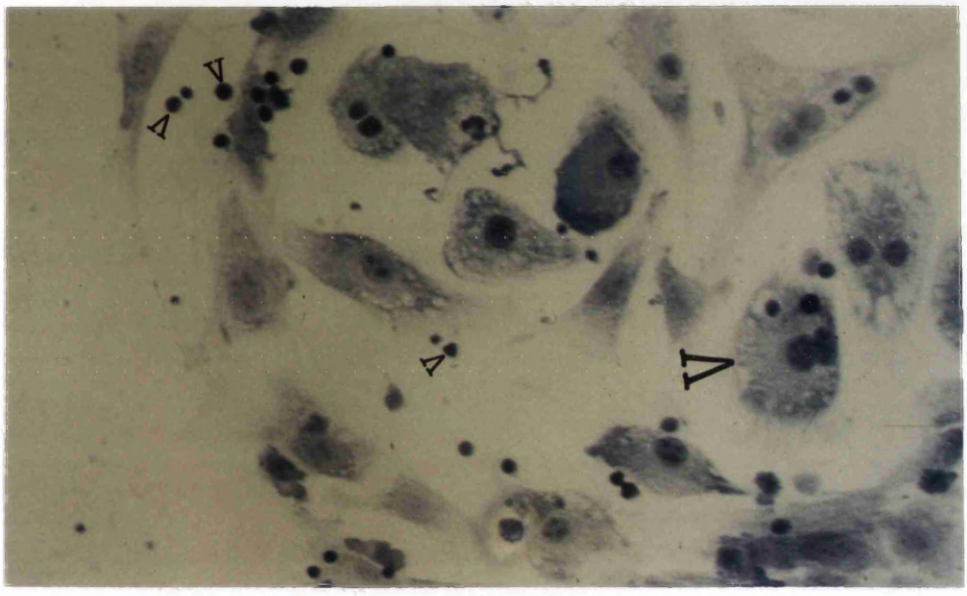
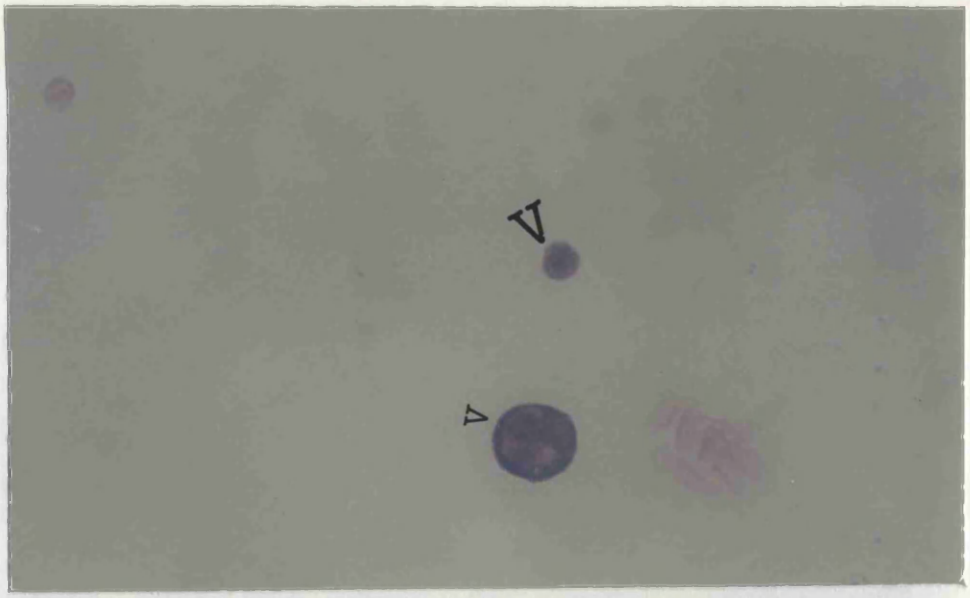


Figure 6.3 Morphological Evidence for Apoptosis of Lymphocytes from Orally Tolerant Animals.

PLN were removed 14d after immunisation of mice with OVA/CFA and cultured in the absence (A & B) or presence (C, D & E) of OVA for 120h. Micrographs show cells pooled from 5 mice per group from animals fed saline (A, C & D) or 25mg OVA (B & E) 10 days before immunisation. (A) Giemsa x1000. (B) The small arrows indicate apoptotic bodies and the large arrow shows an activated macrophage containing apoptotic bodies (Giemsa x100). (C) The small arrow indicates a lymphoblast and the large arrow shows a small resting lymphocyte (Giemsa x1000). (D) Mitotic lymphocyte (Giemsa x1000). (E) Giemsa x1000.

C



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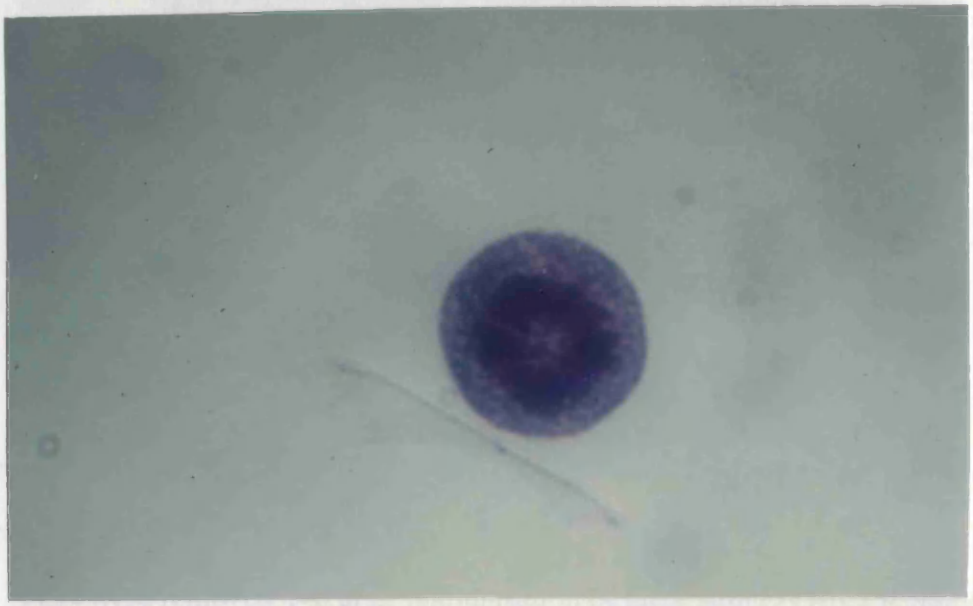

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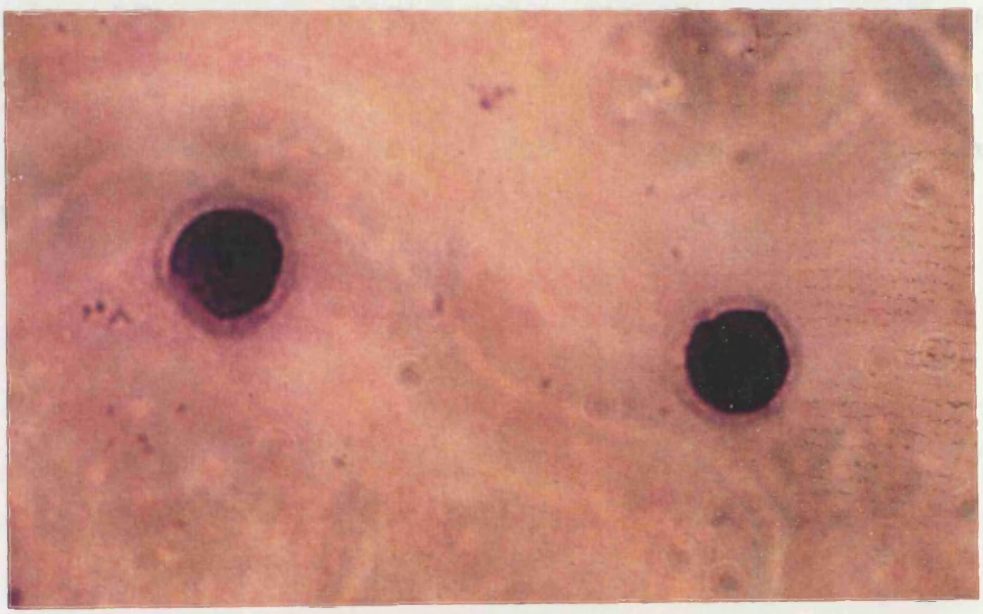


A

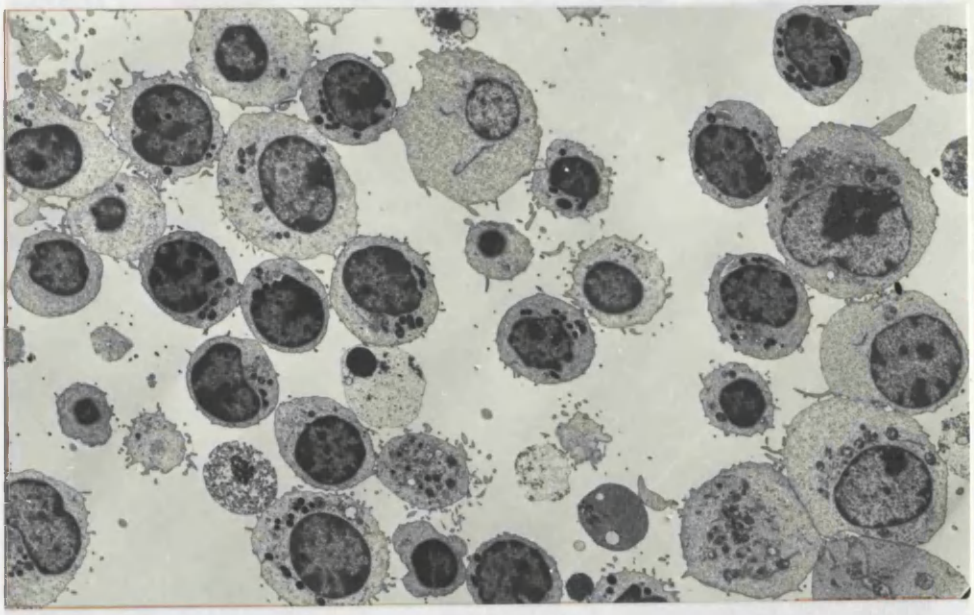
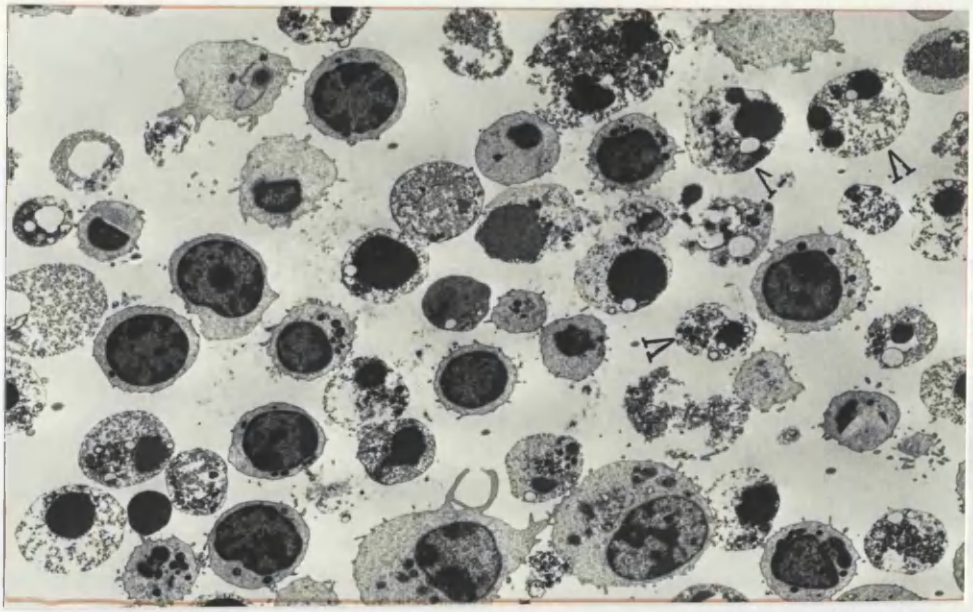


Figure 6.4 Electron Microscopic Appearance of Lymphocytes from Orally Tolerised Animals.

PLN were removed 14d after immunisation of mice with OVA/CFA and cultured in the absence (A&B) or presence (C&D) of OVA for 120h. Electron micrographs show cells pooled from 5 mice per group from animals fed saline (A&C) or 25mg OVA (B&D) 10 days before immunisation. Arrows indicate some of the apoptotic cells present, which have condensed chromatin (x2025).

B



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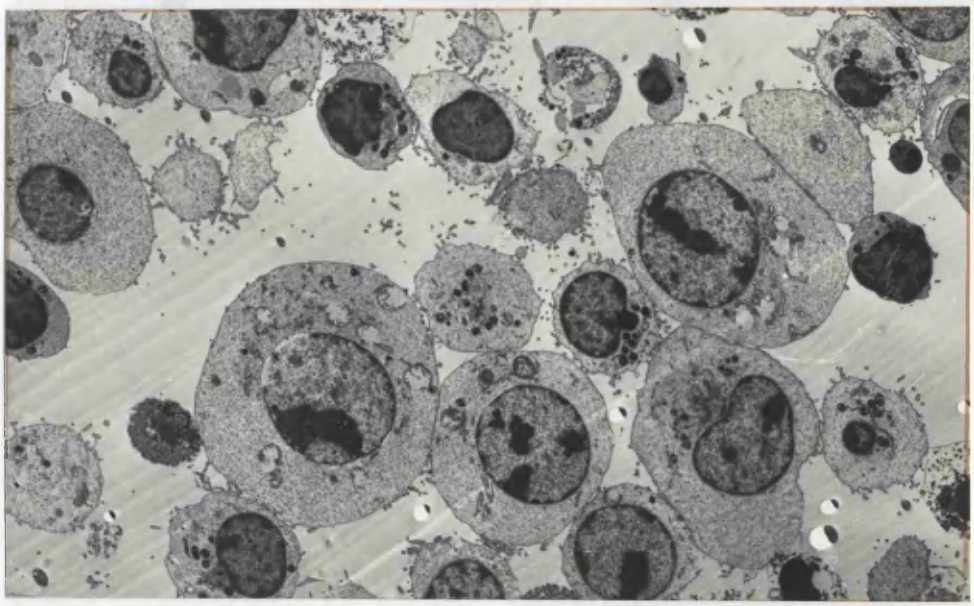

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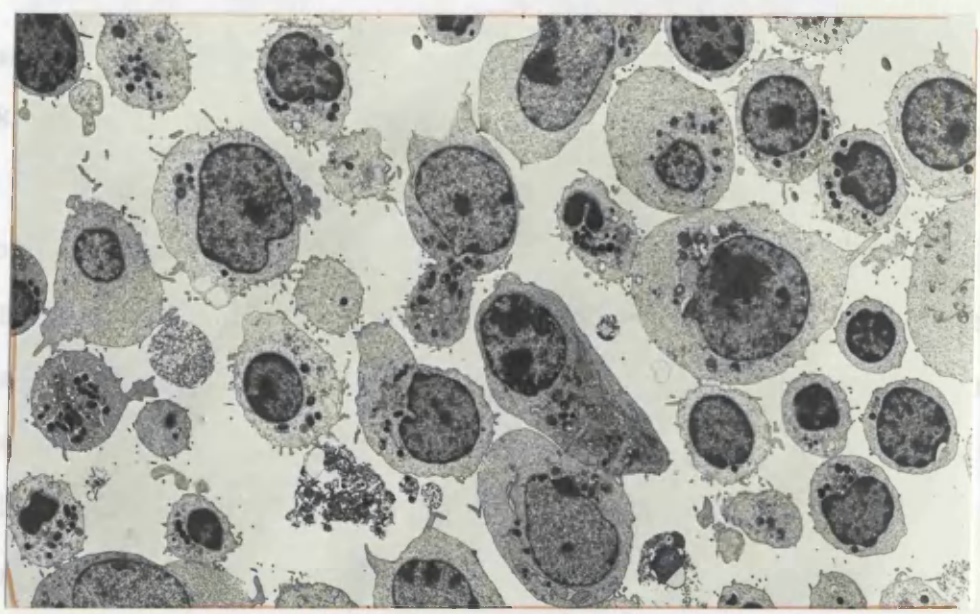


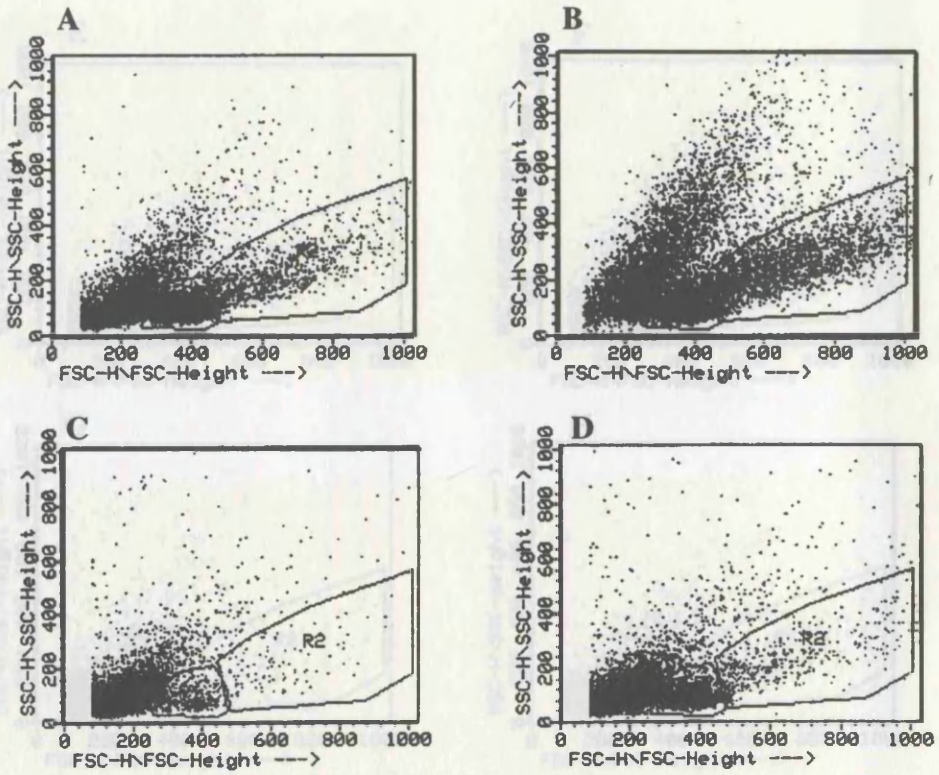
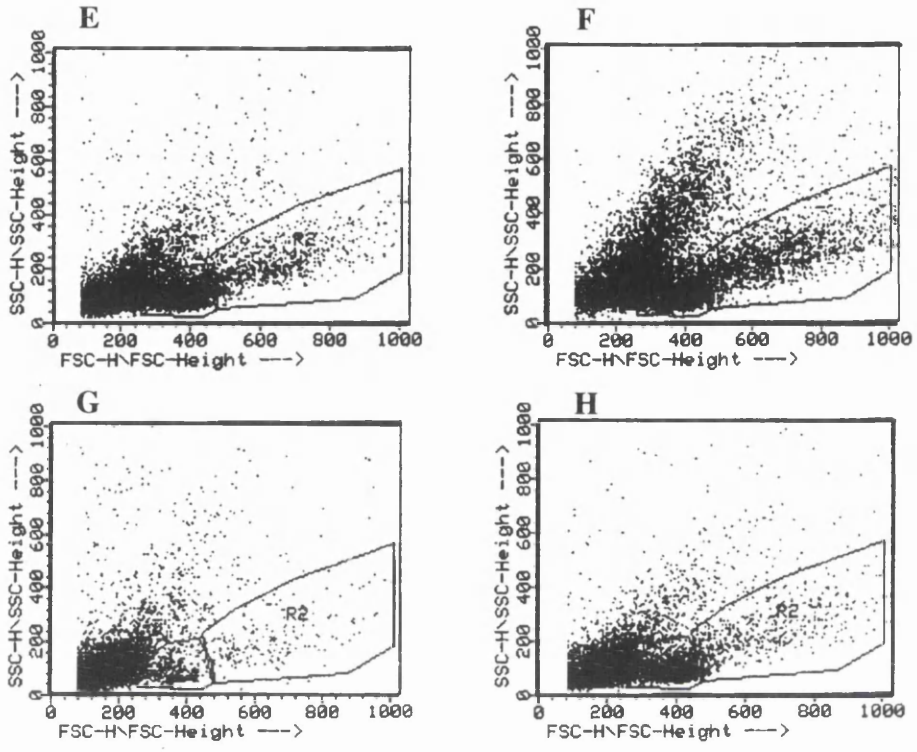


Figure 6.5 High Dose Oral Tolerance Results in Loss of Both CD4$^+$ and CD8$^+$ Lymphocytes During Culture *in vitro*.

Flow cytometric appearance of phenotypically defined populations of PLN cells removed 14d after immunisation of mice with OVA/CFA and cultured for 120h in the absence (A, C, E & G) or presence (B, D, F & H) of OVA (1mg/ml). The plots show the forward and side light scatter of lymphocytes pooled from 5 mice per group for animals fed saline (A, B, E & F) or 25mg OVA (C, D, G & H) and previously gated on the expression of CD4 (A, B, C & D) or CD8 (E, F, G & H). Similar results were obtained in 2 replicate experiments.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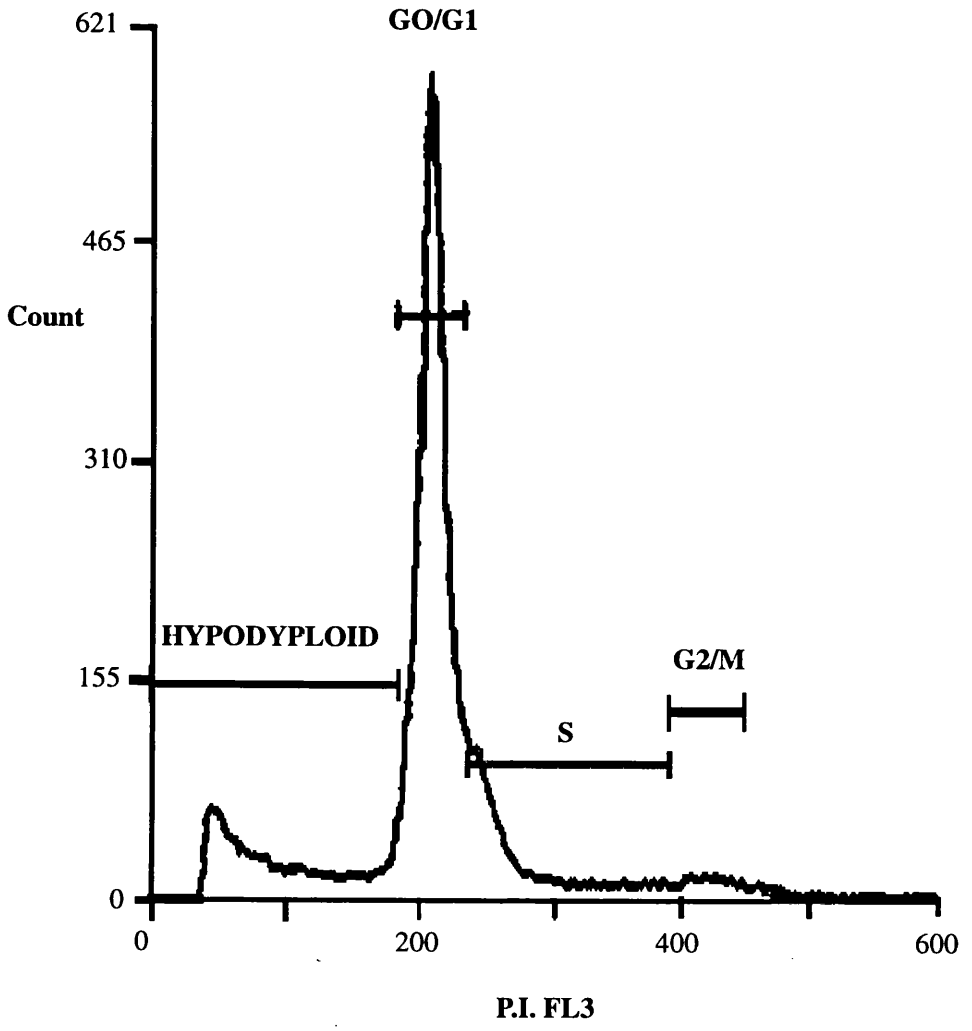


Figure 6.6 Cell Cycle Profile Obtained by Staining with PI.

Flow cytometric cell cycle analysis of normal PLN cells removed 14d after immunisation of mice with OVA/CFA and cultured for 120 in medium alone. The histogram represents cells differentially stained with PI according to their position in cell cycle. In G0/G1 phase, cells are resting and contain normal diploid nuclear DNA (2n). In G2/M phase, cells are undergoing mitosis and have doubled their nuclear DNA content (4n). A nuclear DNA content between 2n and 4n indicates cells positioned in the S phase of cell cycle, while less than 2n DNA represents apoptotic cells with hypodiploid nuclear DNA.

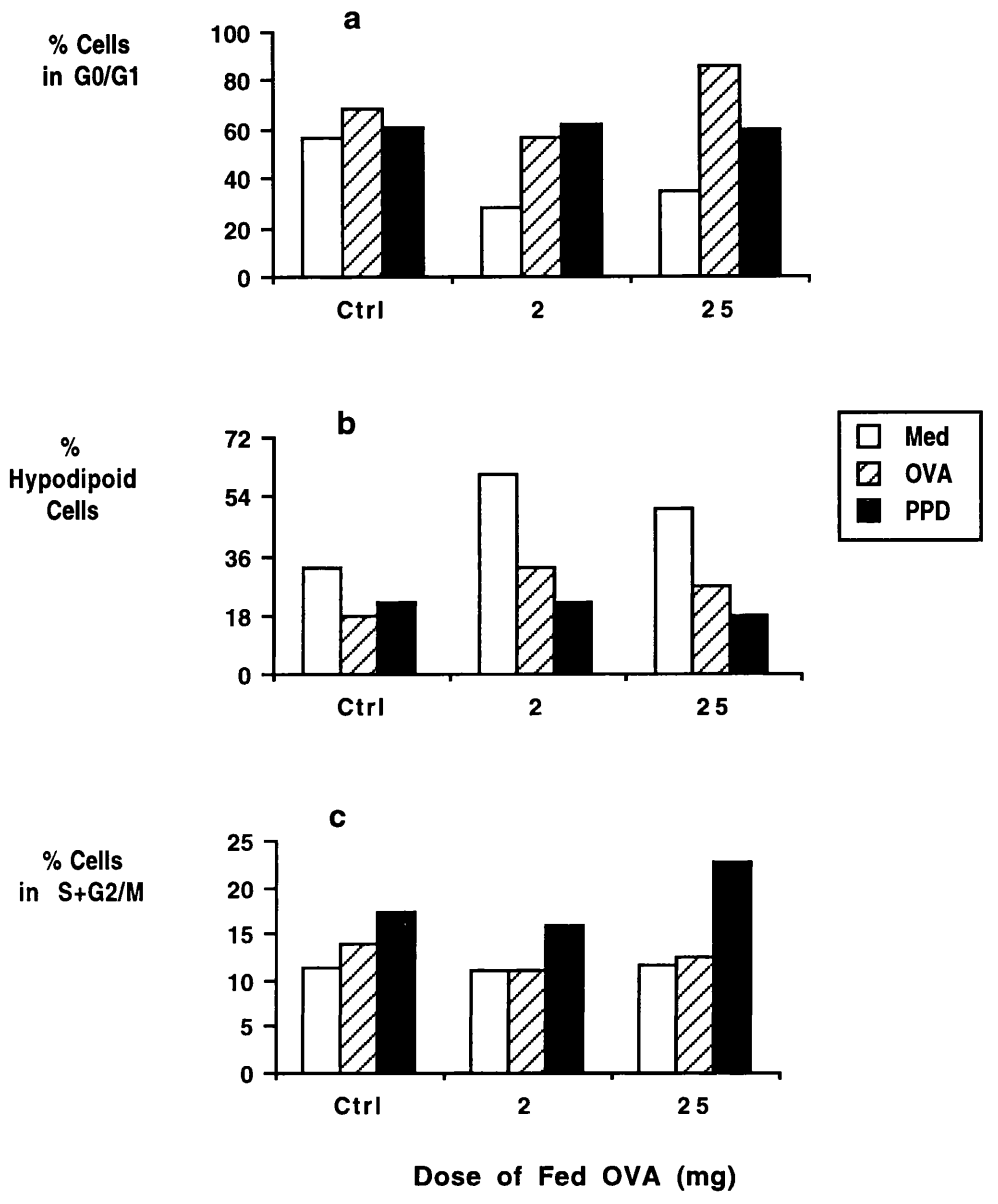


Figure 6.7 Orally Tolerant Cells Undergo DNA Fragmentation *in vitro*.

Flow cytometric cell cycle analysis of PI stained PLN cells removed from mice 14d after immunisation with OVA/CFA and cultured for 72h in medium ± OVA (1mg/ml) or PPD (50μg/ml). The results shown are percentages of cells positioned in (a) G0/G1 and (c) S+G2/M phases of cell cycle or (b) those containing hypodiploid DNA and pooled from 5 mice per group for mice fed saline (Ctrl), 2 or 25mg OVA 10 days before immunisation. Similar results were obtained in three further experiments.

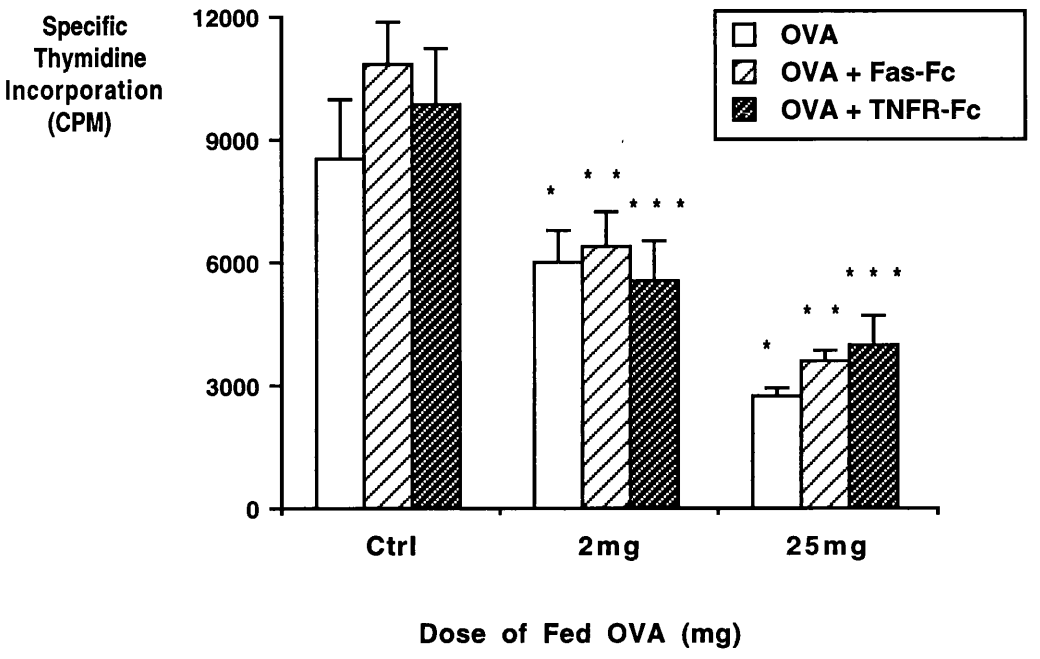


Figure 6.8 Defective Proliferation of Tolerant Lymphocytes is not Fas Dependent.

OVA-specific proliferative responses of PLN cells taken 14d after immunisation of mice with OVA/CFA and restimulated for 72h with OVA (1mg/ml) ± either fas-Fc or TNFR-Fc fusion proteins (both 20μg/ml). The results shown are mean ^{3}H-TdR incorporation (CPM) ± 1 SEM in quadruplicate cultures from cells pooled from 5 mice per group for animals fed saline (Ctrl), 2 or 25mg OVA 10 days before immunisation. (*$p<0.05$ versus OVA-restimulated Ctrl cells. **$p<0.05$ versus OVA-restimulated Ctrl cells in the presence of fas-Fc. ***$p<0.05$ versus OVA-restimulated Ctrl cells in the presence of TNFR-Fc).

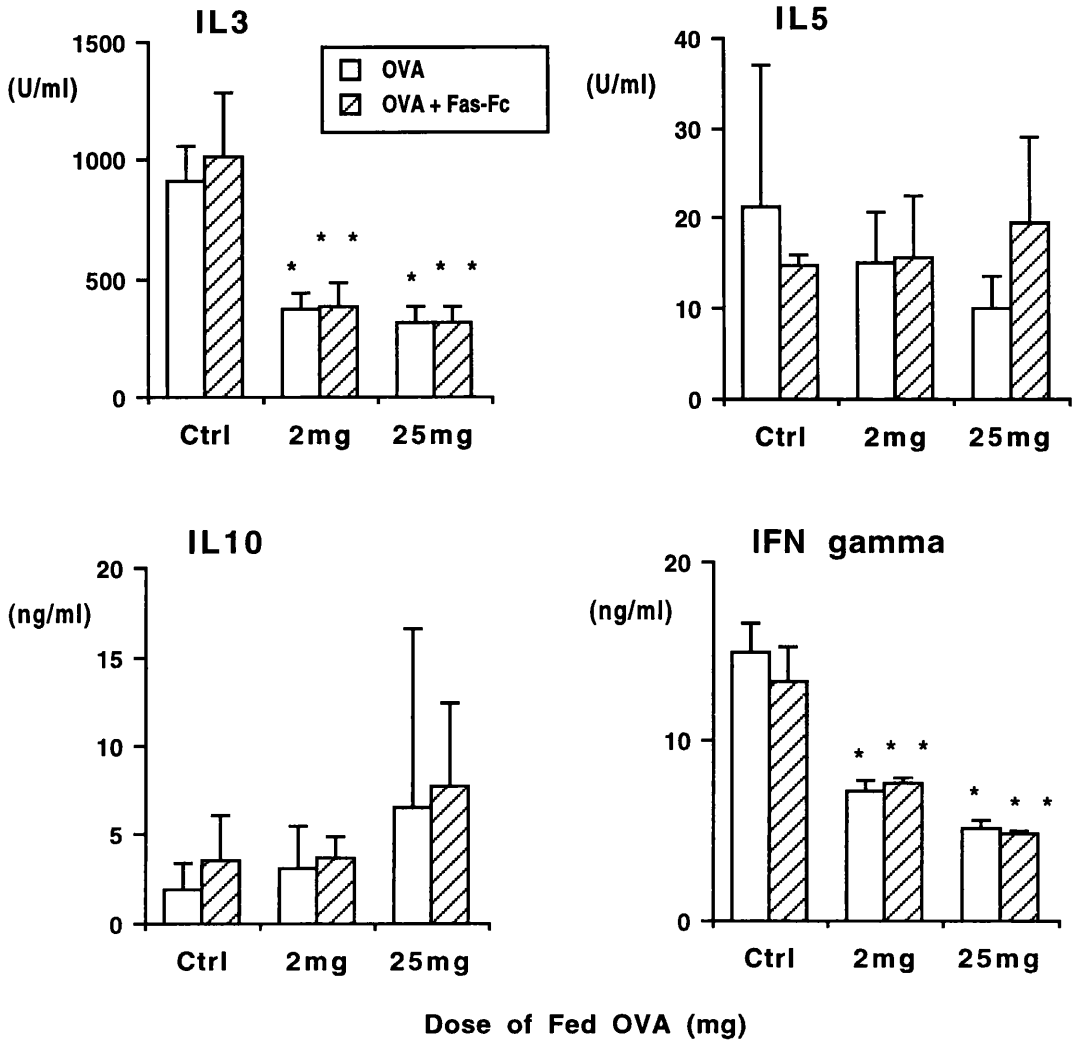


Figure 6.9 Defective OVA-specific Cytokine Production by Tolerant Lymphocytes is not Fas Dependent.

OVA-specific IL3, IL5, IL10 and IFNγ production by PLN cells taken 14d after immunisation of mice with OVA/CFA and restimulated with OVA (1mg/ml) ± fas-Fc fusion protein (20μg/ml) for 72h. The results shown are mean cytokine levels (U/ml or ng/ml) ± 1 SEM from culture supernatants of cells pooled from 5 mice per group for animals fed saline (Ctrl), 2 or 25mg OVA 10 days before immunisation. There was little or no production of any cytokines from cells cultured in the absence of antigen. (*$p<0.05$ versus OVA-restimulated Ctrl cells; **$p<0.05$ versus OVA-restimulated Ctrl cells in the presence of fas-Fc).

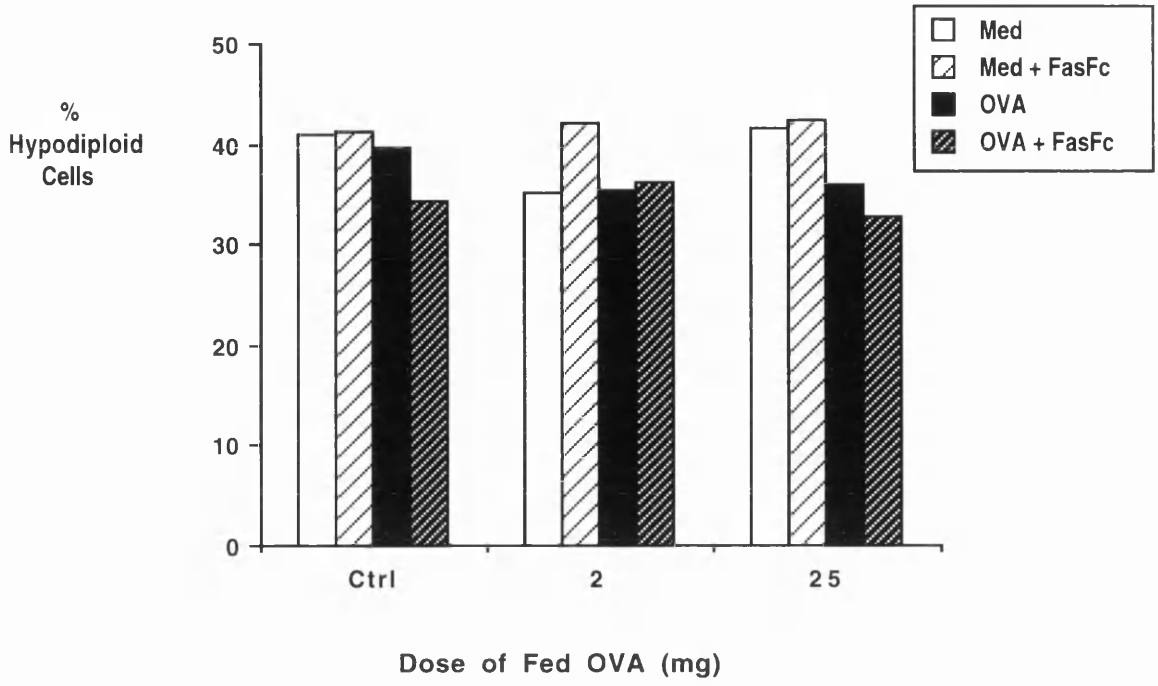


Figure 6.10 Role of Fas in the Predisposition of Tolerant Cells to Apoptose *in vitro*.

Flow cytometric cell cycle analysis of PI stained PLN cells removed from mice 14d after immunisation with OVA/CFA and cultured for 72h in medium ± OVA (1mg/ml) ± fasFc fusion protein (20µg/ml). The results shown are percentages of cells with a hypodiploid nuclear DNA content from cells pooled from 5 mice per group for animals fed saline (Ctrl), 2 or 25mg OVA 10 days before immunisation.

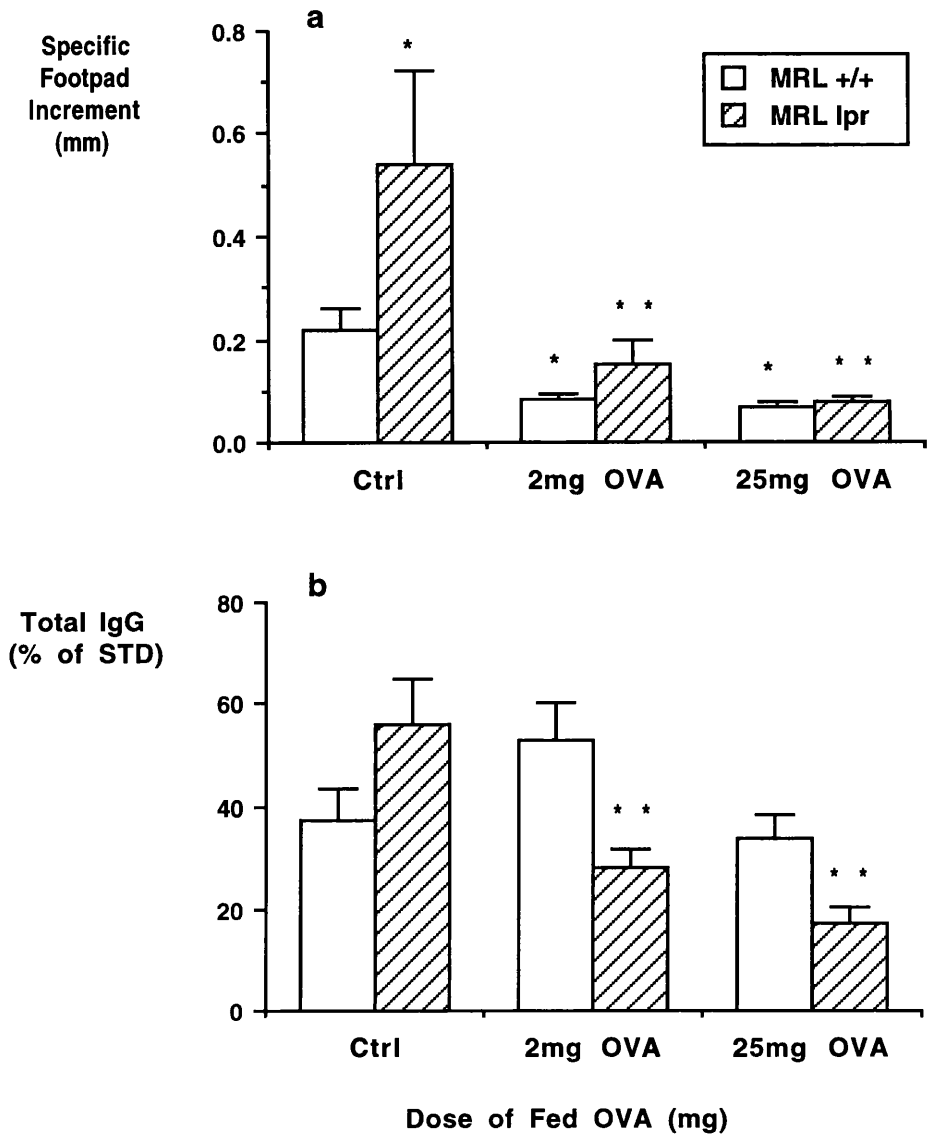


Figure 6.11 Induction of Oral Tolerance is Normal in MRL lpr Mice.

a) OVA-specific DTH responses in mice immunised 21d before with OVA/CFA. Results shown are mean specific increments in footpad thickness ± 1 SEM for 5 mice per group in $MRL^{+/+}$ or MRL lpr animals fed saline (Ctrl), 2 or 25mg OVA. b) Total OVA-specific IgG responses in mice 21d after immunisation with OVA/CFA. The results shown are calculated with reference to a hyperimmunised control serum and are mean % ± 1 SEM for individual sera from 5 mice per group in $MRL^{+/+}$ or MRL lpr animals fed saline (Ctrl), 2 or 25mg OVA 10 days before immunisation. (*$p<0.05$ versus $MRL^{+/+}$ Ctrl; **$p<0.05$ versus MRL lpr Ctrl). Similar results were obtained in a replicate experiment.

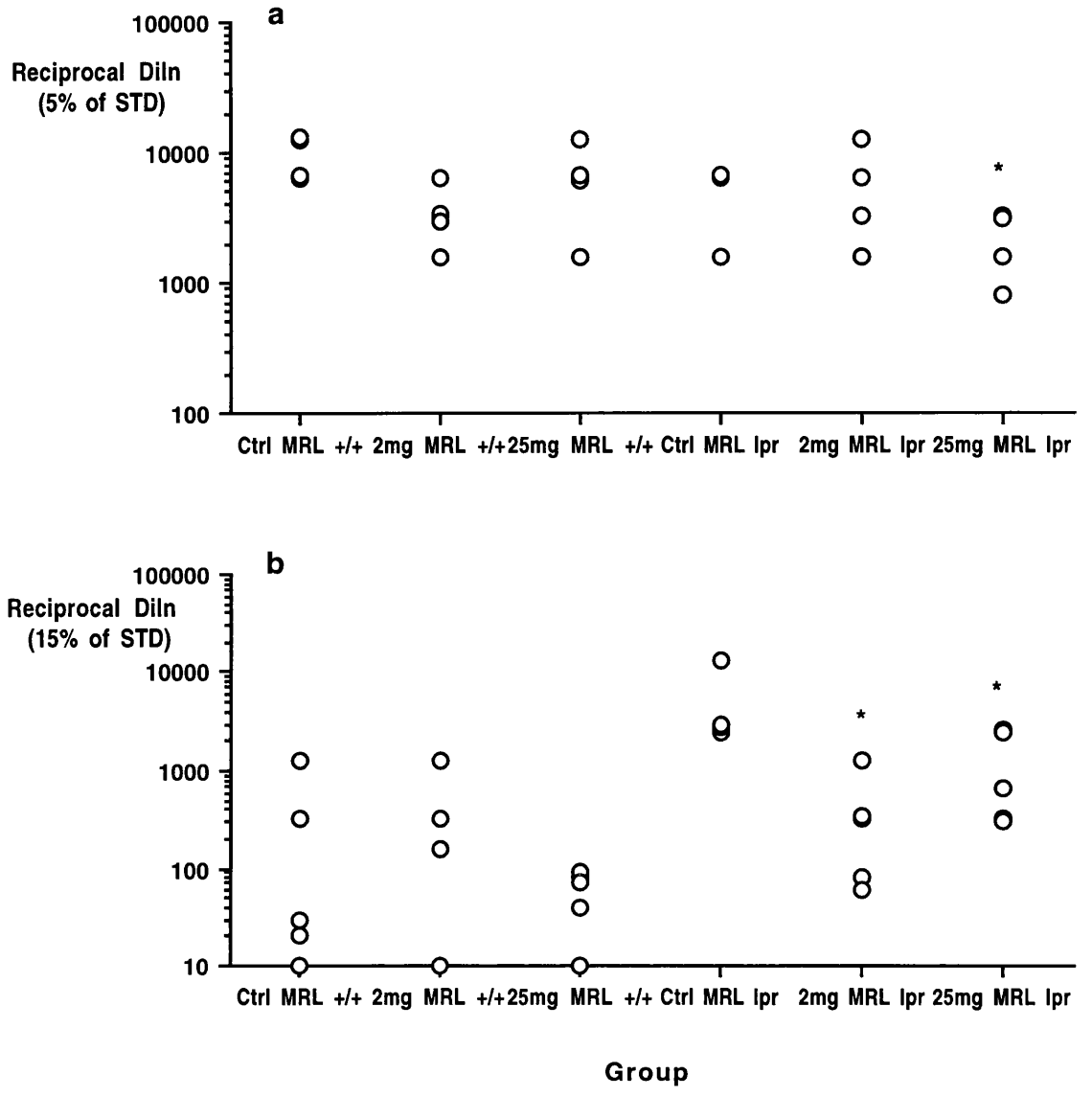


Figure 6.12 Suppression of Systemic Antibody Isotypes is Normal in OVA Fed MRL lpr Mice

OVA-specific serum IgG1 (a) and IgG2a (b) responses in mice 21d after immunisation with OVA/CFA. The results shown are the reciprocal dilutions giving an OD value equivalent to (a) 5% or (b) 15% of a hyperimmunised control serum diluted (a) 1/400 or (b) 1/20 and are for individual sera from 5 mice per group in $MRL^{+/+}$ or MRL lpr animals fed saline (Ctrl), 2 or 25mg OVA 10 days before immunisation. (*$p<0.05$ versus MRL lpr Ctrl). Similar results were obtained in a replicate experiment.

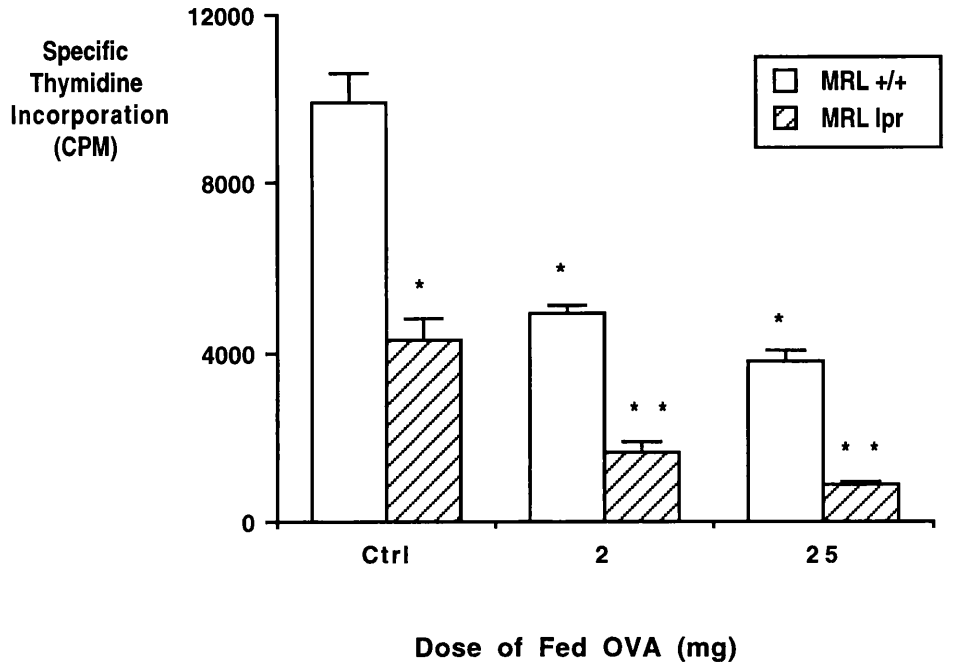


Figure 6.13 Suppression of OVA-specific Proliferation *in vitro* is Normal in MRL lpr Mice.

OVA-specific proliferative responses of PLN cells taken from mice 14d after immunisation with OVA/CFA and restimulated for 96h in OVA (1mg/ml). The results shown are mean ^{3}H-TdR incorporation (CPM) ± 1 SEM in quadruplicate cultures from cells pooled from 5 mice per group in MRL$^{+/+}$ or MRL lpr mice fed saline (Ctrl), 2 or 25mg OVA 10 days before immunisation. (*$p<0.05$ versus MRL$^{+/+}$; **$p<0.05$ versus MRL lpr). Similar results were obtained in a replicate experiment.

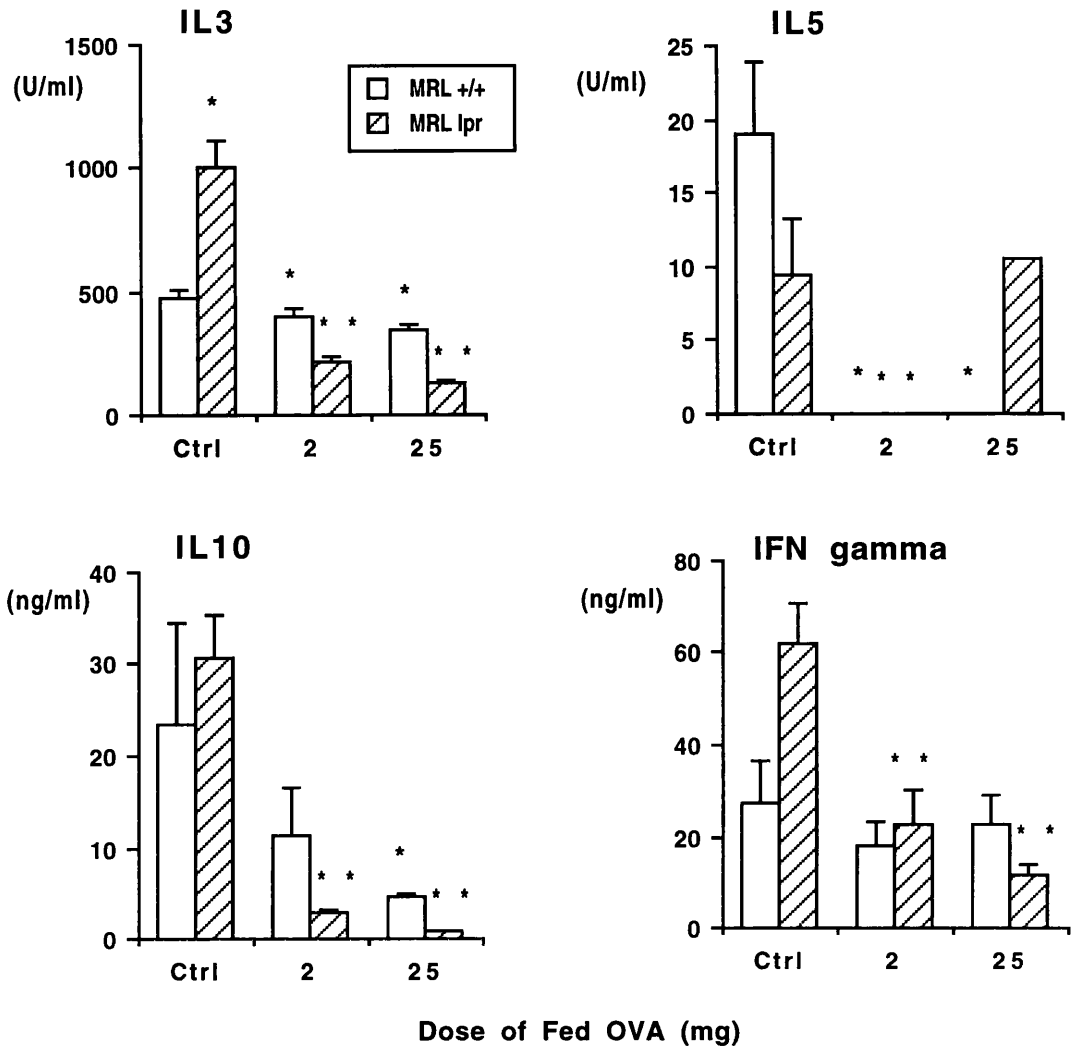


Figure 6.14 Suppression of OVA-specific Cytokine Production *in vitro* is Normal in MRL lpr Mice.

OVA-specific production of IL3, IL5, IL10 and IFNγ by PLN cells taken from mice 14d after immunisation with OVA/CFA and restimulated for 96h in OVA (1mg/ml). The results shown are mean cytokine levels (U/ml or ng/ml) ± 1 SEM in cultures of cells pooled from 5 mice per group in $MRL^{+/+}$ or MRL lpr mice fed saline (Ctrl), 2 or 25mg OVA 10 days before immunisation. (*$p<0.05$ versus $MRL^{+/+}$; **$p<0.05$ versus MRL lpr). Similar results were obtained in a replicate experiment.

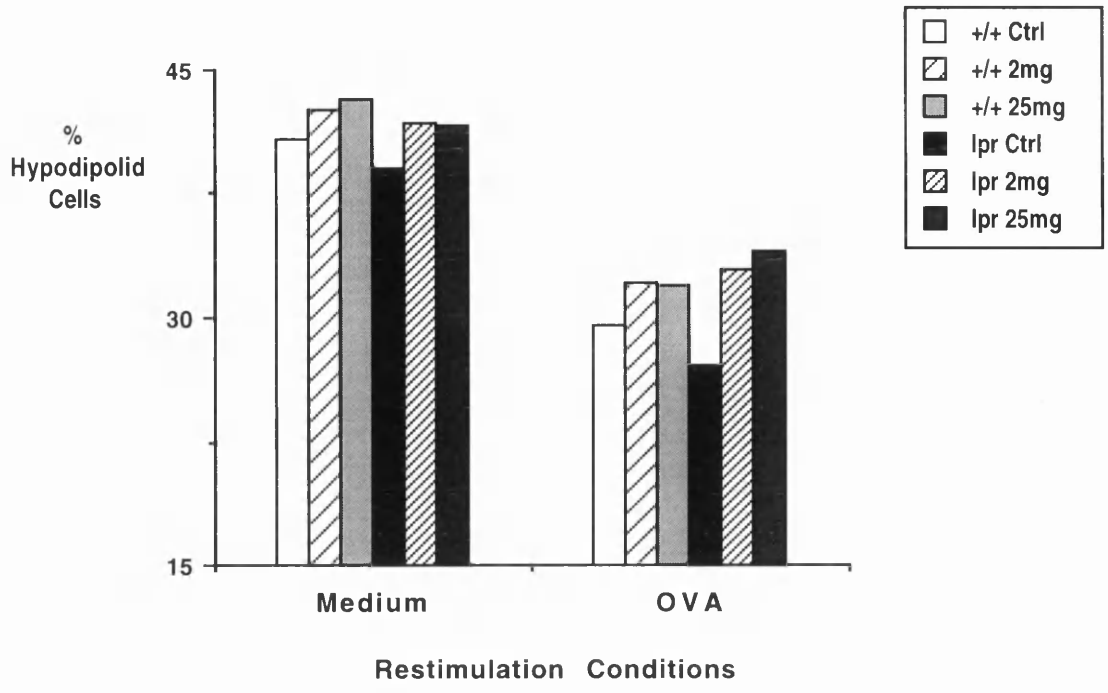


Figure 6.15 Cell Death of Orally Tolerant MRL lpr cells *in vitro*

Flow cytometric cell cycle analysis of PLN cells removed from mice 14d after immunisation with OVA/CFA and cultured for 96h in medium alone or with OVA (1mg/ml). The results shown are the percentages of hypodiploid cells from $MRL^{+/+}$ or MRL lpr mice fed saline (Ctrl), 2 or 25mg OVA 10 days prior to immunisation, as determined by flow cytometric analysis of PI stained cells. Similar results were obtained in a replicate experiment.

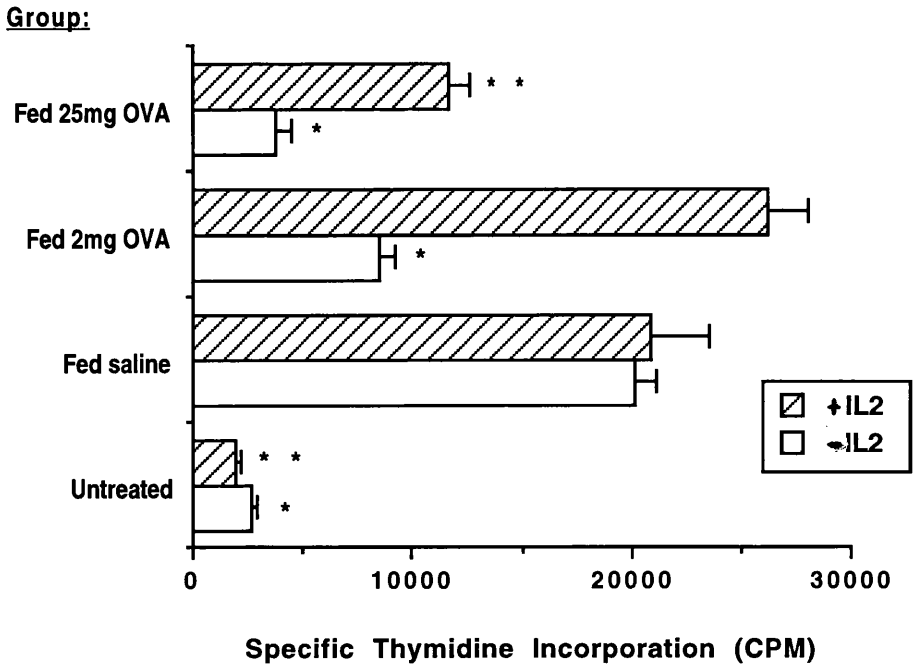


Figure 6.16 OVA-specific Proliferative Responses of Tolerant Cells are Restored by Preculture with IL2.

OVA-specific proliferative responses of PLN cells removed from mice 14 days after immunisation with OVA/CFA and cultured for 5 days ± rIL2 (50U/ml) before washing, resting overnight and culturing with mitomycin C-treated naive spleen cells ($1.25x10^6$ cells/ml) ± OVA (1mg/ml) for 96h. The results shown are mean ^{3}H-TdR incorporation (CPM) ± 1 SEM in quadruplicate cultures of cells pooled from 11 mice per group in animals fed saline, 2 or 25mg OVA 10 days before immunisation or untreated mice. (*$p<0.05$ versus saline fed cells + rIL2; **$p<0.05$ versus saline fed cells - rIL2)

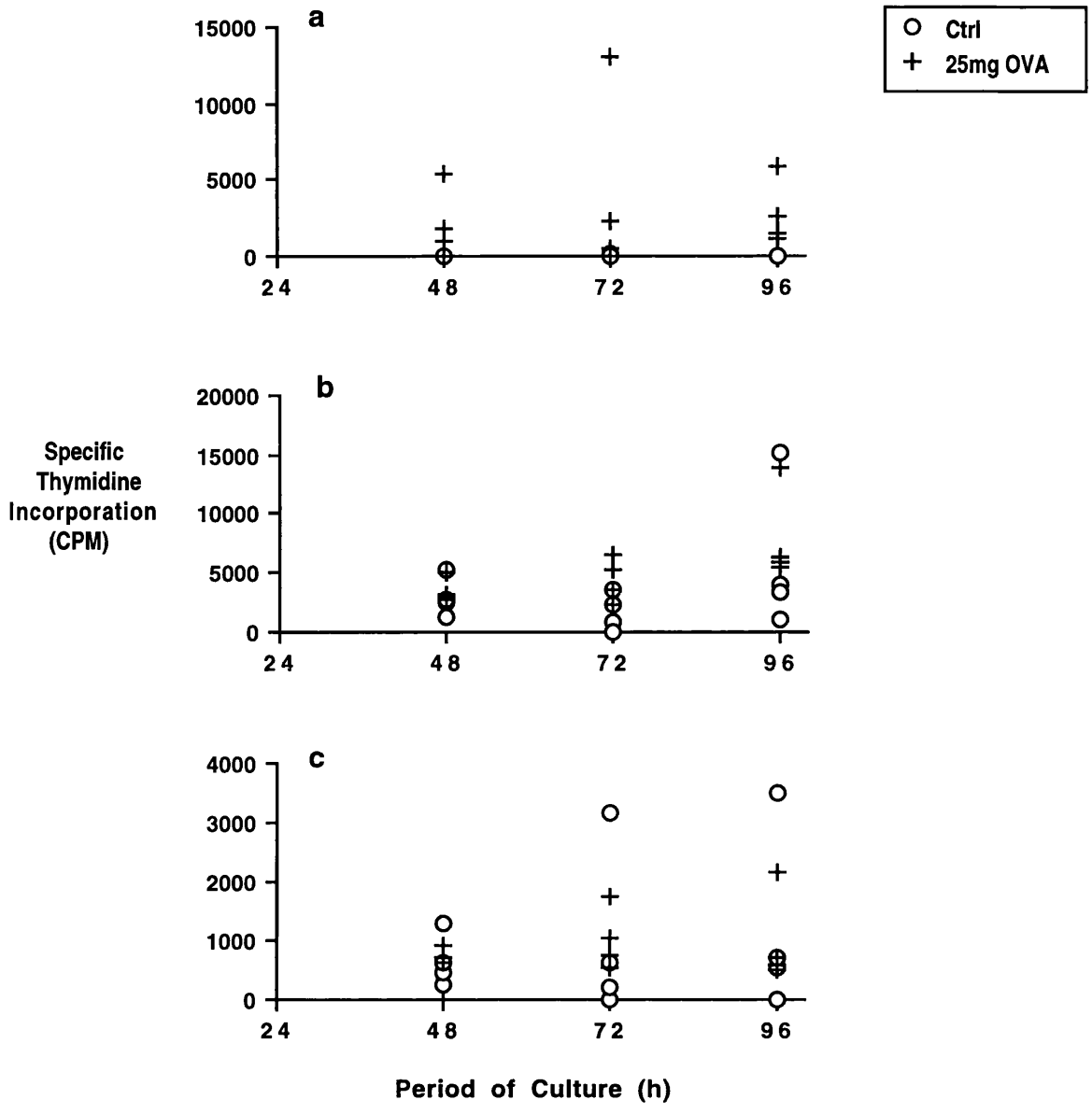


Figure 6.17 Priming of Antigen-specific Proliferation by Feeding a Tolerogenic Dose of OVA.

OVA-specific proliferative responses of spleen cells removed from individual mice (a) 1, (b) 2 or (c) 4 days after feeding either saline (Ctrl) or 25mg OVA and cultured with OVA (1mg/ml) for 96h. The results shown are mean ^{3}H-TdR incorporation (CPM) in quadruplicate cultures of cells from individual mice.

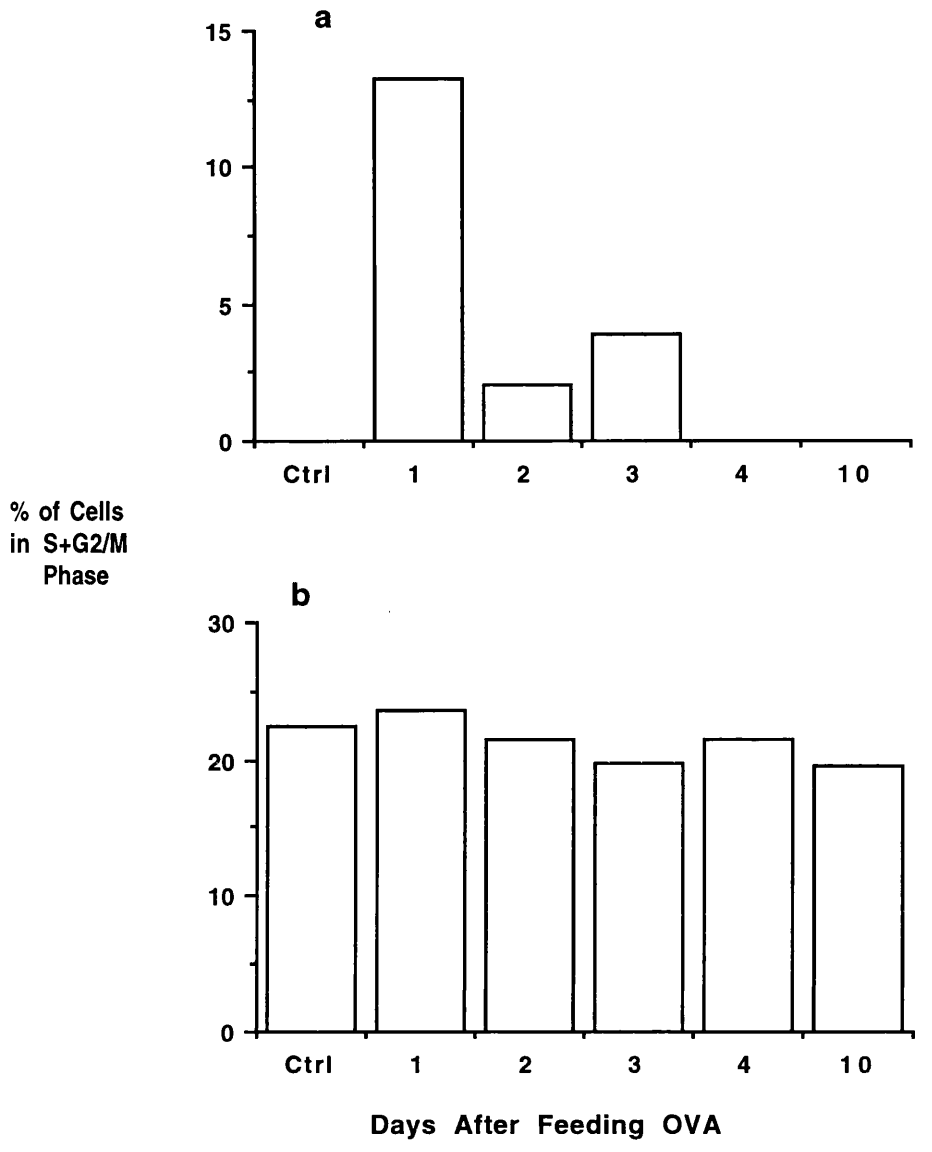


Figure 6.18 Feeding a Tolerogenic Dose of OVA Induces Cell Activation *in vitro*.

Flow cytometric cell cycle analysis of spleen cells removed 1, 2, 3, 4 or 10 days after feeding mice saline (Ctrl) or 25mg OVA and cultured for 72h either with (a) OVA (1mg/ml) or (b) Con A (10μg/ml). The results shown are the percentages of cells in the S and G2/M phases of cell cycle with background levels in medium subtracted, as determined by flow cytometric analysis of PI stained cells.

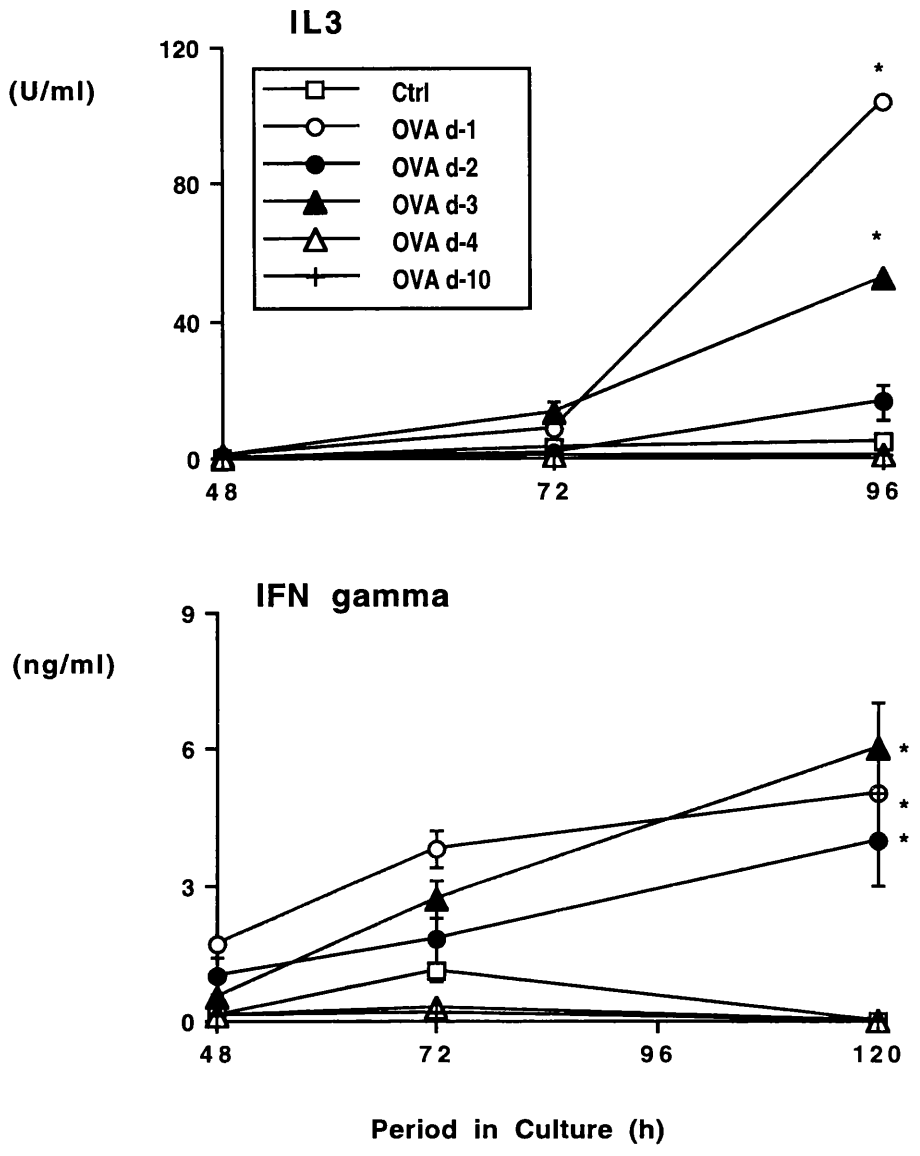


Figure 6.19a Priming of Antigen-specific Cytokine Production by Feeding a Tolerogenic Dose of OVA.

Secretion of IL3 and IFNγ by spleen cells taken from mice 1, 2, 3, 4 or 10 days after feeding saline (Ctrl) or 25mg OVA and cultured with OVA (1mg/ml) for up to 120h. The results shown are mean cytokine levels (U/ml or ng/ml) ± 1 SEM in culture supernatants of cells pooled from 5 mice per group. Negligible levels of OVA-specific IL5 and IL10 were detected by any group examined. (*$p<0.05$ versus Ctrl). Similar results were obtained in three replicate experiments.

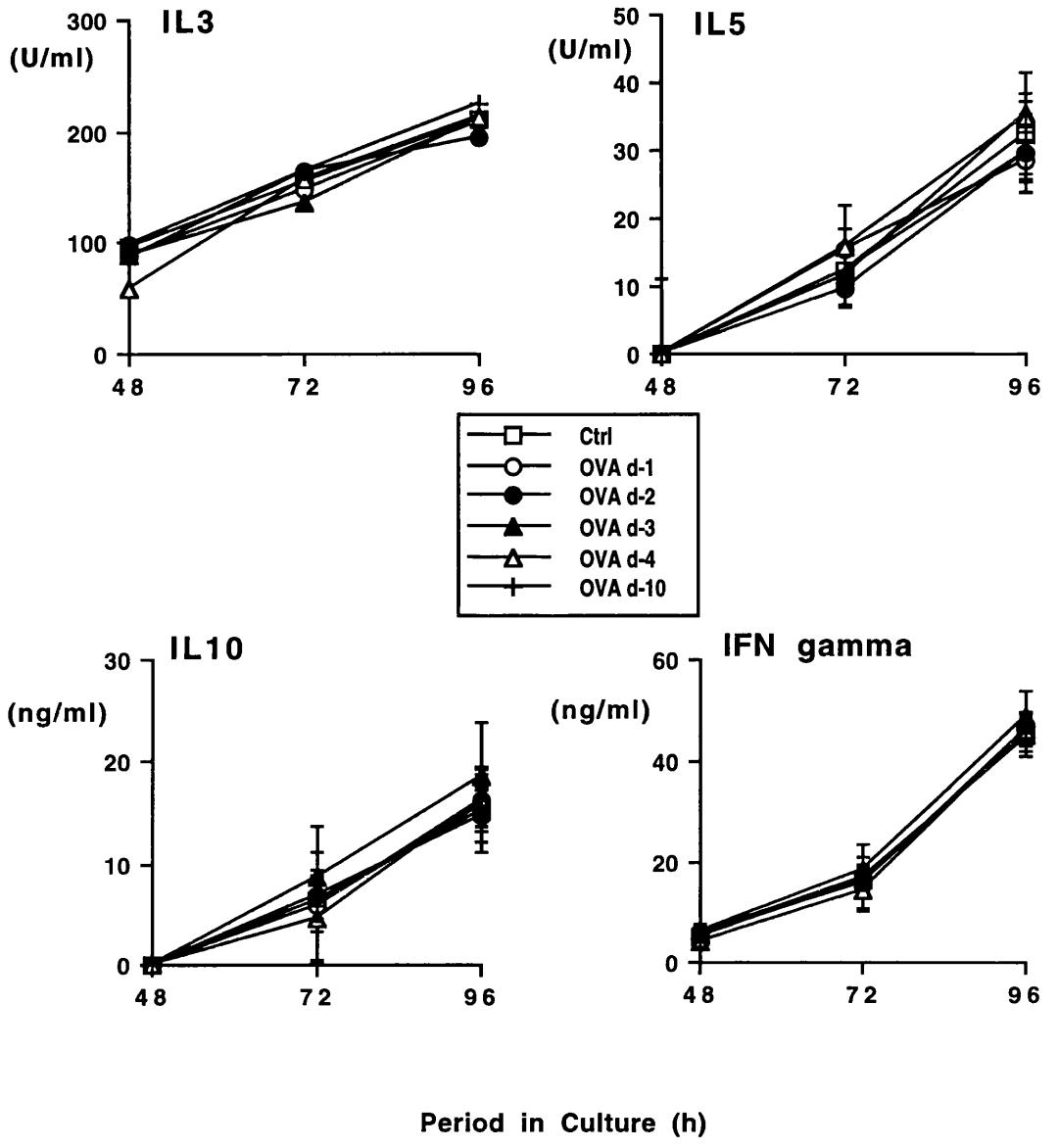


Figure 6.19b Priming of Antigen-specific Cytokine Production by Feeding a Tolerogenic Dose of OVA.

Secretion of IL3, IL5, IL10 and IFNγ by spleen cells taken from mice 1, 2, 3, 4 or 10 days after feeding saline (Ctrl) or 25mg OVA and cultured with Con A (10μg/ml) for up to 120h. The results shown are mean cytokine levels (U/ml or ng/ml) ± 1 SEM in culture supernatants of cells pooled from 5 mice per group. (*$p<0.05$ versus Ctrl). Similar results were obtained in three replicate experiments.

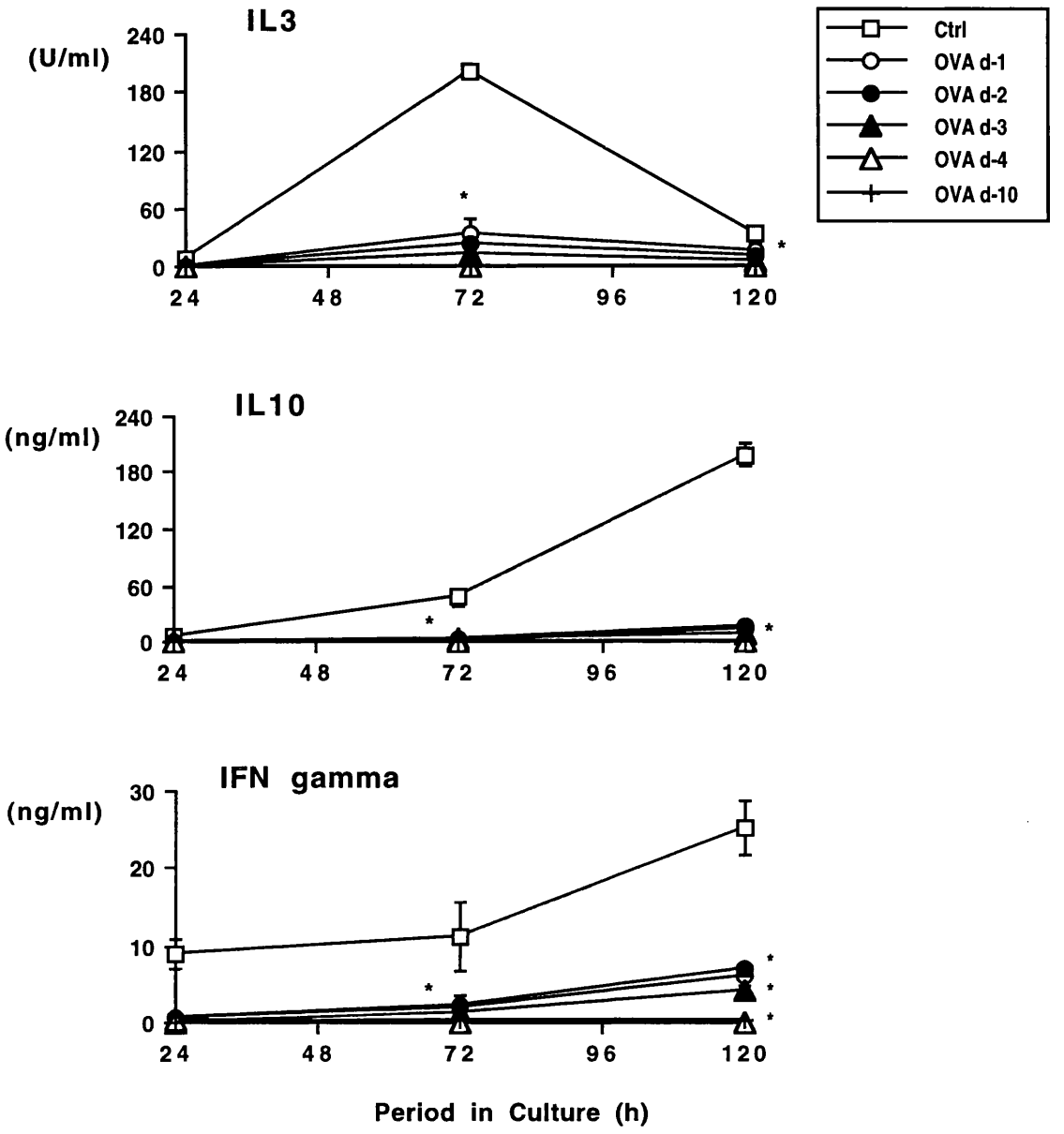


Figure 6.20 Tolerance to Parenteral Challenge is Induced Within a Day of Feeding OVA

OVA-specific production of IL3, IL10 and IFNγ by spleen cells removed from mice 14 days after immunisation with OVA/CFA and cultured for 120h in OVA (1mg/ml). The results shown are mean cytokine levels (U/ml or ng/ml) ± 1 SEM in culture supernatants of cells pooled from 5 mice per group for animals fed 1, 2, 3, 4 or 10 days previously with saline (Ctrl) or 25mg OVA. (*$p<0.05$ versus Ctrl). Similar results were obtained in three replicate experiments.

Table 6.1: % CD4$^+$ and CD8$^+$ Non-viable PLN Cells *in vitro*

i) In the absence of antigen:

Group	CD4$^+$	CD8$^+$
Ctrl	57.2	64.2
25mg OVA	94.6	94.1

ii) In OVA:

Group	CD4$^+$	CD8$^+$
Ctrl	53.7	62.9
25mg OVA	78.7	74.8

Flow cytometric analysis of PLN cells from mice 14 days after s.c. immunisation with 100μg OVA/CFA and cultured for 120h ± OVA (1mg/ml). Results shown are % non-viable cells after 120h culture of cells from mice fed saline (Ctrl) or 25mg OVA 10 days prior to immunisation. Similar results were obtained in 2 repeat experiments.

Table 6.2: Absolute Numbers of $CD4^+$ and $CD8^+$ PLN Cells in Tolerant Cultures

i) In the absence of antigen:

Group	No. $CD4^+$ Cells/ml	No. $CD8^+$ Cells/ml
Ctrl	$6.4x10^5$	$2.5x10^5$
2mg OVA	$2.8x10^5$	$2.4x10^5$
25mg OVA	$1.8x10^5$	$0.84x10^5$

ii) In OVA:

Group	No. $CD4^+$ Cells/ml	No. $CD8^+$ Cells/ml
Ctrl	$8.3x10^5$	$3.3x10^5$
2mg OVA	$6.1x10^5$	$2.4x10^5$
25mg OVA	$3.5x10^5$	$1.63x10^5$

Flow cytometrical analysis of PLN cells removed from mice 14 days after s.c. immunisation with OVA/CFA and cultured at $4x10^6$cells/ml ± OVA (1mg/ml). Results show absolute numbers of $CD4^+$ or $CD8^+$ cells/ml remaining after 120h culture of cells from mice fed saline (Ctrl), 2 or 25mg OVA 10 days prior to immunisation. Similar results were obtained in 2 repeat experiments.

Table 6.3: % Cell Recovery After Exposure to rIL2

Fed	Immunised with	% (-rIL2)	% (+rIL2)
Saline	CFA	16.5	9.6
Saline	OVA/CFA	16.5	15.3
2mg OVA	OVA/CFA	11.1	16.1
25mg OVA	OVA/CFA	9.1	14.5

Cell recovery obtained for PLN cells removed from mice 14 days after parenteral immunisation with CFA ± OVA and cultured for 5 days ± rIL2. The results shown are % total cells present at the start of culture for cells pooled from 11 mice per group in animals fed saline (Ctrl), 2 or 25mg OVA 10 days before immunisation.

Chapter 7: The Longevity of Oral Tolerance

Introduction

Several experimental autoimmune disorders can be suppressed by inducing oral tolerance [94, 105, 193, 336, 337], and this approach is being proposed as a treatment for clinical disease. As most current immunosuppressive therapies are non-specific and have many side-effects, they are unsuitable for long-term use, and an alternative antigen-specific treatment, such as oral tolerance, would be of great benefit if it was sufficiently long-lasting.

The experiments I have described thus far illustrate that the peripheral tolerance induced by feeding OVA is profound, can inhibit effector responses of both Th1 and Th2 cell subsets and may reflect T cell anergy and/or deletion. It might be predicted that this would be likely to have long-lasting effects, an idea consistent with a single previous report that oral tolerance is a stable and persistent phenomenon [120]. However, this earlier work also demonstrated that the tolerance of cell-mediated immunity persisted longer than that of humoral immunity, which was present for only 3 months after feeding antigen. Although this might suggest that the use of oral tolerance as a long-lasting immunotherapy would be problematic, the long term effects of feeding antigen on a wide range of effector responses has never been examined either in experimental models or in clinical practice.

In this chapter, I therefore documented the longevity of oral tolerance both *in vitro* and *in vivo* from 10 days to 18 months after feeding mice a single bolus of OVA. I also examined both 2 and 25mg doses of fed OVA to determine how the feeding dose influenced the longevity of tolerance.

Experimental Protocol

Oral tolerance was induced by feeding BALB/c mice 2 or 25mg OVA and the mice were challenged s.c. with OVA/CFA 10 days, 3, 6, 9 or 18 months later. Control mice were fed saline before immunisation.

Results

Approximately 25% of both OVA fed and control mice did not survive up to 18 months, indicating that I was examining the longevity of oral tolerance virtually over the entire life span of the animals.

(A) Persistence of Oral Tolerance *in vivo*

DTH Responses

The OVA-specific DTH responses of mice fed 25mg OVA were significantly lower than those of immunised controls at every time point examined after feeding (Fig 7.1a), with equivalent tolerance for at least 18 months. This was despite the general decrease in DTH responses displayed by ageing control mice (Fig 7.1a). Although feeding 2mg OVA also induced significant tolerance of DTH responses, this was only present until 9 months after feeding and the responses measured at 18 months were comparable to those of controls (Fig 7.1a).

Serum Antibody Responses

As I found in previous chapters, the serum levels of OVA-specific IgG, IgG1 and IgG2a antibodies could be significantly reduced by feeding either 2 or 25mg OVA before immunisation (Figs 7.1b & 7.2a&b). However, this pattern was only found in animals immunised 10 days after feeding (Fig 7.1b & 7.2a&b), with the exception that IgG1 responses were also significantly tolerised 3 months after feeding 25mg OVA (Fig 7.2a). Thereafter, all antibody responses in OVA fed mice were comparable tothose of controls (Fig 7.1b & 7.2a&b), although it should be noted that the levels of both IgG isotypes in mice fed 25mg OVA were always somewhat lower than controls (Fig 7.2a&b). As with DTH responses, antibody responses declined with age in controls (Fig 7.1b & 7.2a&b).

These results confirm previous findings that oral tolerance of humoral immunity is less long-lasting than that of CMI [120].

(B) Persistence of Oral Tolerance *in vitro*

Proliferative Responses

To examine this phenomenon further, I examined cell-mediated immunity *in vitro* by measuring OVA-specific proliferation and cytokine production by PLN cells. Proliferative responses were tolerised significantly by feeding 2 or 25mg OVA, but unlike DTH responses *in vivo*, this only lasted up to 3 months after feeding either dose of OVA (Fig 7.3).

Cytokine Production

As the different effects of oral tolerance on antibody and DTH responses *in vivo* might suggest the presence of different regulatory mechanisms acting via Th1 and Th2 cells, I examined the cytokine responses of PLN cells taken from groups of mice fed 2 or 25mg OVA and parenterally immunised 10 days, 3, 6, 9 or 18 months later.

10 Days

As I described previously, feeding mice 2 or 25mg OVA 10 days before s.c. immunisation with OVA/CFA reduced the subsequent production of IL3, IL5, IL10 and IFNγ when their PLN cells were restimulated with OVA *in vitro* (Fig 7.4a, b, c & d, respectively). The cytokine responses of mice fed either 2 or 25mg OVA were reduced to a similar extent compared with controls, again consistent with my previous experiments.

3 Months

A similar pattern was found in mice challenged 3 months after feeding, when the production of OVA-specific IL3, IL5, IL10 and IFNγ was significantly reduced in comparison to controls (Fig 7.5a, b, c & d, respectively). This effect was generally similar in extent to that found 10 days after feeding for both Th1- and Th2-dependent cytokines.

6 Months

Oral tolerance of OVA-specific IL3, IL5 and IL10 production was still apparent in PLN cells removed from mice immunised 6 months after feeding 25mg OVA (Fig 7.6a, b & c). This was similar in extent to the inhibition found at earlier times, but was accompanied by a significantly enhanced OVA-specific IFNγ response compared with controls (Fig 7.6d), indicating that tolerance of Th2-dependent cytokines was conserved while Th1-dependent responses may be augmented in these animals. In contrast, by this time there was no longer tolerance of any cytokine responses in mice fed 2mg OVA. The OVA-specific production of IL3, IL5 and IFNγ by PLN cells from these animals was comparable to controls (Fig 7.6a, b & d respectively), while OVA-specific IL10 responses were now significantly increased beyond control levels (Fig 7.6c), suggesting that termination of low dose tolerance was associated with preferential upregulation of Th2 cell activity.

9 Months

A similar pattern was observed in mice challenged 9 months after feeding. Thus, production of OVA-specific IL3, IL5 and IL10 was significantly reduced in mice fed 25mg OVA compared with controls, while production of OVA-specific IFNγ, although no longer augmented, was comparable to control levels (Fig 7.7a, b, c & d, respectively). In contrast, animals fed 2mg OVA showed no evidence of tolerance of any cytokines. Their production of OVA-specific IL3 and IFNγ remained comparable with controls (Fig 7.7a & d), while OVA-specific IL10 continued to be significantly enhanced (Fig 7.7c) and was now accompanied by significantly increased levels of OVA-specific IL5 compared with controls (Fig 7.7b). These findings provide further evidence for a skewed immune response in favour of Th2-dependent cytokine production when low dose tolerance had declined.

18 Months

The OVA-specific production of every cytokine examined in control mice immunised 18 months after feeding saline was very low (Fig 7.8a, b, c & d). However, mice fed 25mg OVA and immunised 18 months later continued to display significant tolerance of OVA-specific IL3, IL5 and IL10 (Fig 7.8a, b & c). This was accompanied by an IFNγ response similar to that observed in controls (Fig 7.8d). All the cytokine responses of animals fed

2mg OVA prior to immunisation were comparable to controls (Fig 7.8 a, b, c & d), indicating that both oral tolerance and skewing towards Th2-dependent cytokine production were no longer present by this time.

Summary and Conclusions

The results presented in this chapter confirm and extend previous studies by demonstrating that oral tolerance is a persistent phenomenon with different long-term effects on individual effector responses. In addition, these effects are dependent on the initial dose of fed antigen.

Peripheral immune unresponsiveness was most persistent when antigen was fed in high doses. Animals fed 25mg OVA showed some degree of tolerance for virtually their entire life-span, with significantly reduced DTH responses *in vivo* and inhibited OVA-specific IL3, IL5 and IL10 production *in vitro* when challenged for up to 18 months after feeding. However, other effector responses recovered more quickly. The tolerance of OVA-specific serum IgG1 antibody production and antigen-specific proliferation did not last beyond 3 months after feeding, while OVA-specific IgG and IgG2a antibodies were only tolerant when animals had been challenged 10 days after feeding. The effects of tolerance were generally less persistent in mice fed 2mg OVA, where DTH responses were not tolerised beyond 9 months after feeding and the tolerance of OVA-specific PLN cell proliferative and cytokine responses wained after 3 months. More strikingly, the inhibition of OVA-specific IgG, IgG1 and IgG2a responses was not observed beyond 10 days after feeding.

The differential persistence of tolerance displayed by individual functions was somewhat unpredictable. Contrary to expectation, the recovery in production of IFNγ in OVA fed mice did not correlate with that displayed by either the DTH or serum IgG2a responses. The reasons for this are unclear, and, although consistent with my previous findings that DTH and IFNγ responses were not always related, they conflict with the general view that IFNγ is critically required for a switch to IgG2a production [338].

Some evidence was provided that the persistence of tolerance may be accompanied by upregulation of certain responses. Thus, the long-lasting tolerance induced by feeding 25mg OVA was associated with a switch to enhanced IFNγ production upon immunisation 6 months later. Although transient, it is possible that this effect might be necessary for the maintenance of high dose oral tolerance. An opposite effect was seen in low dose oral tolerance, since the decline of tolerance in animals fed 2mg OVA was concomitant with increased OVA-specific IL10 production, which was observed at both 6 and 9 months after feeding and was accompanied by enhanced IL5 responses at 9 months. This suggests that the loss of oral tolerance in these mice was followed by a skewing of immune responses in favour of the production of Th2-dependent cytokines and thereby provides further evidence against a suppressive role for Th2 cells in low dose oral tolerance.

In summary, these results highlight that oral tolerance can be manipulated, by altering the feeding dose, to be a long-lasting and dynamic phenomenon. However, its long-term effects on individual responses differ and therefore its use as a therapy for immunopathology will require that individual responses be assessed directly.

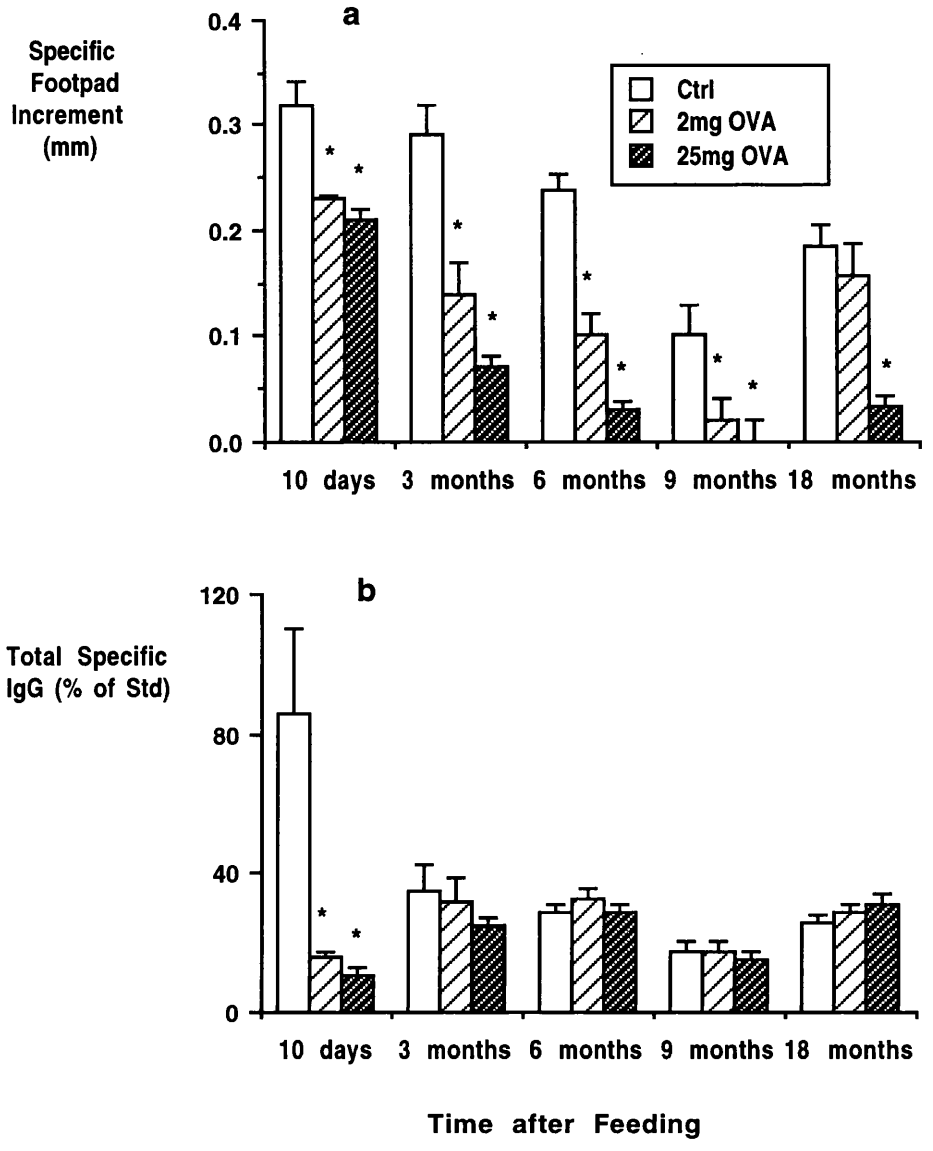


Figure 7.1 Duration of Oral Tolerance *in vivo*.

a) OVA-specific DTH responses in mice 21 days after s.c. immunisation with OVA/CFA. Results shown are mean specific increments in footpad thickness ± 1 SEM for 6 mice per group in animals fed saline (Ctrl), 2 or 25mg OVA and immunised 10 days, 3, 6, 9 or 18 months later. (*$p<0.05$ versus Ctrl). b) Total OVA-specific IgG responses in mice 21 days after immunisation. The results shown are calculated with reference to a hyperimmunised control serum and are mean % ± 1 SEM for individual sera from 6 mice per group in animals fed saline (Ctrl), 2 or 25mg OVA and immunised at the times indicated after feeding. (*$p<0.05$ versus Ctrl).

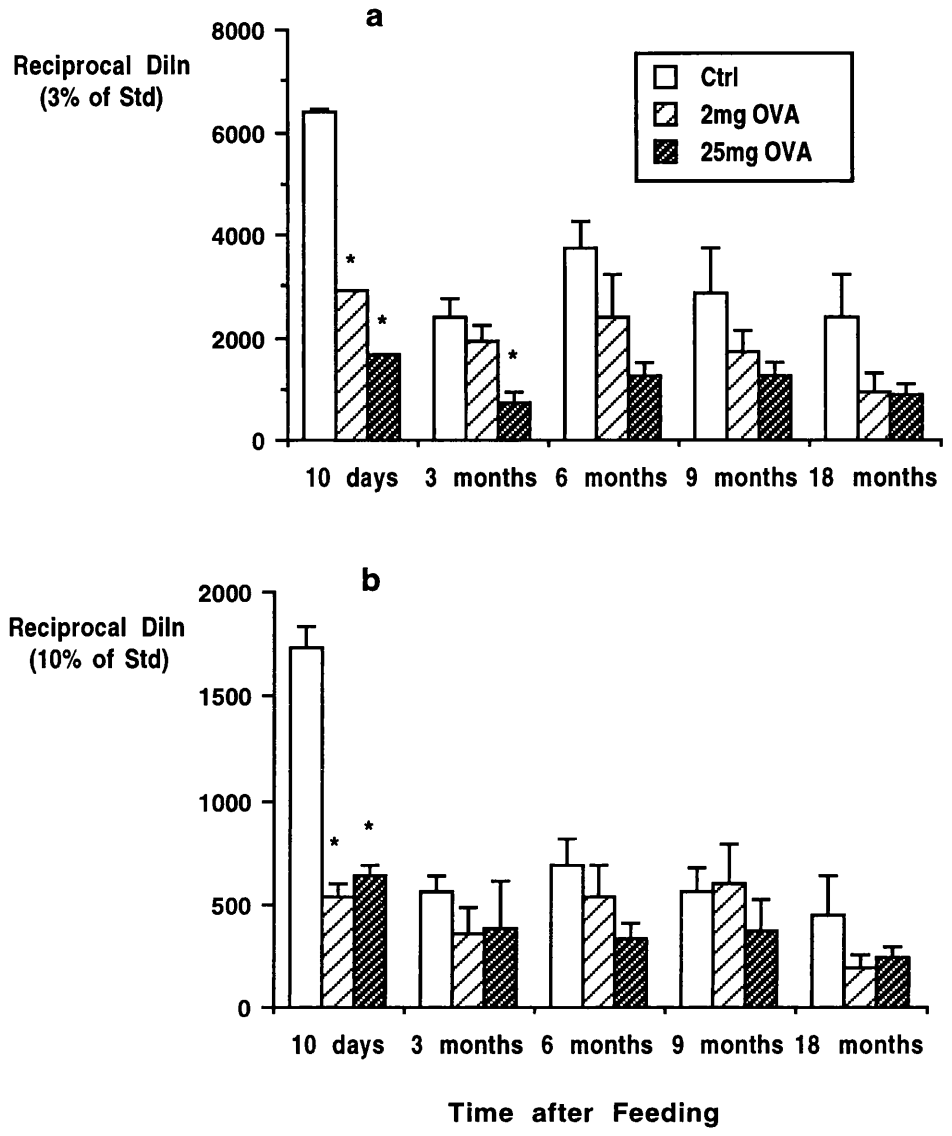


Figure 7.2 Duration of Oral Tolerance of Serum IgG Isotypes.

OVA-specific serum IgG1 (a) and IgG2a (b) responses in mice 21 days after immunisation with OVA/CFA. The results shown are the mean reciprocal dilutions giving an OD value equivalent to (a) 3% and (b) 10% of a hyperimmunised control serum and are for individual sera from 6 mice per group in animals fed saline (Ctrl), 2 or 25mg OVA and immunised at the indicated times after feeding. (*$p<0.05$ versus Ctrl).

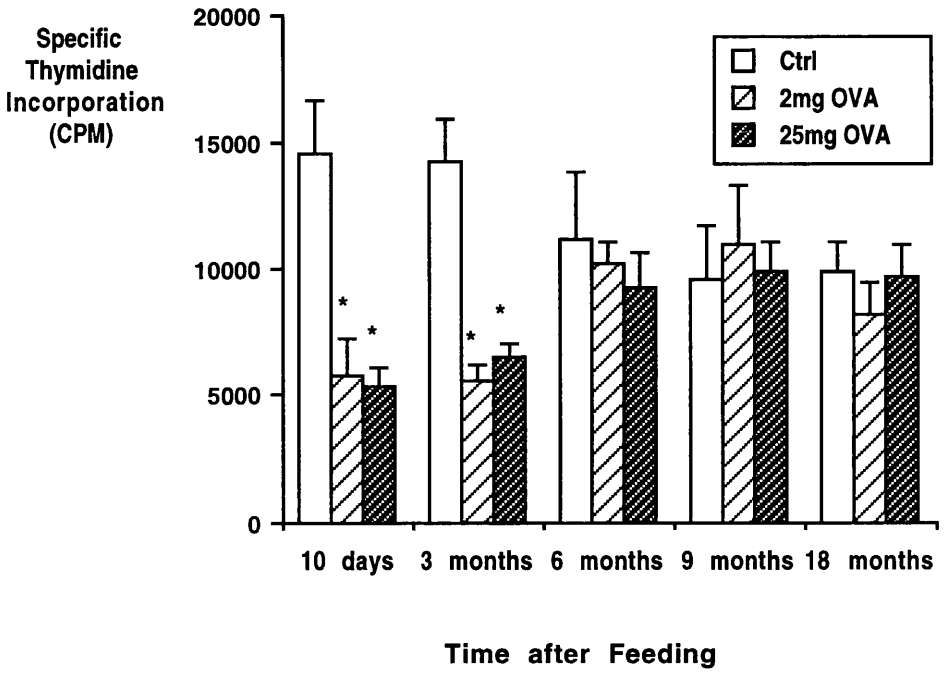


Figure 7.3 Duration of Oral Tolerance of Antigen-specific Proliferative Responses *in vitro*.

OVA-specific proliferative responses of PLN cells taken from mice 14 days after immunisation with OVA/CFA and restimulated with 1mg/ml OVA. The results shown are mean ^{3}H-TdR incorporation (CPM) ± 1 SEM in quadruplicate cultures of cells pooled from 4 mice per group in animals fed saline (Ctrl), 2 or 25mg OVA and immunised at the times indicated after feeding. (*$p<0.05$ versus Ctrl).

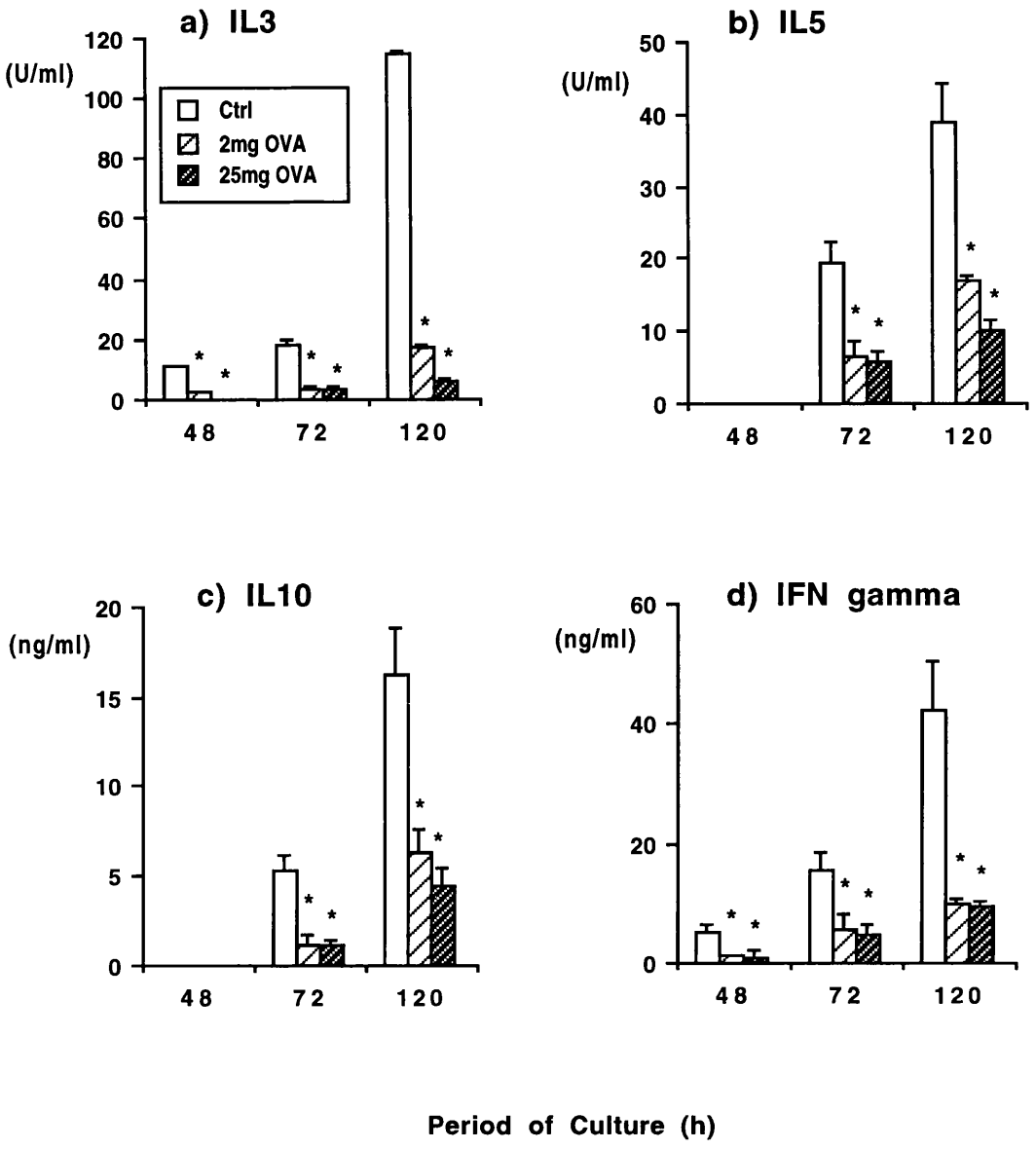


Figure 7.4 Oral Tolerance of Antigen-specific Cytokine Responses 10 Days after Feeding OVA.

OVA-specifc production of IL3, IL5, IL10 and IFNγ by PLN cells taken from mice 14 days after immunisation with OVA/CFA and restimulated with 1mg/ml OVA. The results shown are mean cytokine levels (U/ml or ng/ml) ± 1 SEM for cells pooled from 4 mice per group in animals fed saline (Ctrl), 2 or 25mg OVA and immunised 10 days later. (*$p<0.05$ versus Ctrl).

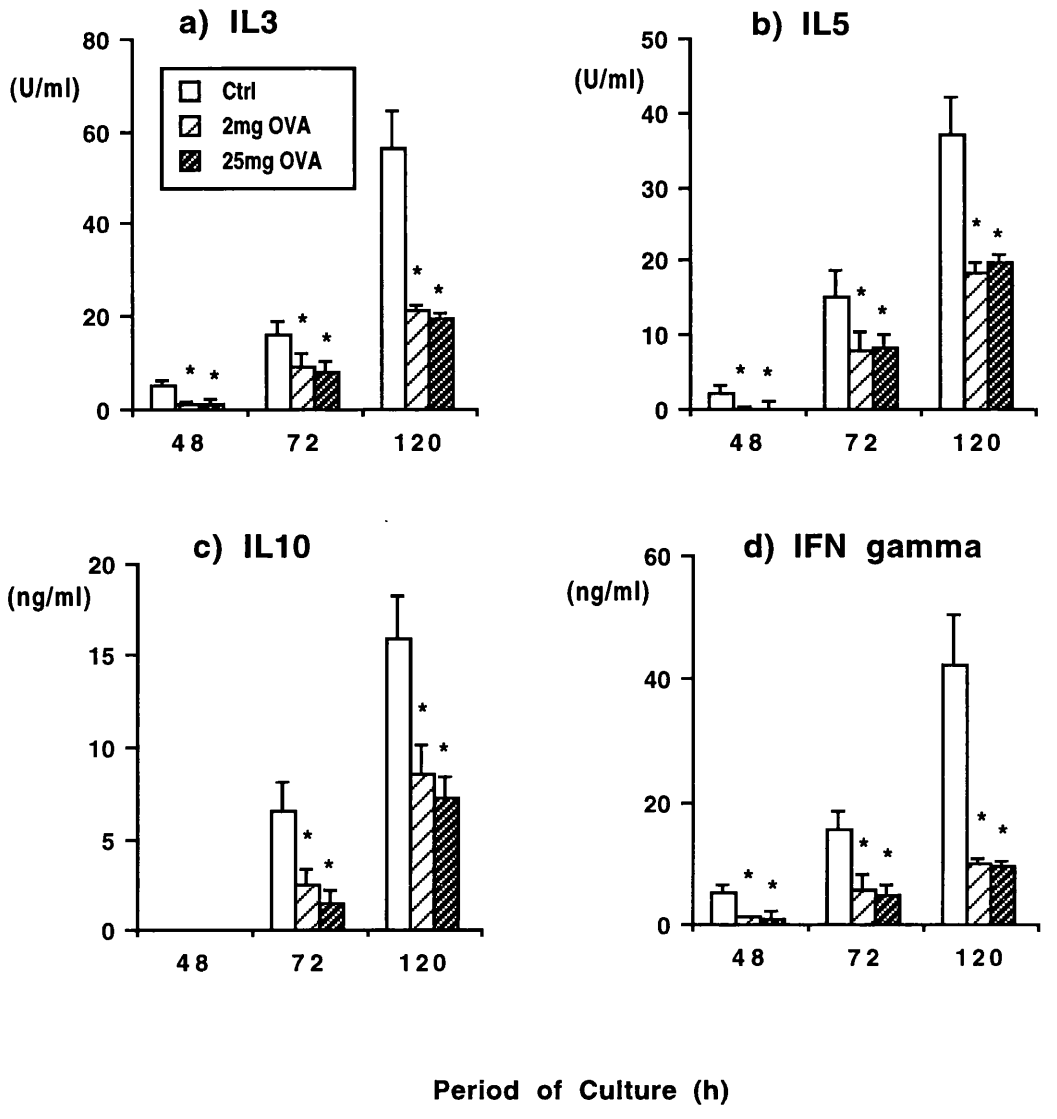


Figure 7.5 Oral Tolerance of Antigen-specific Cytokine Responses 3 Months after Feeding OVA.

OVA-specifc production of IL3, IL5, IL10 and IFNγ by PLN cells taken from mice 14 days after immunisation with OVA/CFA and restimulated with 1mg/ml OVA. The results shown are mean cytokine levels (U/ml or ng/ml) ± 1 SEM for cells pooled from 4 mice per group in animals fed saline (Ctrl), 2 or 25mg OVA and immunised 3 months later. (*$p<0.05$ versus Ctrl).

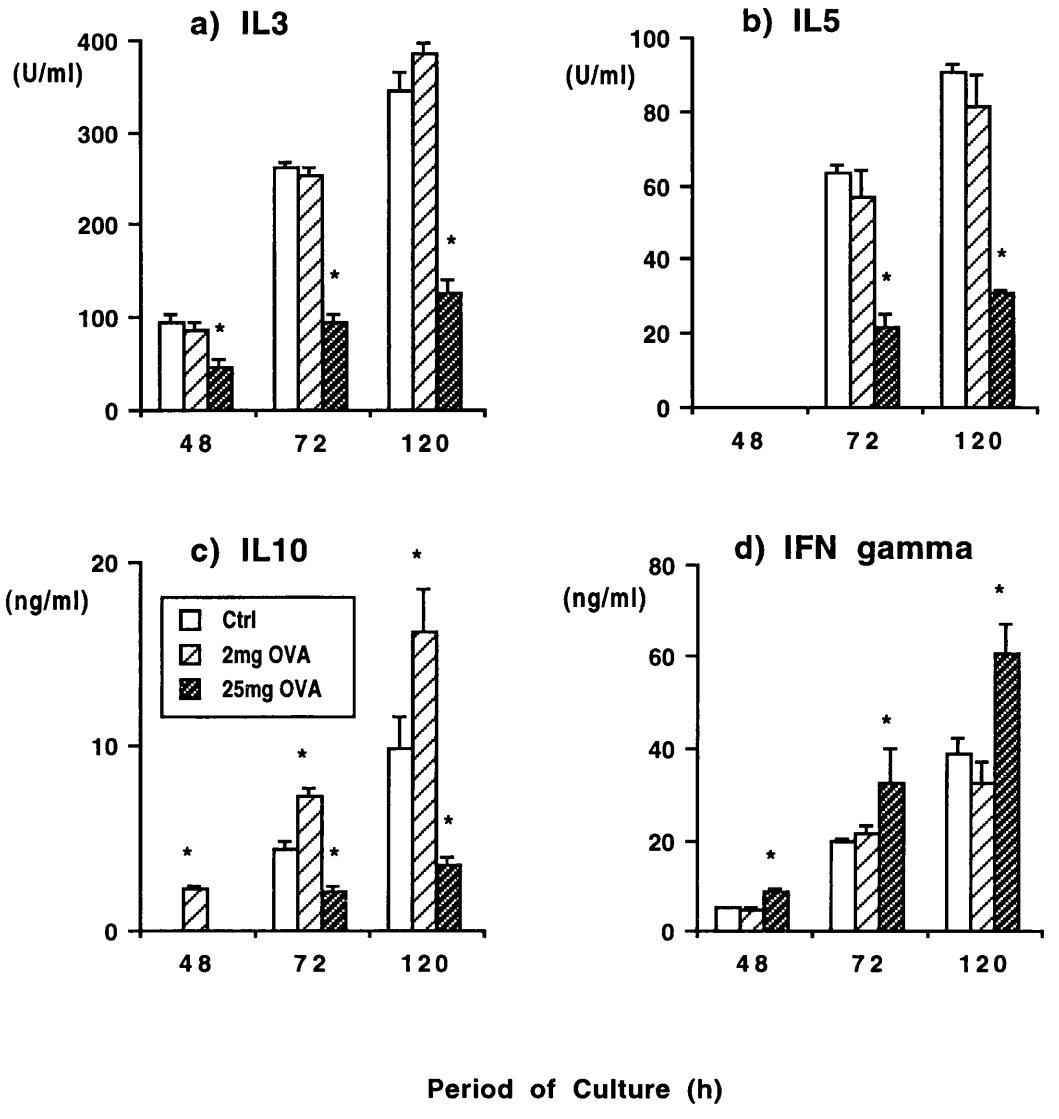


Figure 7.6 Oral Tolerance of Antigen-specific Cytokine Responses 6 Months after Feeding OVA.

OVA-specifc production of IL3, IL5, IL10 and IFNγ by PLN cells taken from mice 14 days after immunisation with OVA/CFA and restimulated with 1mg/ml OVA. The results shown are mean cytokine levels (U/ml or ng/ml) ± 1 SEM for cells pooled from 4 mice per group in animals fed saline (Ctrl), 2 or 25mg OVA and immunised 6 months later. (*$p<0.05$ versus Ctrl).

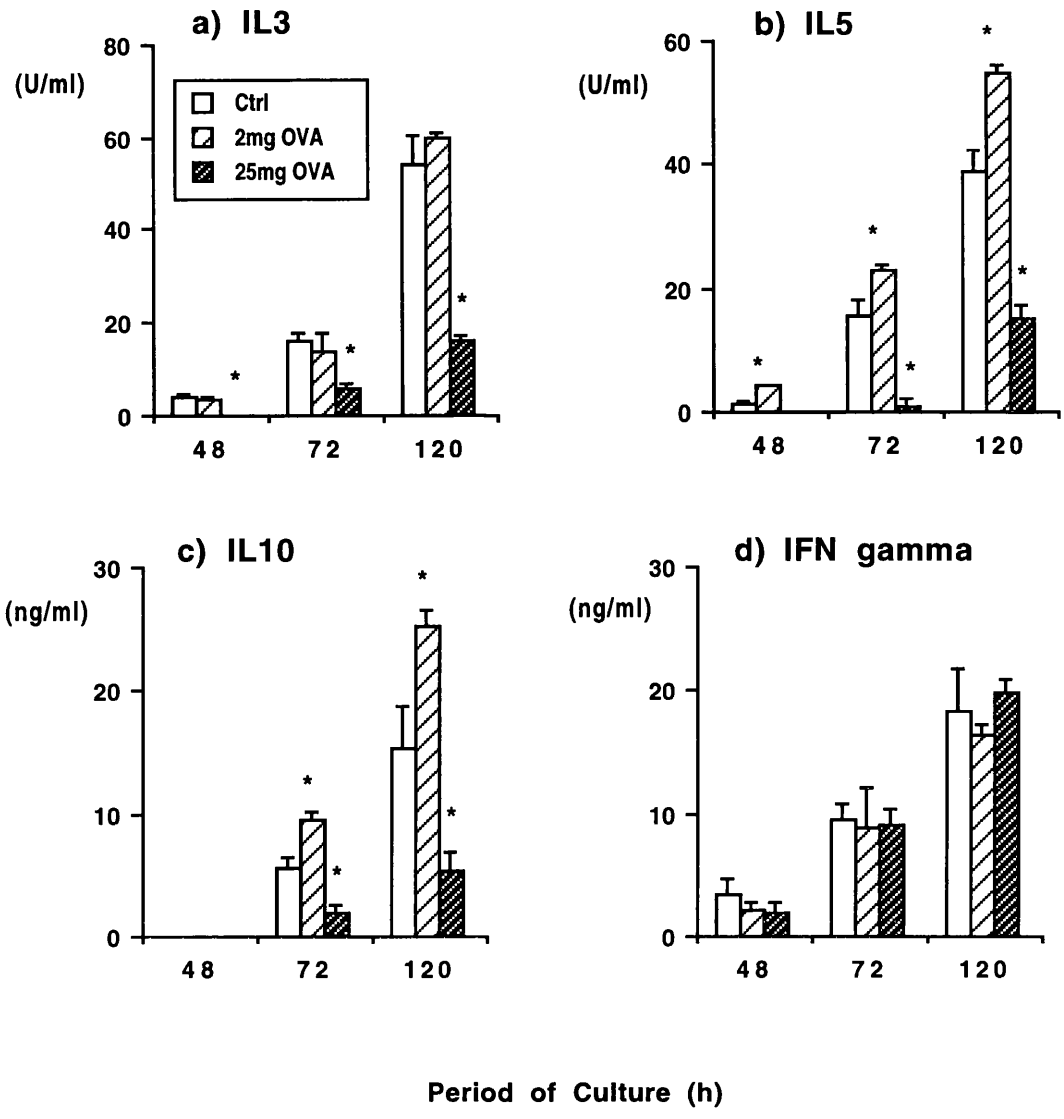
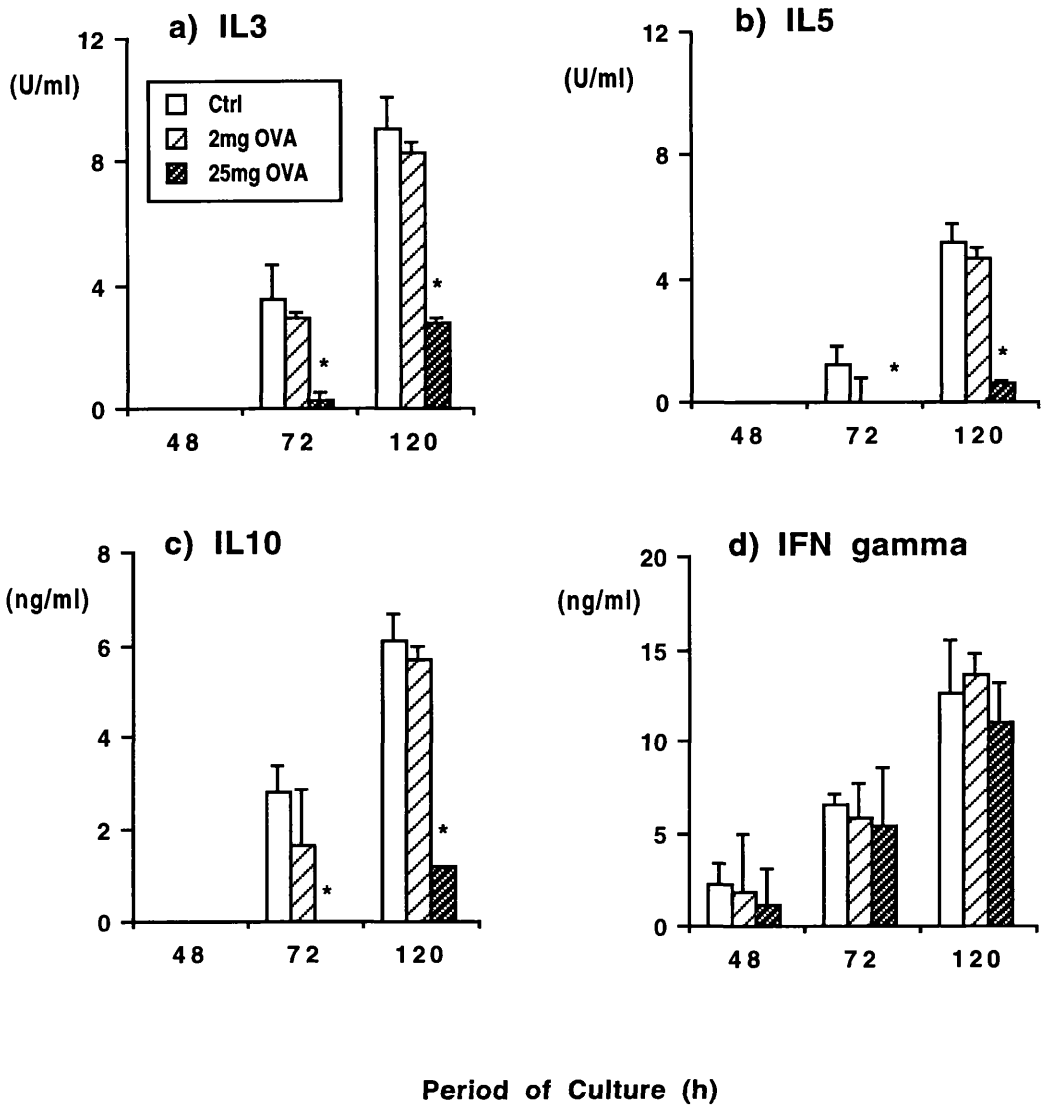


Figure 7.7 Oral Tolerance of Antigen-specific Cytokine Responses 9 Months after Feeding OVA.

OVA-specifc production of IL3, IL5, IL10 and IFNγ by PLN cells taken from mice 14 days after immunisation with OVA/CFA and restimulated with in 1mg/ml OVA. The results shown are mean cytokine levels (U/ml or ng/ml) ± 1 SEM for cells pooled from 4 mice per group in animals fed saline (Ctrl), 2 or 25mg OVA and immunised 9 months later. (*$p<0.05$ versus Ctrl).


a) IL3
(U/ml)
12
8
4
0
Ctrl
2mg OVA
25mg OVA
*
*
48
72
120
b) IL5
(U/ml)
12
8
4
0
*
*
48
72
120
c) IL10
(ng/ml)
8
6
4
2
0
*
*
48
72
120
d) IFN gamma
(ng/ml)
20
15
10
5
0
48
72
120
Period of Culture (h)

eriod of Culture (h)

Figure 7.8 Oral Tolerance of Antigen-specific Cytokine Responses 18 Months after Feeding OVA.

OVA-specifc production of IL3, IL5, IL10 and IFNγ by PLN cells taken from mice 14 days after immunisation with OVA/CFA and restimulated with 1mg/ml OVA. The results shown are mean cytokine levels (U/ml or ng/ml) ± 1 SEM for cells pooled from 4 mice per group in animals fed saline (Ctrl), 2 or 25mg OVA and immunised 18 months later. (*$p<0.05$ versus Ctrl).

Chapter 8: Discussion

Introduction

At the time I began my study, the dogma in oral tolerance was that distinct regulatory mechanisms could predominate depending on the dose of fed antigen administered [132, 136, 138]. However, as the findings made in a number of different systems were not always consistent in every aspect, a clearer understanding of the immunological basis of the phenomenon was required to determine its full potential as a clinical therapy for immunopathological disorders. The results presented in this thesis have used a well established model of tolerance induced by feeding OVA in single doses to confirm and extend many of the previous findings concerning the regulatory mechanisms of oral tolerance and the factors influencing them. This work involved study of a number of features, including the dose-dependency of tolerance, the requirement for individual suppressive cytokines, regulatory lymphocytes and direct T cell inactivation in the expression of unresponsiveness, as well as the longevity of the phenomenon. Since the dose of antigen was a critical component of most of the experiments described in this thesis, I shall first consider the work designed to assess this aspect before discussing the experiments relating to regulatory mechanisms involved in the induction and persistence of oral tolerance.

The Effect of Antigen Dose in Oral Tolerance

It was known from early reports that the effects of oral tolerance could be influenced by the dose of antigen [131]. In light of the recent studies confirming this, it had become generally accepted that low doses of fed antigen induce active suppression, while high doses inactivate T cells directly [132]. However, this view was based on investigations made with no more than two antigen doses and with a limited number of functional readouts, which were not consistent in every aspect [132, 136]. In models of active regulation, several

different T cell subpopulations had been implicated and proposed to exert suppression by a variety of means [117, 129, 281], while the studies demonstrating direct T cell inactivation after feeding antigen had failed to agree on whether this was mediated by anergy [118, 305] or deletion [300], or both [303, 329]. As will be discussed more fully below, active regulation and direct T cell inactivation will have different implications for the practical application of oral tolerance. Thus, I considered it important to clarify the regulatory mechanisms induced after feeding antigen in a broad range of doses (100μg-25mg OVA).

My first important finding was that individual effector responses became more susceptible to tolerance induction with increasing doses of fed OVA. Thus the two highest doses of fed OVA (10 and 25mg) significantly reduced every OVA-specific immune response examined, including DTH and IgG responses *in vivo* and PLN cell proliferation and cytokine production *in vitro*. These results confirmed previous *in vivo* findings made with the same doses of OVA [120, 131] and extended them by showing that the activity associated with both Th1 and Th2 cells was tolerised by high doses of fed OVA, including Th1-dependent DTH, IgG2a, proliferation and IFNγ responses and Th2-dependent IgG1, IL5 and IL10 production. Under these cirmcumstances, it seems unlikely that either T cell subset was mediating the unresponsiveness of the other. Although it remains feasible that another, as yet unidentified, suppressive factor may have been involved, my findings are consistent with the increasing evidence that a high dose of fed antigen preferentially induces T cell inactivation directly [132, 305] and further evidence for this will be discussed below.

Individual effector responses were differentially susceptible to the tolerance induced by the range of doses lower than those described above. This appeared to follow a pattern generally consistent with individual T helper cell subsets, with Th1-dependent responses, such as DTH, IgG2a and IFNγ production, being particularly susceptible to tolerance induction, whereas IgG and Th2-dependent IgG1 and IL10 responses remained resistant to inhibition until ≥10mg OVA was fed. This finding is consistent with reports that Th2 cells are less susceptible to tolerance induction than Th1 cells [254] and may explain previous *in vivo* observations that the humoral arm of the immune response was more difficult to tolerise than CMI responses [131]. In addition to finding that Th2-dependent responses were

resistant to oral tolerance induced by 100μg-5mg fed OVA, I showed that IgG1 and IL10 responses were actually enhanced by feeding 100μg-1mg OVA. Taken together, these results suggested that Th2 cells could play an active role in low dose oral tolerance via Th2-dependent crossregulation of Th1 cells. This type of regulatory activity has been shown in other studies of T cell activation [221, 291] and has been postulated to explain previous observations of upregulated Th2-dependent cytokines in oral tolerance induced by low doses of fed antigen [285, 313]. However, this interpretation is not consistent with my additional finding that IL5 production was readily inhibited by all tolerising doses of fed antigen. IL5 is a classical Th2 cell product [220] and its susceptibility to oral tolerance induction suggests that the production of individual cytokines may be regulated independently by different doses of fed antigen, rather than following a strict Th1/Th2 dichotomy. Although the reasons for this will be discussed more fully in a mechanistic review below, it is noteable that others have recently reported differential production of individual cytokines using TcR transgenic T cells *in vitro* [315]. This study showed that, in the absence of accessory molecules, it was possible to generate IL4-producing "Th2 cells" which did not secrete IL5. It would be interesting to determine if a similar situation arose when T cells were stimulated by a low dose of fed antigen. This could be achieved by direct examination of cytokine production by individual T cells, using ELISPOT or intracellular FACS analysis of TcR transgenic mice.

Although my results were partly consistent with the presence of Th2-dependent crossregulation of Th1 cells after feeding low doses of OVA, I was unable to detect evidence for the non-specific bystander suppression that is reported to accompany this form of tolerance [139, 284]. Under these circumstances, regulatory mechanisms induced initially in an antigen-specific manner inhibit subsequent responses to an unrelated antigen, provided it is presented to the immune system at the same time as the original antigen and within the same microenvironment [339]. To examine for the presence of bystander suppression in my experiments, I assessed the responses of orally tolerised cells when restimulated *in vitro* with the antigen PPD, which had been present in the CFA used for parenteral challenge with OVA. However, I found no evidence of suppressed PPD responses when cells from OVA

fed mice were restimulated with PPD either in the absence or presence of OVA and irrespective of the dose of OVA used to induce tolerance. These findings indicate that the Th2 cell activity that could be primed by feeding low doses of OVA was unable to mediate bystander suppression. This discrepancy with other reports may reflect differences in the protocols used for assessing bystander suppression. The phenomenon has been demonstrated *in vitro* using either culture supernatants from orally tolerised cells to suppress the antigen-specific responses of T cell lines [117, 274] or transwells separating the orally tolerised cells from responding T cells, but permitting transfer of a soluble suppressive factor released from the tolerant cultures upon antigen stimulation [271]. As it has been suggested that cognitive interactions prevent bystander suppression by inhibiting TGFβ release from tolerant cells *in vitro* [304], this may explain why I was unable to demonstrate evidence for the phenomenon in my experiment, where the T cells specific for both PPD and OVA were together in the same culture well. However, it is equally possible that bystander suppression was not a feature of oral tolerance in my system. The presence of bystander suppression has been documented in models of oral tolerance induced by repeated administration of low antigen doses, rather than the single dose, I used [271, 284]. Therefore, it may be possible that the tolerance induced by multiple and single doses of fed antigen reflects distinct mechanisms. In support of this theory, a recent study found that multiple, but not single, doses of fed antigen could protect mice from relapsing chronic EAE [340]. The authors suggested that protection from the disease might require upregulation of TGFβ production as this activity has been implicated in the suppression of immune responses to the multiple autoantigens, which arise through epitope spreading in chronic forms of autoimmunity [339] but was not detected in their system after feeding antigen in single doses. Other work in oral tolerance has shown that multiple feeding regimes are required for enhanced TGFβ production [300]. However, this contrasts with the findings made in my lab where enhanced TGFβ production could be detected early during culture of cells from animals tolerised by a single dose of fed OVA. Taken together this work highlights the need for further detailed examination of the regulatory factors induced by feeding single and multiple doses of antigen.

Unlike other groups, I did not examine for bystander suppression *in vivo.* The phenomenon has been previously demonstrated by feeding an antigen to induce suppression of subsequent responses to a third party antigen when administered parenterally along with the originally fed protein [271]. Therefore, similar studies of bystander suppression *in vivo* would be required to confirm my lack of support for the phenomenon *in vitro.*

Priming of Systemic Immunity by Feeding Low Doses of Antigen

As part of my dose response study, I also assessed the systemic effects of feeding very low doses of antigen as previous reports had found that 10-50μg fed OVA could preferentially prime CMI *in vivo* [120, 131]. However, the immunological basis of oral priming had not been investigated and this would be desirable for oral vaccine development. The usual requirement for T cell priming is that antigen is presented by "professional" APC delivering the necessary costimulation required to activate responding T cells [249]. This effect can occur with antigens delivered via the intestine, provided that they are administered in a particulate or viable form likely to direct their passage across the intestine to M cells [17]. Thus, different forms of fed antigen may produce different outcomes for systemic immune responses by being presented in different microenvironments or by distinct APC. The reasons why both tolerance and oral priming can be induced by feeding an antigen in the same form but in different doses is intriguing and will be discussed more fully in a mechanistic review below.

I was unable to reproduce previous findings of oral priming with 10-50μg fed OVA [120, 131] unless I used a lower challenge dose of OVA in adjuvant. This immunisation protocol induced suboptimal responses in control mice and augmented both DTH responses and serum levels of IgG1 in OVA fed mice, although their total OVA-specific IgG production was not altered. These results confirmed and extended previous findings that different limbs of the immune response differ in their susceptibility to oral priming [120, 131] by indicating that, under the appropriate circumstances, both CMI and certain aspects of the humoral response could be primed by oral administration of protein.

Individual immune responses varied in their susceptibility to upregulation by fed OVA, such that DTH, proliferation and IFNγ responses were enhanced by feeding 10-50μg OVA, while upregulation of IgG1, IL3 and IL5 responses required feeding 50μg OVA. Although this pattern appears to reflect a dichotomy in the sensitivity of Th1 and Th2 cells to different doses of OVA, this is inconsistent with the observed OVA-specific IgG2a production, which did not behave in the same way as the other Th1-dependent responses measured. Serum levels of this secondary antibody were unaltered by either dose of fed OVA and inspite of the enhanced IFNγ production. The dose-dependent sensitivity of individual effector functions in oral priming would be especially useful for oral vaccination strategies as it might allow targetted upregulation of the protective responses associated with pathogens of different etiology. However, as the extent of systemic priming appeared to be relatively weak in my study, it would be important to determine if this could be improved by increasing the frequency of oral administration using a boosting regime. In addition, as the effects of oral priming will vary with different antigens, it will be necessary to characterise the dose-dependent effects for individual proteins prior to their clinical application.

My results also highlight the potential hazards of feeding antigen in doses too low to tolerise. In terms of immunotherapy, an insufficient amount of fed antigen might exacerbate immunopathology rather than suppress it. This effect has been reported in an experimental model of EAE [340] and could explain a recent report where the development of autoimmune diabetes was prevented by feeding antigen to chimeric mice expressing OVA in the islet β cells of the pancreas until they were bone marrow reconstituted with OVA-specific TcR transgenic $CD8^+$ T cells [341]. When the proportion of autoaggressive $CD8^+$ T cells was enhanced in this way, the dose of fed antigen was not increased to compensate and therefore it may have been more equivalent to the very low doses of fed antigen associated with oral priming. Taken together with my own results, these studies highlight the importance of establishing optimal tolerising doses for individual antigens before their use in clinical practice.

The Role of Cytokines in Oral Tolerance

The results discussed above suggested that oral tolerance may involve different mechanisms depending on the dose of fed antigen administered and that Th2 cell activity was somewhat resistant to tolerance induction. I therefore examined whether Th2-dependent cytokines played a direct role in oral tolerance and I explored the requirement for other potentially suppressive cytokines in the induction and/or maintenance of the unresponsiveness after feeding a high (25mg) or low (2mg) dose of OVA .

i) Oral Tolerance does not Require Th2-dependent Cytokines

Of the many interleukins associated with regulatory functions in the immune system, IL4 has been demonstrated to play a central role [220]. In particular, it has been found to be responsible for the generation of Th2 functions in $CD4^+$ T cells [287, 342] and the subsequent downregulation of Th1-dependent responses [221, 291]. Moreover, IL4 has been associated with peripheral T cell tolerance [289] and reports of increased IL4 mRNA in the lymph nodes of orally tolerised mice suggest that IL4 may also have a regulatory function in oral tolerance [283]. However, my experiments in $IL4^{-/-}$ mice showed for the first time that oral tolerance could be induced and maintained normally despite a lack of IL4 expression and hence of Th2 cells [306].

As expected, these animals produced very low or negligible levels of OVA-specific IgG1, IL4, IL5 and IL10 after immunisation with OVA/CFA. Instead, their Th1-dependent responses were augmented so that both PLN cell proliferation and IFNγ production were higher than found in wild type controls. Others have also reported that mice lacking IL4 exhibited Th1-dominated immune responses with strong IgG2a/IgG3 and low or no IgG1 and IgE antibodies [343, 344], consistent with the idea that Th2 cells normally crossregulate Th1 activity [291]. More surprisingly, and despite the enhanced IFNγ production, I found that $IL4^{-/-}$ mice displayed lower DTH responses than their wild type littermates after parenteral immunisation, indicating that some Th1-dependent functions were not augmented in the absence of IL4. This finding suggests that IFNγ may not be as critical a factor for DTH as is often assumed and agrees with its presence in the $IFN\gamma R^{-/-}$ mice (see below).

Feeding OVA to IL4$^{-/-}$ mice induced systemic unresponsiveness of every OVA-specific effector function examined, including DTH, IgG and IgG2a responses *in vivo* and PLN cell proliferation and production of IL3 and IFNγ production *in vitro*. This indication that oral tolerance may be induced in Th1 cells in the complete absence of Th2 cells or their associated cytokines is consistent with findings from a similar study of oral tolerance in IL4$^{-/-}$ mice [345] and with the recent report that mice treated with anti-IL10 remain permissive to the induction and maintenance of oral tolerance to OVA [292]. The unresponsiveness induced in IL4$^{-/-}$ mice was irrespective of the dose of fed OVA used, indicating that the preservation or priming of Th2-dependent cytokines after feeding low antigen doses, is not required for oral tolerance to occur. A similar situation has been reported in another model of peripheral tolerance induced by continuous administration of low doses of soluble proteins, delivered subcutaneously by mini-osmotic pump [346]. In this study, unresponsiveness was also characterised by down regulation of Th1-dependent cytokines and a concomitant priming of Th2 cells. However, inhibition of endogenous IL4 did not restore Th1 cell responsiveness despite preventing Th2 cell expansion, indicating that the development of Th2 cells and the inhibition of Th1 cells were independent pathways. My findings indicate that this is also the case for oral tolerance and the possible mechanisms will be discussed later.

ii) Oral Tolerance is Induced in the Absence of IFNγ

Although the results described above showed that IL4 and Th2 cells played no role in either the induction or maintenance of oral tolerance, it remained possible that the unresponsiveness was being mediated by another suppressive cytokine. IFNγ has well known cytostatic properties [293, 319] and its production is relatively preserved in mice tolerized by i.v. injection of staphylococcal enterotoxin B [228]. Furthermore, IFNγ is required for the tolerance induced in rats by intranasal administration of OVA [128]. However, most studies of oral tolerance report that IFNγ production is particularly sensitive to inhibition and therefore a regulatory role for this cytokine is not usually considered. Due

to my finding that IFNγ was transiently upregulated during the first few days after feeding OVA, its potential as a mediator of oral tolerance required further assessment.

After neutralising endogenous IFNγ, OVA-specific DTH and serum IgG antibodies, including both IgG1 and IgG2a isotypes, were inhibited by feeding mice 25mg OVA, suggesting that IFNγ is not required for the induction of high dose oral tolerance. But as it was difficult to prove that all active IFNγ had been neutralised in this experiment, I also examined IFNγR$^{-/-}$ mice, which lack the receptor for IFNγ and hence are unresponsive to the cytokine [307]. Unfed IFNγR$^{-/-}$ mice had normal serum IgG and IgG2a levels after parenteral immunisation, but displayed impaired DTH, IgG1 and IFNγ responses. In parallel, they exhibited augmented PLN cell proliferation and production of IL3, IL5 and IL10 *in vitro*. Although most of these findings were as expected, the IgG isotype results were surprising, since IFNγ is believed to be involved in Ig isotype regulation [338] and other reports have shown that IFNγR$^{-/-}$ mice are impaired in their IgG2a response [307]. A corresponding decrease in IgG1 might also have been expected, but I found the opposite effect, despite the fact that other Th2-dependent responses, including IL5 and IL10, were augmented. As I am unable to explain these findings, the effects of oral tolerance on IgG isotypes were difficult to interprete.

Despite their unusual immunological phenotype, IFNγR$^{-/-}$ mice displayed normal oral tolerance, irrespective of the dose of OVA fed. This included inhibition of OVA-specific DTH, IgG, IgG1 and IgG2a responses *in vivo* and PLN cell proliferation and production of IL3, IL5 and IL10 *in vitro*. These findings clearly show that a lack of responsiveness to IFNγ does not prevent the normal induction and maintenance of oral tolerance and confirm my antibody depletion results. My findings are consistent with the fact that IFNγ production is particularly sensitive to inhibition by oral tolerance and imply that my later observation of preferential release of this cytokine during the first few days after feeding OVA is unlikely to play a pivotal role in the induction of oral tolerance.

iii) The Role of TGFβ in Oral Tolerance

As the experiments described so far appeared to exclude a role for conventional cytokine mediators produced by either Th1 or Th2 cells, I went on to examine the possibility that an alternative cytokine, TGFβ, might be important. TGFβ can function as a potent immunosuppressive factor either by direct cytostatic effects on lymphocytes or by inhibition of functional activities such as IFNγ production, cytolysis or expression of adhesion molecules [347]. The importance of these inhibitory functions is underscored by the finding that $TGF\beta_1$ null mice exhibit multiorgan inflammation, lymphocytic infiltrates, and early death [348, 349]. Abundant in the normal intestine [296-298], TGFβ has been found to downregulate the immunopathology of murine colitis [350, 351] and intestinal graft-versus-host reaction (GvHR) [320]. In addition, TGFβ has been implicated as a mediator of oral tolerance in rodents. $CD4^+$ T cell clones [282] and $CD8^+$ populations [274, 285] derived from mice fed MBP have been reported to inhibit the induction of EAE by a mechanism that involved TGFβ. Moreover, others in this lab have detected TGFβ production when tolerised PLN cells were restimulated *in vitro* [321].

I assessed the role for TGFβ in oral tolerance by neutralising its production *in vivo*. This treatment failed to prevent the induction of oral tolerance of most aspects of the immune response, including OVA-specific DTH responses and IL5, IL10 and IFNγ production, indicating that endogenous TGFβ was not required for tolerance of CMI *in vivo* or of Th1 or Th2 effector functions *in vitro*. I obtained similar findings irrespective of the dose of OVA fed and these results disagree with the adoptive transfer studies described above. In addition, they contrast with another study in which neutralisation of TGFβ *in vivo* abrogated the protection from EAE and the suppression of DTH responses which were induced by oral administration of low doses of MBP [274]. These discrepancies are likely to reflect differencies in the protocols used both for depleting TGFβ and for feeding antigen, but may also reflect the effects of residual TGFβ *in vivo,* as I was unable to prove that all active TGFβ was neutralised in my experiment. This could have been achieved by measuring the release of TGFβ *in vitro* using a sandwich ELISA, bioassay or by examining the binding of ^{125}I-labelled TGFβ to splenocytes from TGFβ-depleted or control mice [352], while

immunoperoxidase staining for TGFβ would have revealed its presence *in vivo* [353]. This latter technique would also address whether TGFβ had been depleted from the intestinal tissues, which are a potential site for oral tolerance induction [35].

One aspect of the immune response which did appear to require endogenous TGFβ for its suppression in my hands was serum IgG production. Oral tolerance of this response was prevented in mice treated with anti-TGFβ, although it should be noted that there was still slight suppression of IgG responses in TGFβ-depleted mice. If TGFβ was preferentially required for oral tolerance of humoral immunity, it might explain previous reports that the cellular and humoral limbs of the immune response are differentially regulated by fed antigen [131]. However, this differential effect has been associated primarily with tolerance induced by low doses of antigen, whereas my findings were similar irrespective of whether I fed a high or low dose of OVA, implicating a potential role for TGFβ in both situations. Indications that TGFβ could be involved in high dose oral tolerance contrast with previous reports that increasing the dose of fed antigen inactivates T cells directly [118, 132, 300] and reduces the amount of TGFβ secreted [284]. Therefore my results require further investigation, particularly as they were somewhat variable and could not be repeated due to the limited supply of reagent. An alternative approach to address this issue using TGFβ mice$^{-/-}$ might not be feasible because of their lethal multiple organ disease [348, 349]. However, the technique of recombination-activating gene (RAG)-2-deficient blastocyst complementation [354], in which mouse embryonic stem cells disrupted in both copies of the gene of interest are injected into RAG-2-deficient blastocyts, may allow the generation of a chimeric mouse with disrupted TGFβ expression targetted to the T and B cell compartments for direct examination of a role for TGFβ in oral tolerance.

Another aspect of oral tolerance which appeared to be modulated by neutralisation of endogenous TGFβ was OVA-specific PLN cell proliferation and indeed TGFβ-depleted mice fed 25mg OVA displayed enhanced proliferative responses *in vitro*. However, the response was very low in control immunised mice that had been depleted of TGFβ and it was therefore difficult to interpret the additional effects of feeding OVA.

Neutralisation of TGFβ production *in vitro* showed that this cytokine was not required to maintain oral tolerance of several OVA-specific responses, including proliferation and production of IL3, IL5 and IL10. These data provide further evidence against an exclusive role for TGFβ in the expression of oral tolerance. However, its role in the suppression of IFNγ production remains to be determined, as every culture treated with anti-TGFβ contained IFNγ beyond the level of detection by ELISA making it difficult to discern the effects of feeding OVA. Only cells from mice fed 2mg OVA appeared to produce less IFNγ than controls in the presence of anti-TGFβ.

Overall, the results above do not support a crtical role for TGFβ in the induction of oral tolerance *in vivo* or for its expression *in vitro.* If time had permitted, I would also have examined its role in the maintenance phase of unresponsiveness *in vivo* by neutralising endogenous TGFβ production around the time of parenteral immunisation.

Taken together, these parts of my study do not support the hypothesis that the induction of oral tolerance induced in mice by feeding single doses of antigen is critically dependent on the presence of suppressive cytokines. However, this work does not preclude the possibility that another form of active regulation might be involved and I will now discuss the findings from experiments designed to address this issue.

The Role of $CD8^+$ T cells in Oral Tolerance

Early studies of oral tolerance, in which suppression was transferrable to naive recipients with $CD8^+$ T cells [93, 97, 109, 324], were the first to implicate a regulatory role for this T cell subset. Although the presence of functional $CD8^+$ T_S cells remains controversial, more recent studies of oral tolerance confirm that $CD8^+$ T cells can transfer suppression [117] and that this lymphocyte subpopulation may be primed in the lamina propria by fed antigen [35]. $CD8^+$ T cells can play a regulatory role in a number of immune responses, either through classical cytotoxic effects on APC [275] or via the production of suppressive cytokines such as IFNγ [128] and TGFβ [274]. As my findings above were inconsistent with a role for either of these cytokines in oral tolerance, I was particularly

interested in the possibility that suppression was associated with the cytotoxic properties of $CD8^+$ T cells.

In the first set of experiments, I confirmed that a single s.c. administration of OVA ISCOMS generated a wide range of OVA-specific effector responses, including DTH, serum IgG and class I MHC-restricted splenic CTL activity [325]. I then showed for the first time that the responses induced by this adjuvant could be suppressed by feeding low or high doses of OVA. Most importantly, complete abrogation of OVA-specific CTL, which have been shown to be $CD8^+$ class I MHC-restricted T cells, extends the range of immune responses which can be modulated by oral tolerance. Similar findings have recently been made for the $CD8^+$ CTL responses induced by parenteral administration of either EL4 cells or spleen cells loaded with OVA by osmotic lysis [341, 355].

However, all the regimes described above for priming CTL *in vivo* are known to be dependent on the presence of $CD4^+$ T cells [326, 341] and I thought it important to determine whether the suppression of CTL responses in OVA fed mice was due to direct tolerization of OVA-specific $CD8^+$ T cells or if it was secondary to inhibition of the IL2-producing $CD4^+$ T cells necessary for the induction of CTL [356]. Unfortunately my attempts to examine whether the tolerance of $CD8^+$ effector CTL activity could be overcome *in vitro* by addition of functional OVA-specific $CD4^+$ T cells were unsuccessful. An alternative approach to address this issue could have involved administering exogenous IL2 to the CTL cultures in an attempt to overcome oral tolerance of cytotoxicity by this means. Time restraints prevented me from performing this work. Instead, I decided to explore the issue by taking advantage of a recent report that CD4 independent OVA-specific CTL could be primed by immunisation with OVA/CFA [327]. I was able to confirm these findings by showing that parenteral immunisation with OVA/CFA induced OVA-specific class I MHC-restricted splenic CTL activity which developed normally in mice depleted of $CD4^+$, but not $CD8^+$, T cells. I then showed that those CTL responses were not altered by feeding OVA prior to OVA/CFA immunisation. This was irrespective of the antigen dose used and despite the presence of tolerised antibody production. This finding that fed antigen cannot influence the CD4-independent cytotoxic activity generated by OVA/CFA contrasts with another report

showing that CTL responses primed in exactly the same way were tolerised by feeding multiple, low doses of OVA [355]. I did not have time to repeat this work and it would be important to determine if the discrepancies reflect different feeding regimes or not.

As the results discussed above indicated that a subset of $CD8^+$ T cells might remain resistant to inhibition by fed antigen, I therefore assessed their requirement in the induction of oral tolerance by feeding OVA to animals depleted of $CD8^+$ cells around the time of feeding. In parallel, I used the same approach to explore the requirement for $CD4^+$ T cells in the phenomenon. Despite the fact that the antibody regimes produced successful systemic depletion of the appropriate T cell subset, the OVA-specific DTH and serum IgG responses generated after challenge with OVA/CFA were entirely normal, indicating that the T cell populations had recovered by the time of immunisation, 10 days after the final dose of antibody was given. Feeding either a high or low dose of OVA induced entirely normal tolerance of both the DTH and IgG responses in CD8-depleted mice, indicating that $CD8^+$ T cells were not required for the induction of unresponsiveness. These findings have recently been confirmed by others using mice depleted of $CD8^+$ cells either by antibody [281, 357] or by genetic manipulation [345]. In addition, one study has also shown that oral tolerance is unaffected if $CD8^+$ cells are depleted after feeding [357].

At face value, these findings suggest that $CD8^+$ T cells play no role whatsoever in oral tolerance. Moreover, as CTL responses were neither primed nor tolerised by fed OVA and as others in this lab have been unable to prime CTL after feeding alone, the class I MHC-restricted $CD8^+$ T cells may be ignorant of fed OVA , perhaps because it does not enter the class I pathway for presentation. However, there is increasing evidence to suggest that this idea warrants reexamination. Primed CTL responses have been recently reported in mice fed OVA [341] and tolerance induced by feeding MBP or by nasal administration of OVA has been found to be transferrable with $CD8^+$ T cells which secrete TGFβ or IFNγ, respectively [128, 274]. In addition, intestinal lamina propria $CD8^+$ T cells from antigen fed mice have been shown to secrete IFNγ and TGFβ, and to transfer tolerance to naive recipients [35]. As I only confimed the efficacy of the depletion in my experiments using splenocytes, it cannot be ruled out that intestinal $CD8^+$ T cells remained in my mice and

could have contributed to the continuing tolerance. Consistent with this idea, mucosal $CD8^+$ T cells expressing the γδ form of the TcR have been implicated both in the model of nasal tolerance mentioned above [129, 276] as well as in oral tolerance of experimental autoimmune uveitis [277]. In addition, a report that the induction and maintenance of oral tolerance was blocked in OVA fed mice administered with anti-γδ antibody [278] suggests that γδ T cells may be important in the phenomenon. Indeed γδ T cells have already been found to downregulate αβ T cell responses [358], although the immunological basis of this remains to be elucidated. Unfortunately, time restraints prevented me from assessing the role of γ/δ cells in my system of oral tolerance.

Despite these provisos, the overriding feature of my experiments was that $CD4^+$ T cells were essential for the induction of oral tolerance, as mice depleted of $CD4^+$ cells prior to feeding had DTH and IgG responses comparable with unfed mice. This finding has since been confirmed [357] and is consistent with the fact that oral tolerance of CD4 independent hapten-specific effector $CD8^+$ T cell responses requires $CD4^+$ T cells [359].

Thus, $CD4^+$ T cells are not only the principal target of oral tolerance, but may also be required to mediate it. That this effect does not require Th2-dependent cytokines, TGFβ or cytotoxicity suggests that oral tolerance may be induced by direct T cell inactivation rather than by an active regulatory mechanism. To examine this issue more closely, I examined the potential role of anergy and deletion in oral tolerance.

The Role of Cell Death and Anergy in Oral Tolerance

Clonal deletion is an important mechanism of central tolerance to self antigens [360] and is also found when peripheral tolerance is induced by exogenous superantigens [328] or after administration of conventional antigens to TcR transgenic animals [330]. Cell death by apoptosis has recently been demonstrated *in situ* after oral tolerance induction in TcR transgenic mice [300], but it remains to be identified in oral tolerance to nominal antigens in normal animals. In my first set of experiments to examine for direct T cell inactivation, I showed that PLN cells removed from orally tolerised mice after parenteral immunisation had compromised viability when cultured in the absence of antigen *in vitro.* This cell death

affected up to 90% cells and involved both $CD4^+$ and $CD8^+$ T lymphocytes, implying that it was unlikely to be antigen-specific. The dying lymphocytes displayed morphological features indicative of apoptosis, including nuclear fragmentation and membrane blebbing, and these changes were accompanied by an increased proportion of cells with hypodiploid DNA content in the tolerant cultures, again consistent with apoptosis. An additional aspect of my experiments was that, unlike immunised control cells, lymph node cells from naive unimmunised mice showed a similar susceptibility to die *in vitro*. This suggests that the induction of oral tolerance renders antigen-experienced cells unresponsive to the growth/survival signals which primed cells normally react to.

Although these results could imply that deletion of OVA-specific T cells was occurring *in vivo,* the cell death was more extensive than would be anticipated from this. In addition, antigen-specific cells must still have been present at the time of *in vitro* culture, as the addition of OVA rescued a proportion of orally tolerised cells from dying *in vitro.* Although the cell viability failed to return to control levels under these conditions, the same phenomenon was not observed with naive lymph node cells. Thus, if deletion had occurred *in vivo*, it could not have been complete. It is also possible that cell death may only occur *in vitro*, with the presence of antigen and the lymphoid microenvironment allowing tolerant cells to remain alive but anergic *in vivo.* It would therefore be interesting to examine for apoptotic cells in tolerised mice using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labelling (TUNEL) method, which detects apoptotic cells by virtue of their DNA strand breaks [361]. However, the frequency of antigen-reactive T cells in normal animals may be too low to allow detection of the apoptotic proportion by TUNEL and preliminary experiments performed to address this issue in the lab have been inconclusive. An alternative approach would involve exploitation of a recent model in which normal animals are given a defined number of TcR-transgenic T cells, which can then be traced with the appropriate anti-clonotypic ab [362]. In this way, the proportion of antigen-reactive T cells might be tailored to a level suitable for TUNEL detection of any apoptosis in antigen fed mice.

To try and investigate whether apoptosis might be important in the development of oral tolerance *in vivo* and to examine the molecular nature of the cell death described *in vitro,* I decided to assess the role of fas. Fas-mediated apoptosis has been implicated in peripheral T cell tolerance induced by the superantigen SEB [334] or by crosslinking of TCR-CD3 molecules [363], as well as in tolerance within immunologically privileged sites, such as the eye [364]. Moreover, T cell death in the human lamina propia has been reported after fas cross-linking [365], outlining a potential role for this mechanism in oral tolerance.

In the first series of experiments, I found that tolerance of OVA-specific effector functions *in vitro* was not altered by addition of fas-Fc fusion protein, which has recently been demonstrated to block fas-dependent cell death [244]. Cells from orally tolerant mice remained impaired in their proliferation and production of IL3 and IFNγ, suggesting that fas-dependent apoptosis was not involved in this phenomenon. Unfortunately, I was unable to determine conclusively if the enhanced apoptosis in cultures from orally tolerised mice was fas-dependent. Although the addition of fas-Fc fusion protein did not influence the apoptosis observed in control and tolerant cultures, in this experiment, the levels of apoptosis were comparable for all groups, irrespective of whether the animals had been fed OVA. Although this result was found at a time point in culture when I usually observe enhanced apoptosis in tolerant cultures, this feature normally increases with time and therefore it is possible that differences in control and tolerant cultures might have been observed had the supply of fusion protein allowed a full kinetic study of apoptosis to be performed. It will be important to confirm this in future studies. It would also be important to confirm that the fas-Fc fusion protein was indeed effective in blocking fas-dependent apoptosis *in vitro.* Previous reports have shown that fas-Fc fusion protein can prevent the early fas-dependent cell loss induced by anti-CD3 treatment [335] and it would have been important for me to perform a similar test to confirm that the fas-Fc fusion protein was working in my experiments. Unfortunately, a limited supply of the fusion protein prevented this.

In the second series of experiment, I examined if fas-mediated apoptosis was required for oral tolerance *in vivo,* using fas-deficient MRL lpr mice [235]. I found that

many of the immune responses generated by s.c. immunisation with OVA/CFA differed between MRL lpr and $MRL^{+/+}$ control mice. OVA-specific DTH responses, serum IgG and IgG2a antibodies and PLN cell production of IL3 and IFNγ were augmented in MRL lpr mice, while IgG1 and IL5 responses were unaltered, indicating that disrupted fas expression may have skewed the immune response to a Th1-like phenotype. This is consistent with previous observations in MRL lpr mice [366] and is likely to reflect the differential expression of fasL by Th1 and Th2 cells, as activated Th1 cells express more fasL and display an enhanced susceptibility to fas-mediated cell death [244]. The altered immune phenotype of MRL lpr mice may reflect an attempt to downregulate effector responses in the absence of fas-dependent apoptosis via the release of inhibitory cytokines such as IFNγ, which was enhanced in my study, or TGFβ, which has recently been reported to be upregulated in MRL lpr mice [367]. These features might also explain my additional observation that the cells from immunised MRL lpr mice displayed a reduced capacity for OVA-specific proliferation.

Despite these abnormalities in systemic responsiveness, I found that MRL lpr mice displayed normal oral tolerance. Feeding OVA prior to immunisation reduced every effector response examined in MRL lpr mice, indicating that fas-dependent apoptosis was not critical for either the induction or maintenance of the phenomenon. This effect was irrespective of the antigen dose used to tolerise. Of particular importance was my finding that cells from orally tolerant MRL lpr mice also displayed an enhanced propensity to die in the absence of antigen *in vitro*. Although these findings exclude a role for fas in the *in vivo* or *in vitro* consequences of oral tolerance, they do not rule out the possibility that clonal deletion could occur via a fas-independent mechanism, as is the case for central tolerance [368]. One mechanism of this could be apoptosis via TNFR, which has been implicated in the deletion of CTL *in vivo* [247]. However, recent preliminary experiments performed by others in the lab suggest that oral tolerance is normal in $(p55 \times p75)^{-/-}$ mice, which lack both chains of the TNFR (Mowat, unpublished observations).

The absence of a role for fas, together with the evidence for residual OVA-specific T cells at the time of *in vitro* culture, highlight the possibility that anergy rather than deletion

was occurring in tolerant mice. This has been reported previously with only high doses of antigen [118, 132, 304, 305]. To investigate this issue further, I attempted to restore responsiveness by culturing cells from orally tolerant mice in rIL2, a procedure reported to reverse anergy [258]. The OVA-specific proliferative responses of PLN cells from mice tolerised by 2mg OVA were reversed completely by IL2. This contrasts with the idea that has become increasingly accepted that oral tolerance induced by low doses of fed antigen is mediated entirely by active suppression [132, 136, 271, 274, 369], whereas high dose tolerance is due to anergy [118, 132, 304, 305]. However, since these reports relate to multiple, rather than to single, low doses of fed antigen, my novel finding implies that different forms of regulation may be induced depending on the frequency of feeding.

rIL2 also enhanced the proliferation of cells from mice fed 25mg OVA, but this effect was incomplete since the response failed to return to control levels, indicating that although oral tolerance induced by high doses of fed antigen may involve anergy, additional factors may also be operating. These findings are consistent with other reports of anergy in high dose oral tolerance [118, 132, 304, 305], but more so, with the idea that both anergy and partial deletion may occur in SEB-induced oral tolerance [329].

The fact that IL2 reduced cell loss in the tolerant cultures may also support a possible association between anergy and deletion in my system of oral tolerance. IL2 may either have prevented the death of inactivated cells or may have preferentially induced clonal expansion. The first possibility would be predicted if T cell anergy was followed by apoptosis, as observed in other tolerance models [370], while the second idea would be consistent with reports that anergic cells can proliferate to exogenous IL2 because they express IL2R [258]. Further studies on the effects of IL2 treatment on cell death and proliferation could discriminate between these possibilities. That the cell death I observed in orally tolerant cultures might reflect the eventual apoptosis of anergic cells is consistent with a recent report that anergized lymphocytes can undergo apoptosis [370]. The mechanism of this was also found to be fas-independent and this is consistent with the fact that fas-sensitivity may require IL2-driven proliferation [371] which is impeded in anergic T cells [262].

Feeding Tolerogenic Doses of OVA Primes T Lymphocytes *in vivo*

In other models of peripheral tolerance, anergy and deletion have been reported to follow a transient period of T cell activation [328, 331, 332]. This may reflect incomplete T cell signalling, as others have shown that partial T cell activation can occur during the process of tolerance induction, such as T cell stimulation in the absence of appropriate costimulation [372] or by an altered peptide ligand [260, 261]. In my experiments, I found that feeding a tolerogenic dose of OVA primed spleen cells for OVA-specific proliferation, entry into cell cycle and production of IL3 and IFNγ within the first 3 days of feeding, but not thereafter. I was unable to detect OVA-specific IL5 or IL10 production, suggesting that fed OVA had preferentially generated a Th1-dependent response. Smilar findings have been made in a study of nasal tolerance, where Th1-dependent cytokines were also transiently upregulated after peptide administration [332] and in a study of superantigen-induced T cell apoptosis, where exposure to BrdU *in vivo* revealed that transient proliferation was perequisite for the effect [373].

The restricted and transient cytokine production observed in OVA fed mice which were tolerant to challenge *in vivo* with antigen at this time is consistent with the induction of anergy after partial activation, in which T cells are unresponsive to further antigenic stimulation provided in an immunogenic manner [257]. These findings provide further support for the involvement of anergy in oral tolerance.

In summary, my results in chapter 6 highlight the possibility that fed antigen might induce anergy, perhaps along with a degree of deletion, in antigen-reactive T cells.

The Longevity of Oral Tolerance

In the final part of my study, I examined the long-term effects of feeding antigen and explored if the tolerance of different aspects of immunity *in vivo* persisted for equivalent periods of time.

These experiments confirmed earlier reports that feeding a high dose of OVA induced long lasting oral tolerance *in vivo*, which persisted for the entire life-span of the mouse [120]. This was despite the fact that the magnitude of the systemic immune responses

generated by parenteral immunisation tended to decline with age. Thus, Th1-dependent responses, such as DTH and IFNγ, diminished progressively in ageing control animals, although their IL5 and IL10 responses remained reasonably constant for up to 9 months, consistent with previous studies showing dysregulation of Th1 and Th2 cells during senescence [374].

The longevity of the tolerant state depended on the dose of fed antigen. Mice fed 25mg OVA prior to immunisation showed some degree of tolerance for virtually their entire life-span, with impaired DTH responses *in vivo* and suppressed OVA-specific IL3, IL5 and IL10 production *in vitro* when challenged for up to 18 months after feeding. In contrast, feeding 2mg OVA induced a less persistent form of oral tolerance, which was no longer detectable when mice were challenged beyond 3 months after feeding. Taken together with a recent report that the persistence of IgG and IgE tolerance is dependent on the dose of fed protein [151], these results imply that, like other forms of peripheral tolerance, oral tolerance requires the continued presence of antigen to be maintained [263]. The ability of a fed antigen to withstand degradation will probably depend on the initial dose administered, such that high antigen doses may persist for longer than lower doses. Although it may be impossible to confirm this issue directly due to difficulties in locating and quantifying the amount of antigen remaining *in vivo* at different times after feeding, it would be important to determine if oral tolerance required the continued presence of antigen, perhaps by "parking" T cells from an orally tolerised mouse for different times in a naive recipient before assessing antigen-specific immune responsiveness.

I also found that individual effector responses recovered at different times after feeding. This was particularly apparent in comparisons of cellular and humoral immunity, where DTH responses remained inhibited for at least 9 months, whereas serum IgG antibodies recovered to control levels after only 3 months. This is consistent with previous reports [120] and supports my earlier findings that individual immune responses differ in their sensitivity to be modulated by different doses of fed antigen. As in my dose response study, individual responses recovered from tolerance without a clear pattern. Contrary to expectation, the recovery in production of IFNγ did not correlate with that of DTH or serum

IgG2a responses. The reasons for this are unclear, but are consistent with my own previous findings that DTH and IFNγ responses are not always related. However, since IFNγ is important for a switch to IgG2a production [338], my finding that only low levels of IgG2a antibodies were produced in mice primed for IFNγ production *in vitro* is confusing. If confirmed, these findings could mean that IFNγ production *in vitro* does not correlate with that *in vivo.* Alternatively, the cytokine may be produced *in vivo* but fails to reach the environment of the germinal centres, where immunoglobulin class switching occurs. This might be predicted if orally tolerised T cells behaved in a manner similar to peripherally tolerised T cells, which are unable to enter germinal centres [362]. It would be interesting to investigate this by direct examination of IFNγ production *in vivo,* using *in situ* hybridisation techniques.

Some evidence was obtained that the maintenance of the tolerant state was accompanied by upregulation of IFNγ production. This occurred 6 months after feeding, but only in mice receiving 25mg OVA. Oral tolerance could no longer be detected in mice fed 2mg OVA at this time. As I had shown previously that IFNγ production is particularly sensitive to oral tolerance and can even be reduced by low antigen doses, the enhanced release of this cytokine in the presence of tolerance of other responses, was surprising and and could imply a late role for IFNγ-mediated active regulation. This requires confirmation by examining the longevity of oral tolerance in IFNγR$^{-/-}$ mice. As IFNγ is known to mediate Th2 cell crossregulation [319], the absence of upregulated IFNγ production might explain why tolerance waned quickly in mice fed 2mg OVA, with an associated skewing towards Th2-dependent functions, including OVA-specific IL10 and IL5 production.

In summary, my results highlight the long-lasting effects of oral tolerance and show that this affects individual immune functions differently and in a dose-dependent manner. Moreover, they suggest that different mechanisms may come into play at different times during the expression of unresponsiveness, highlighting that oral tolerance is a dynamic phenomenon, even after a single dose of fed antigen.

Mechanisitic Review of Oral Tolerance

The oral administration of soluble protein antigen normally results in a profound immunological tolerance. Contrary to popular belief, I found that, in my hands, this did not involve active regulatory mechanisms and was probably mediated by clonal inactivation mechanisms such as anergy or deletion. As anergy results from TcR engagement in the absence of costimulation [254, 255, 257], my findings support the view that oral tolerance occurs because fed protein associates with "non-professional" APC, which are capable of processing and presenting antigen in the context of class II MHC molecules but cannot provide the costimulation necessary for complete T cell activation [375]. Consistent with this possibility is the finding that oral tolerance can be circumvented if the fed protein is administered along with inflammatory adjuvants, such as cholera toxin [376], muramyl dipeptide [166] or ISCOMS [377], which are likely to upregulate costimulatory molecules [378]. However, a number of important questions about the role of antigen processing and presentation remain, including the location of antigen presentation, the cells responsible and whether tolerance is induced locally in the gut or systemically. A role for entirely local presentation in oral tolerance is supported by the findings that lamina propria APC can present antigen in a manner that results in T cell unresponsiveness [35]. In order for intestinal APC to mediate the induction of systemic tolerance, these cells, which are as yet unidentified, would probably have to migrate from the intestine to peripheral tissues, since the only alternative would be that all antigen-specific T cells would have to pass through the gut to be tolerised and this is incompatible with the current understanding of lymphocyte recirculation [379]. Although gut-associated dendritic cells isolated from the thoracic duct of rats fed OVA carry immunologically relevant antigen [171, 172], these cells are thought of as professional APC and indeed were found to prime T cells when transferred into naive recipients [172]. However, the possibility remains that the dendritic cells were activated during the isolation procedure and might have been contributing to the induction of oral tolerance *in vivo*. Alternatively, fed antigen might associate with other APC, such as resting B cells, unactivated macrophages or epithelial cells, either locally or peripherally, to be presented to T cells in the absence of costimulation thereby inducing anergy.

A further property of adjuvants is their ability to promote slow and persistent release of antigen [380] and recent studies show that rapid access to APC may prevent sustained presentation of peptide-class II MHC complexes [381], which normally have a half-life of 6 hours *in vivo* [382]. That transient T cell stimulation might induce functional unresponsiveness is consistent with the fact that anergy results when T cells receive TCR signals but do not proliferate extensively [383]. Thus oral tolerance could reflect the fact that fed antigen gains access to the systemic circulation very rapidly [138] and this is supported by previous studies correlating oral tolerance with the clearance of antigen from the circulation [384].

A critical role for anergy as a mechanism of oral tolerance would be consistent with a number of my *in vitro* findings, including the ability of antigen to rescue tolerised cells from apoptosis and to induce the partial persistence of some cytokines, but not IL2. Furthermore, the differential effects of different feeding doses on Th1 and Th2 cell functions may reflect the fact that Th2 cells may be more resistant to anergy than Th1 cells [254].

The dose-dependent effects of oral tolerance could also reflect differences in the extent of TcR ligation. Recent studies of T cell activation show that the ability of different doses of systemic antigen to prime Th1/Th2 cells correlates wtih the affinity of the resulting TcR-MHC-antigen interaction [173, 174]. This is believed to act by stimulating different levels of cytokine gene transcription, with high antigen doses resulting in high affinity interactions which induce Th1 cell priming, whereas low doses were presented with a lower affinity which was optimal for priming Th2 cells. Thus, I would suggest that low doses of fed antigen may result in low ligand density antigen presentation in the absence of costimulation. This might be anticipated to favour Th2 cell activation and/or anergy. At the other end of the spectrum, high doses of fed antigen may swamp the immune system to be presented by many different types of APC at a high ligand density. If all T cells were of high affinity, this might lead to clonal exhaustion due to the synchronous activation of all specific T cells, as has been shown in TcR transgenic systems [229]. However, in normal mice, the TcR heterogeneity would result in both high and low affinity interactions producing a mixture of deletion and anergy. This would be consistent with my finding that

oral tolerance induced by high doses was only partly reversed by IL2 treatment, whereas low doses completely reversed T cell unresponsiveness.

The capacity of very low antigen doses to induce oral priming rather than tolerance might reflect the limited availability of antigen, which would be presented in a more selective manner. This might favour antigen uptake by antigen-specific B cells which are the most potent APC at capturing antigen by virtue of their surface Igs [385] and can induce priming of naive T cells [386]. In addition, T cells with the highest affinity TcR would outcompete with others to interact with the peptide-class II MHC complexes. During these high affinity interactions, it would be anticipated that Th1 responses might be preferentially augmented [173, 174] and this is consistent with my own findings.

Overall, my hypothesis above provides a theoretical basis to explore factors such as APC type, ligand density and TcR-MHC-peptide affinity in oral tolerance induced by different antigen doses. This might be achieved by using combinations of mice deficient in particular APC populations and transgenic for T cells with different TcR affinities for antigen.

Oral Tolerance as a Therapeutic Strategy

The results I have presented in this thesis have a number of implications for the potential role of oral tolerance as a therapy for immunopathological disorders. As noted earlier, an effective therapy of this type should possess at least 3 important properties: it must be able to effectively inhibit all aspects of the immune response which would normally be involved in a particular disorder; it should be able to target these pathological immune functions without influencing other protective responses generated against unrelated antigens; and it must be able to mediate its effects for long enough to provide long-term therapy. Although, my results show that the regulatory factors governing oral tolerance can be manipulated to fulfill these factors, they also highlight a number of possible problems.

A highly beneficial characteristic of oral tolerance is that it can modulate a wide range of CD4-dependent effector functions in a dose-dependent manner. My results showed that different doses of fed antigen allowed Th1 cell activity to be suppressed alone or in

conjunction with Th2-dependent immune functions. In this way, both Th1-dependent autoimmunity and Th2-dependent allergic reactions might be treated by oral tolerance induction. However, the main feature of my results was the variability of individual effector functions, implying that it will be difficult to find a single regime with readily predictable effects for a range of different antigens.

The evidence that T cell anergy was induced by single doses of fed antigen would predict that oral tolerance should be antigen-specific and therefore superior to the other forms of immunosuppression currently used in immunopathological disorders. However, tolerance due to anergy carries the risk that the residual lymphocytes could be reactivated in the presence of inflammation, which would provide an environment rich in IL2 and other costimulatory cytokines. This potential complication would not arise if oral tolerance was mediated by active suppression, where further exposure to antigen would reactivate the regulatory T cells to maintain tolerance.

Another theoretical concern over anergy induction in therapeutic strategies for autoimmunity would be the potential hazard that thymic emigrants would not be tolerised unless the antigen was administered repeatedly. Thus, autoreactive T cells would accumulate and the autoimmune disorder might recur in susceptible individuals. However, my findings indicate that this problem need not arise, since oral tolerance could be demonstrated for the entire life-span of mice fed a high antigen dose. The persistence of unresponsiveness appeared to be associated with a switch to upregulation of IFNγ production, which might have prevented the activation of naive OVA-specific T cells via its cytostatic properties [293, 319]. Regulation by IFNγ might even have allowed bystander suppression to develop in oral tolerance induced by a single high dose feed, but this feature was not investigated.

In summary, my results have highlighted that oral tolerance has several advantages over the existing forms of immunosuppressive therapy. It induces a long-lasting effect without toxicity and can inhibit a wide range of systemic immune responses in an antigen-specific manner, allowing protective responses against unrelated antigens to be generated if and when required. The unresponsiveness induced by feeding can be manipulated, by

altering the dose of antigen, to target particular aspects of the immune response and therefore, oral tolerance may form the basis of future therapies designed for the treatment of a variety of immunopathological disorders. However, it is important to point out the need to examine individual functions for each dose of each antigen, as very low doses may induce priming rather than tolerance and therefore exacerbate immunopathology. In addition, the results from this study were of naive T cell tolerance and previous findings in primed animals suggest that oral tolerance is more difficult to induce in the presence of antigen-experienced T cells [177]. This is consistent with other models of peripheral tolerance [175] and, as the principal application of oral tolerance would be for the treatment of patients with ongoing immunological disorders, my findings now need to be extended to tolerance of primed animals.

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