Intervention in Established Immune Responses by Feeding Protein Antigens

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Summary

The gastrointestinal (GI) tract is exposed continuously to a variety of foreign antigens that range from soluble dietary proteins to numerous pathogens and is thus the major site of antigenic challenge in the body. Whereas infection with a mucosal pathogen results in an active primary immune response followed by memory to subsequent exposure, oral administration of soluble antigens results in a suppressed response to subsequent systemic exposure to the antigen. This phenomenon is termed oral tolerance and its physiological role may be to prevent food hypersensitivities. In addition it may provide a potent therapy for a variety of autoimmune and inflammatory disorders. However, oral tolerance is also a major obstacle to the development of oral vaccines.

Although there is a substantial amount of evidence that suggests that suppression of autoimmunity by orally administered antigen is a feasible and therapeutic option, most existing studies of oral tolerance have explored the mechanisms responsible for the ability of fed antigen to prevent subsequent systemic immune responses in previously naive animals. Little is known about the mechanisms which determine the induction of oral tolerance in the primed immune system.

Thus the aims of my studies were to define feeding regimes for inducing optimal oral tolerance in primed mice, to establish the effects of feeding antigen to primed mice on a variety of parameters of systemic immunity *in vivo* and *in vitro* and to investigate the mechanisms of oral tolerance in primed mice. I used a model antigen in my studies so that I could exploit experimental systems already developed in the laboratory for the induction and assessment of oral tolerance to OVA in naive mice. Thus I examined a variety of antigen doses administered at different times after priming with OVA/CFA and investigated the scope of responses influenced by the tolerance. I first confirmed that it is possible to induce tolerance by feeding antigen to primed mice, with DTH responses, proliferation and IFNγ production being readily tolerised. However, the degree and scope of tolerance was less than that found in an

equivalent dose fed before immunisation. For example, TH2 cytokine production and antibody responses were not as readily tolerised when antigen was fed after priming compared with feeding antigen before priming. In contrast to previous reports, I found that only a brief time window was available after priming when mice were susceptible to induction of tolerance, which in my hands, was in order of a week. In an attempt to enhance tolerance, I fed higher single doses of antigen and increased frequency of feeding. Generally this did result in more intense and wider tolerance, although this was still less than in mice fed before priming. Together, these results illustrate the difficulty in tolerising a primed immune system and in subsequent chapters, I went on to examine the reasons why this should be the case and what the mechanisms responsible for the tolerance might be.

Previous studies in naive mice found that several parameters of the systemic immune responses remained tolerant for up to 17 months after feeding and so I investigated how long tolerance persisted after a single feed of 25mg OVA given 7 days after priming. The results indicated that the oral tolerance induced when antigen is fed after immunisation is not as long lasting as that found in naive mice. *In vivo* tolerance was only found early after feeding, when antigen specific DTH responses were suppressed compared with controls. DTH responses were normal at later time points and antibody responses were not tolerised at any time after feeding, confirming my initial findings. Overall, these results suggest that only some aspects of the established immune response can be tolerised for any length of time by a single feed of antigen. It seems that as in other forms of oral tolerance, IFNγ production is particularly sensitive to feeding in primed mice, although my results suggest that other aspects such as IL5 might also be tolerised at different times. Thus oral tolerance is a dynamic phenomenon but there is surprisingly little correlation between those aspects which can be tolerised at different times. Although this is consistent with previous results in the lab in naive mice, it emphasises the unpredictability of tolerance in primed animals.

These experiments showed that it was impossible to tolerise antibody responses and in some cases, IL5 production was more difficult to tolerise than IFNγ and could suggest that T_H2 responses are unusually resistant to oral tolerance induction in primed mice. Thus, I decided to investigate whether Th2 cells were necessary for oral tolerance in primed mice by examining the effects of feeding OVA to $IL4^{-/-}$ mice which had been immunised with OVA/CFA. The results confirmed previous findings from our laboratory that feeding OVA to naive $IL4^{-/-}$ mice results in oral tolerance. I also extended these studies by showing that primed $IL4^{-/-}$ mice develop a pattern of oral tolerance similar to that found in normal animals, with suppression of DTH *in vivo* and antigen-specific proliferation *in vitro*, as well as some suppression of IFNγ and IL5 production *in vitro*, but no effects on serum antibodies. Thus the partial effects of oral tolerance in primed mice do not depend on the presence of IL4 dependent T_H2 cells, but could reflect another mechanism such as clonal anergy/deletion or an alternative active suppressor mechanism.

An alternative active suppressor mechanism that I studied was TGFβ. To do this, I made use of the suggestion that TGFβ production is downregulated by IL12 and IFNγ and examined the induction of tolerance in $IL12^{-/-}$ mice. Certain aspects of tolerance were enhanced in $IL12^{-/-}$ mice compared with wild type mice, namely IgG1 antibody levels. However, other components of tolerance such as total OVA-specific IgG antibody levels were normal and tolerance of OVA-specific DTH levels were reduced, and no clear pattern emerged. Therefore, it is probably true to conclude that the absence of IL12 has no overall effect on the induction of tolerance induced either before or after immunisation, with no reproducible enhancement of tolerance in primed animals. These results suggest that it is unlikely that the main mechanism of oral tolerance in primed mice is active suppression via TGFβ, however, it would be important to measure TGFβ production directly in such animals.

As oral tolerance in primed mice was not as profound or wide ranging as that found when equivalent amounts of antigen are fed to naive mice, I was interested in finding an alternative strategy for enhancing tolerance in primed animals. Recent work

has shown that administration of Flt3L to mice enhances the induction of oral tolerance in naive mice and I decided to test the idea that expansion of DCs by Flt3L might also extend the scope of oral tolerance in primed mice. The rationale for this effect appears to be that Flt3L expands the numbers of resting dendritic cells in the gut which then present fed antigen without costimulation to T cells, resulting in more profound anergy and/or clonal deletion, and thus function of tolerance. The results showed that expanding DCs with Flt3L does not interfere with the induction of oral tolerance in primed mice and Flt3L actually enhanced the induction of tolerance in some instances and occasionally allowed tolerance of some responses not normally susceptible to tolerance, such as antibody production. However, the effects of Flt3L were quite variable, probably reflecting the influence that administration of Flt3L had on the level and the speed of the systemic immune responses after challenge with OVA/CFA.

Next I attempted to examine the induction of oral tolerance in primed T cells using the adoptive transfer of Ag-specific transgenic T cells as I thought this might be an appropriate means of studying fate of antigen-specific T cells in primed mice fed OVA. In the first experiments, I found that feeding soluble OVA after priming could alter the kinetics of T cell expansions, but this only occurred if feeding was delayed until 10 days after immunisation. This did not simply appear to be due to re-exposure to antigen, as there was no equivalent effect of feeding OVA in an immunogenic manner with CT. In addition, the inhibited expansion caused by feeding soluble antigen after priming was accompanied by suppressed proliferative responses to OVA. These results contrasted with my earlier findings in normal mice, where tolerance could be induced early, but not late after priming. To try and investigate why this might be the case, I fed a higher dose of OVA, as I thought the increase in numbers of Ag-specific T cells in transfer recipients might complicate the system. These experiments again showed that tolerance might occur late after priming, but there was still no effect earlier and also functional tolerance was variable in that proliferative responses were easier to tolerise than cytokines responses.

I then examined some of the possible reasons underlying the resistance of the primed immune system to oral tolerance. In the first experiment, I found that feeding OVA to mice with a fully established immune response did not improve tolerance. Indeed, feeding OVA at this time appeared to stimulate a secondary response for some aspects of the systemic immune response. As I thought this long term resistance to oral tolerance might reflect the depot effect of this adjuvant, I decided to prime mice with OVA and LPS as a means of administering antigen in an adjuvant without long term depot effects. I found that feeding OVA early after immunisation with OVA/LPS results in a pattern of tolerance similar to that found when mice are primed using CFA as the adjuvant. Next, I went on to show that feeding OVA to mice with a fully established immune response induced with OVA/LPS did not improve tolerance. Thus, I concluded that the difficulty in inducing oral tolerance six weeks after priming was not due to a depot of antigen or adjuvant, but was rather due to the presence of an established immune response. Therefore, I decided to test the idea that activated/memory CD4 T cells were inherently resistant to tolerance induction. In this experiment, I found evidence that oral tolerance could be induced when I fed OVA to mice that were transferred with antigen experienced "memory" cells. Thus, I concluded that activated/memory CD4 T cells are not inherently resistant to orally induced tolerance and that their resistance in the intact animal might be due to their being exposed to persistent antigen in the context of continual costimulation.

In conclusion, the results presented in this thesis have confirmed and extended previous findings on the induction of oral tolerance by feeding antigen after priming. However, they have highlighted several important features of the phenomenon including the fact that antibody responses are not readily tolerised in primed mice and that higher doses of antigen are required to obtain tolerance equivalent to that found in naive mice. My study does not allow me to make firm conclusions whether similar mechanisms underly the oral tolerance induced by feeding antigen before or after systemic immunisation. Although I would propose that anergy and/or clonal deletion is the main mechanism of oral tolerance in primed mice, there is need for careful

definition of the effects of different dose regimes and timing of feeding regimes if this approach is to be used therapeutically. These results have implications not only for the use of oral tolerance in the treatment of inflammatory diseases, but also for understanding the regulation of immune responses to protein antigens *in vivo* in that they highlight the differences in the response of a naive and a primed immune system, and therefore subsequent consequences, to antigen given via a tolerogenic route.

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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:

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Abbreviations

APC	Antigen Presenting Cell(s)
BALT	Bronchial-Associated Lymphoid Tissue
CFA	Complete Freunds' Adjuvant
CMI	Cell Mediated Immunity
CT	Cholera Toxin
DC	Dendritic Cell(s)
DTH	Delayed type hypersensitivity
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence-Activated Cell Scanner
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
GALT	Gut-Assoociated Lymphoid Tissue
HAO	Heat-Aggregated OVA
HEV	High Endothelial Venule(s)
IBD	Inflammatory Bowel Disease
IEL	Intra-Epithelial Lymphocyte(s)
IFNγ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
ISCOMS	Immune Stimulating Complexes
LP	Lamina Propria
MALT	Mucosa-Associated Lymphoid Tissue
MHC	Major Histocompatability Complex
MLN	Messenteric Lymph Node
OVA	Ovalbumin
PBS	Phosphate Buffered Saline
PE	Phycoerythrin

PLN	Popliteal Lymph Node
PP	Peyer's Patch
SA	Streptavidin
SB	Staining Buffer
S.D.	Standard Deviation
TCR	T Cell Receptor
TGFβ	Transforming Growth Factor-β
T_H1	T-helper 1
T_H2	T-helper 2
TMB	3,3'5,5'-tetramethylbenzidine
TNFα	Tumour Necrosis Factor-α
TGF	transforming growth factor
Tg	transgenic

Chapter 1 Introduction

1.1 Oral Tolerance

The gastrointestinal (GI) tract is exposed continuously to a variety of foreign antigens that range from soluble dietary proteins to numerous pathogens and is thus the major site of antigenic challenge in the body. Although the gut-associated lymphoid tissues (GALT) contains 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. More importantly, it might incur food hypersensitivities such as coeliac disease (1,2). T-cell mediated responses to wheat gluten are believed to cause the severe enteropathy found in coeliac disease and similar hypersensitivity to harmless intestinal antigens from the gut flora may be responsible for inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis. However, such diseases are relatively uncommon because the intestinal immune system distinguishes between harmless antigens and those of pathogenic importance (3-5). Whereas infection with a mucosal pathogen results in an active primary immune response followed by memory to subsequent exposure (6), oral administration of soluble antigens results in a suppressed response to subsequent systemic exposure to the antigen (2-5). This phenomenon is termed oral tolerance and its physiological role may be to prevent food hypersensitivities. In addition it may provide a potent therapy for a variety of autoimmune and inflammatory disorders (7,8). However, oral tolerance is also a major obstacle to the development of oral vaccines.

1.2 Anatomy of Mucosal Immune Responses

Mucosal immune responses differ from those in the periphery in many ways and it seems probable that these distinctive features may account for some of the unusual responses to intestinal antigens.

The gut-associated lymphoid tissues (GALT) forms the major part of the mucosa-associated lymphoid tissue (MALT) (2) and comprise lymphocytes and other cells scattered through the mucosa, as well as the organised tissues of the Peyer's patches and mesenteric lymph nodes (MLN). Peyer's patches are organised secondary lymphoid organs separated from the intestinal lumen by a single epithelial layer. Although Peyer's patches have a typical secondary lymphoid structure, they differ from other secondary lymphoid organs because they lack afferent lymphatics. However, the dome area of the Peyer's patch is covered by a unique epithelium enriched for specialised antigen-sampling cells, known as microfold (M) cells (9). The luminal surface of M cells may function to transfer antigen directly from the intestinal lumen to underlying macrophages and dendritic cells. M cells do not express class II MHC antigens and thus primarily perform a transport function (9). 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 (7). Also, M cells may transport antigens to the T and B cells in the dome and underlying follicles.

Peyer's patches are believed to be the primary area of the GALT where specific immune responses are generated. Their germinal centres are the major source of IgA producing B cells (10) and the parafollicular region is rich in mature $\alpha\beta$ T cells of both $CD4^+$ and $CD8^+$ phenotype. $CD4^+$ cells of both T_H1 and T_H2 phenotype are found in the dome region (7). 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 which can suppress immune responses to dietary antigens can be generated (11-13). Draining the Peyer's patches via the lymphatics are the MLN which are identical in overall structure to other peripheral lymph nodes (9) and may function as a cross-over point between the peripheral and mucosal immune systems.

In addition to the organised tissues of the Peyer's patches and the MLN, the villus/crypt units of the intestine contain large numbers of scattered lymphocytes, both in the epithelium itself and in the deeper layer of the lamina propria. In contrast to the Peyer's patches and the MLN, which probably act as inductive sites, these scattered lamina propria and intraepithelial lymphocytes (IEL) are the effector arm of the local response, providing a means for generating disseminated immunity throughout the length of the intestine (2). The epithelium and lamina propria represent highly distinct compartments of the immune system, despite being separated by only a thin layer of basement membrane.

The lamina propria contains most components of the immune system, with large numbers of B cells, plasma cells, macrophages, dendritic cells and T cells of both the $CD4^+$ and $CD8^+$ subsets. Mucosal inflammatory cells such as eosinophils and mast cells are also present (2).

Intraepithelial lymphocytes are adjacent to the columnar epithelial layer of small intestinal villi and are one of the largest populations of lymphocytes in the body (14). Virtually all are T cells and 80% or more are $CD8^+$ expressing a unique integrin molecule, $\alpha_E\beta_7$. Ligation of $\alpha_E\beta_7$ by its counterstructure, E-cadherin, on enterocytes is generally considerd to be crucial for tethering IEL within the epithelial layer (2). A further unusual feature of IEL in many species is that they are relatively enriched for $\gamma\delta$ TCR expressing T cells, although $\alpha\beta$ TCR IEL are also abundant (15). All $\gamma\delta$ TCR expressing IEL and around 50% of $\alpha\beta$ TCR expressing IEL express the CD8 α chain without the β chain (ie they are CD8 $\alpha\alpha^+$). Despite this knowledge, the function of IEL is largely unknown though they may constitute a primitive population specialised for immune surveillance of epithelial surfaces (14). The TCRs expressed on IEL appear to be polyclonal, based on junctional sequences. $\alpha\beta$ TCR IELs also appear to express a restricted set of V regions preferentially. However, skewing toward particular V region usage appears to be the result of oligoclonal expansion of certain IEL clones, perhaps in response to a limited array of antigens. IELs have also been shown to be activated and cytolytic, but they proliferate poorly. One possibility is that IEL may play

a role in cellular immune defense of the intestinal epithelial barrier via their production of a variety of cytokines, including IL2, IFNγ, IL5 and TGFβ (16,17).

Recirculation of lymphoid cells into and within mucosal tissues is also controlled by unique mechanisms and by the unusual anatomy of the GALT. T and B lymphocytes encountering the appropriate antigen in the Peyer's patch or lamina propria, exit via the lymphatic network in the mucosal wall and drain to the MLN and thence via the efferent lymphatics and connecting thoracic duct to the bloodstream, before recirculating back to the effector sites of mucosal tissues (2,18,19). These recirculation properties of mucosal lymphocytes reflect the fact that, after activation in the GALT, lymphocytes decrease their expression of L-selectin, the adhesion molecule which allows them to interact with high endothelial venules (HEV) in peripheral lymph nodes, and up-regulate the $\alpha 4\beta 7$ integrin whose ligand, mucosal addressin cell adhesion molecule (MAdCAM-1) is expressed by blood vessels in mucosal tissues (2, 20-22). This recirculation pathway differs from the route taken by lymphocytes activated in peripheral organs, which do not enter the mucosae as they do not express $\alpha 4\beta 7$.

The most obvious feature of the mucosal immune system is the predominance of IgA as the major class of antibody present in secretions. This IgA is derived from plasma cells present in the lamina propria, which themselves are derived from IgA-producing B cells in the Peyer's Patches. The overall function of secretory IgA remains somewhat unclear, but it is important for neutralization of viruses (23) and toxins (24). 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 (25), or promoting their entrapment in the mucus (26) and subsequent agglutination (27). This function is known as immune exclusion and a similar mechanism may also reduce the absorption of dietary and respiratory antigens (28), possibly accounting for the increased absorption of food antigens (29) and increased susceptibility to food hypersensitivity (20) found in patients with selective IgA deficiency. However, most patients with this common immune deficiency have no symptoms. It is important to

note that there is no correlation between immune exclusion and oral tolerance in experimental studies (21), while 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.

Both the organised and diffuse compartments of the GALT contain conventional APC of many kinds, including dendritic cells, activated B cells and macrophages. The relative roles of these different APC types are unknown, but it seems increasingly possible that each may be involved in different aspects of the local immune response, such as determining whether active immunity or tolerance is induced. The small surface area of M cells suggests that not all antigen can be taken up in this way and it seems likely that soluble antigens also enter the mucosa by being endocytosed across the absorptive gut epithelium via conventional enterocytes, either by paracellular routes or via the enterocytes themselves (2). The antigens may then be processed and presented by local MHC class II-expressing cells of the underlying lamina propria, such as macrophages, B cells and dendritic cells. MHC class II molecules are also present on the basolateral membrane of enterocytes and may allow these cells to play a role in processing and presenting antigen to T cells of the lamina propria or to IEL within the epithelium itself (30). Although supported by *in vitro* studies which have demonstrated that isolated enterocytes from rat and human small intestine can present antigens to appropriately primed T cells (31-34) it is not known if this functions *in vivo*. Some antigens entering the mucosa via this route also gain access to the systemic circulation and hence are likely to enter peripheral lymphoid tissues.

1.3 Regulation of Immune Responses to Dietary Antigens in Oral Tolerance

1.3.1 History

The first evidence that fed antigens could suppress systemic immune responses was an anecdotal report 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 (35). The author and scientist H.G.Wells in 1911, was the first to conduct formal studies of the phenomenon of oral tolerance and showed that anaphylactic reactions to OVA and other proteins in guinea pigs were prevented by prior feeding with hen egg proteins (36). The immunological nature of the phenomenon was first established by the later experiments of Chase in 1946 (37) and studies of oral tolerance during the 1970's and 1980's established that all aspects of the immune response could be tolerised by feeding antigen. Since then, the protocols of inducing oral tolerance and the mechanisms involved have attracted considerable attention, not only from mucosal immunologists, but also from individuals interested in using this system as a model of immunoregulation or as a therapy for immunopathology.

1.3.2 Scope & Longevity of Oral Tolerance

The induction of oral tolerance has been documented widely in many species, including man (38,39). However, species differences do occur and oral tolerance is not induced at all in ruminants (40). Most work has examined responses to proteins, but oral tolerance can be induced to a wide range of other types of antigen, including peptides (41), contact sensitizing agents (42), heterologous red blood cells (43), allogeneic leukocytes (44) and inactivated viruses (45) or bacteria (46).

Feeding antigen can tolerise almost all aspects of the immune response. These include IgM, all IgG isotypes and IgE antibody responses (47-52), as well as cell mediated immune (CMI) responses measured by delayed-type hypersensitivity (DTH) or contact sensitivity *in vivo* (53-55) and lymphocyte proliferation (56,57), cytokine production (57,58) and CD8 cytotoxic T-cell responses *in vitro* (59). One exception may be the production of IgA in the gut, which has been reported to be primed (60), tolerized (11), or unaffected (61) by different regimes of oral tolerance. Although antibody responses to soluble antigens are generally more difficult to suppress than CMI, IgE responses seem to deviate from this rule, being remarkably susceptible to induction of oral tolerance (62-63). This highlights the important biological role of oral

tolerance, as it is interesting to note that both IgE and CMI responses are frequently associated with pathological food hypersensitivity disease.

Systemic tolerance can be demonstrated if animals are challenged within 1 to 2 days of a single feed of antigen (60) and is at its maximum during the first few weeks after feeding. Oral tolerance has been shown to last for 18 months in laboratory mice, although the longevity of tolerance varies for the different facets of the immune response (64). Tolerance of T cell functions is very long lasting, whereas tolerance of IgG responses may wane within 6 months (64). In view of the fact that the average life-span of a laboratory mouse is little more than 2 years, these findings highlight the potency and stability of this immunoregulatory phenomenon. In addition, these data are further evidence that systemic T-cell responses are affected more readily by feeding antigen than humoral immunity. Although the longevity of oral tolerance has not been addressed formally in humans, food hypersensitivities rarely begin in adulthood, indicating the likely persistence of long-term functional unresponsiveness once it has been established (5).

1.4 Clinical and Practical Relevance of Oral Tolerance

1.4.1 Physiological Prevention of Intestinal Hypersensitivity

A number of important chronic diseases of intestinal mucosal surfaces appear to have an immunopathological basis. This idea is consistent with observations of mucosal pathology 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 (65-68). In each case, the features of disease induced are similar to those found in naturally occurring food sensitive enteropathies (FSE) (69,70). One example is coeliac disease where patients have T cell mediated hypersensitivity to the dietary antigen gluten from wheat perhaps reflecting a breakdown in oral tolerance. Recent studies of transgenic and knockout rodents suggest that a similar defect in immunoregulation within the

intestine can result in pathological hypersensitivity to gut flora, with the development of inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis. Examples of the different models of IBD are HLA-B27-transgenic rats (71), human CD3ε chain transgenic mice reconstituted with normal bone marrow (72) and mice with genetically determined knockouts of a number of immunologically relevant genes, including IL2 (73), IL10 (74), α chains of the TCR (75), or Gi2α (76), an inhibitor of G proteins. The induction of IBD in all the models requires intestinal flora, as it does not occur in animals bred in germ-free conditions or given gut sterilising antibiotics. These models indicate that inflammatory T cells capable of being activated by endogenous antigens are present in the normal mucosal immune system and are usually held in check by regulatory cells (see below). Therefore, it is generally concluded that suppression of the immune response to ubiquitous luminal bacteria may be critical for maintaining mucosal homeostasis and thus preventing enteropathy.

1.4.2 Immunotherapy

The oral route offers a convenient and highly acceptable means of administering therapeutic agents and several studies have demonstrated the effectiveness of orally administered antigen in rat and mouse models of autoimmune disease (77,78). This work has stimulated trials of oral tolerance as an immunotherapy for human disease (79-81) and a number of such studies are currently underway. Although early reports were encouraging, more-recent studies have highlighted difficulties. In one study of rheumatoid arthritis, clinical effects and a reduction in collagen type II antibodies were associated with a high collagen intake (82), whereas the original study by Weiner and colleagues suggested positive responses in patients who had received a low dose. In a multiple sclerosis study, clinical response was associated with an increase of myelin-specific TGFβ-secreting regulatory T cells (83). There are also clinical trials being conducted in patients with insulin-dependent diabetes mellitus, myaesthenia gravis and uveitis, but no published data are available yet. 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.

1.5 Factors Influencing Immune Responses to Fed Antigen

1.5.1 Nature and Dose of Antigen

A wide range of antigens are capable of inducing oral tolerance. Tolerance can probably be induced to all thymus-dependent soluble antigens (84,85), but particulate or replicating antigens (ie pathogens) often induce active immunity instead of tolerance. This could reflect preferential uptake by M cells overlying the Peyer's patches, resulting in more efficient antigen processing (86). In addition, the provision of an inflammatory stimulus also prevents the induction of tolerance, and when antigens such as OVA are coupled to immune stimulating complexes (ISCOMs) or to bacterial toxins (87), local and systemic immunity are likely (88).

A wide range of doses and regimens of single and multiple feeds of antigen can induce systemic unresponsiveness. However, there may be dose-dependent differences in the extent to which individual systemic responses are tolerized. There is some evidence that single administration of high doses of antigen induces suppression of virtually all responses by direct inactivation of T cells (57,58,89,90), whereas multiple low doses are more likely to generate regulatory cells (91,92), with more variable effects on individual responses. Continuous exposure of antigen in the drinking water, which is the most physiological route, may lead to more-profound tolerance, even when corrected for the administered total antigen dose (93,94). However, it should be noted that very low doses given orally have been shown to prime the animal for subsequent systemic and local immune responses (95). Although, these conclusions have been drawn from studies in rodents, similar findings have been made in man and a recent report has shown that the duration of feeding alters the mechanisms of tolerance induced by soluble protein antigens (96).

1.5.2 Host Factors

Several host factors influence oral tolerance induction, including genetic background, host age, intestinal flora, intestinal absorption and antigen uptake and presentation.

Most mouse strains are tolerizable to a large number of antigens and, although there are some exceptions, there are no clear linkages to major histocompatibility complex (MHC) haplotype, IgE responder status or differences in antigen uptake via the gut. However, the rate of antigen clearance from the circulation is influenced by genetic differences and can affect the degree of tolerance induction (21).

Food hypersensitivities are most common in infants, particularly at or near the time of weaning, suggesting that oral tolerance is defective during these periods. Intragastric antigen administration to neonatal rodents during the first 7-10 days of life does not suppress systemic immunity and may prime for later systemic immunity and autoimmune responses (97,98). It seems that this inability to induce tolerance is not merely a result of the immaturity of the digestive system or antigen-handling capacity of the neonatal gut (99), but is more likely to be due to an as-yet-uncharacterized regulatory imbalance that can be partially restored with adult spleen cells. An analogous deviation from tolerance induction occurs during weaning (100,101). At the other end of the age spectrum, ageing mice have been reported to become increasingly resistant to the induction of oral tolerance by feeding OVA (102).

The extent and nature of intestinal colonization by bacterial flora can effect the outcome of oral administration of antigen. Although lipopolysaccharide (LPS)-unresponsive mice have defective oral tolerance to sheep red blood cells (SRBCs), tolerance to proteins is normal. Indeed, if given at the time of feeding, LPS may enhance the induction of T-cell tolerance in normal mice fed OVA (103). However, further indirect support for the idea that LPS may play a modulatory role in regulating immunity to dietary antigens comes from the observations that tolerance to OVA may be relatively short-lived in germ-free mice (104).

1.5.3 Antigen Uptake and Processing

Antigen handling and processing have important roles in oral tolerance induction. Aberrant presentation of antigen by APCs lacking a full range of costimulatory molecules is thought to be the cause of clonal anergy of T cells in many other forms of tolerance (105). The fact that stimulation of the RES can prevent the induction of oral tolerance (66,67,106) indicates that antigen presentation may also be important in regulating responses to a fed antigen.

The site and nature of the relevant APC involved in the induction of oral tolerance remain to be identified. There are a number of possibilities. If oral tolerance is induced entirely at local sites in the gut, this might imply a role for MHC class II$^+$ epithelial cells or non-professional APC either in the Peyer's patches or lamina propria (31). The enterocytes that make up the lining epithelium of the intestine express MHC class II and can present antigen to primed T cells *in vitro* (31,107,108). However, enterocytes do not express ICAM-1 or B7-1 (105) and so presentation of antigen by these cells would be expected to lead to anergy of naive CD4$^+$ T cells. B cells are numerous in the gut and antigen presentation by resting B cells has been shown to be a potent inducer of T cell tolerance *in vitro*. However, peripheral T-cell tolerance can be induced in B-cell-knockout mice (109) which may suggest that B cells are not major players in oral tolerance. Dendritic cells (DCs) are abundant in the gut and have been shown to migrate to the MLN from the gut after intra-luminal or intra-gastric challenge (110,111). Potent presenters of soluble protein, dendritic cells (DC) are normally associated with T cell activation (112). However, recent work suggests that dendritic cells can present antigen in a tolerogenic fashion (113) and it has been shown recently that administration of the DC growth factor Flt3L to mice expands the number of DCs in the intestine and other lymphoid organs, and increases the susceptibility to induction of tolerance by feeding OVA (114). DCs recruited in the intestine by administration of the growth factor Flt3L express only low levels of B7-1 or CD40 costimulatory molecules, supporting the view that intestinal DCs may normally be in a resting state *in situ* without the ability to prime T cells (114). Thus, it could be proposed that DCs are

one of the major gatekeepers of the mucosal immune system, sustaining the induction of T cell tolerance to soluble proteins or noninvasive microorganisms, and generating active immunity only when confronted by potentially harmful antigens in the context of appropriate secretion of cytokines or other inflammatory signals.

One problem raised by the idea that oral tolerance occurs after entirely local presentation of antigen by DCs or epithelial cells is that T cells tolerised in the intestine should recirculate preferentially back to mucosal sites. Thus it would be difficult to explain the systemic nature of the immune unresponsiveness and this may mean that presentation of fed antigen must also occur in the periphery (19). This could be achieved if mucosally situated APC acquired antigen in the gut and then dispersed to the periphery to present antigen in a tolerogenic manner. Alternatively, fed antigen absorbed into the blood might associate with non-professional APCs in the periphery (115-117). In support of this, oral tolerance is associated with the presence of tolerogenic antigen which appears in the circulation from 15-60 minutes after feeding and which can suppress systemic delayed-type hypersensitivity when transfered in to naive recipients (55,89,118,119). As the amount of fed antigen absorbed intact varies widely, between 0.001% and 1% of the administered dose, it seems likely that differences in absorption could influence oral tolerance. Whether this is influenced by intralumenal digestion is controversial. Attempts to analyse the requirements for intralumenal digestion by oral administration of serine protease inhibitors prior to feeds have been inconclusive (119-121). However, it is possible to induce systemic tolerance by colonic antigen administration (122), suggesting a single filtration mechanism may be invloved.

1.5.4 Immunological Status of Host

There is a substantial amount of evidence that suggests that suppression of autoimmunity by orally administered antigen is a feasible and therapeutic option. However, most existing studies of oral tolerance have explored the mechanisms responsible for the ability of fed antigen to prevent subsequent systemic immune

responses in previously naive animals and little is known about the mechanisms which determine the induction of oral tolerance in the primed immune system. If treatment of humans is to become a therapeutic reality, it will be necessary to re-establish tolerance in a previously sensitized host.

It is well known that it is difficult to induce systemic tolerance in animals that have been previously primed to the antigen (123). Despite this, several investigators have shown that oral tolerance can be induced in parenterally primed mice (124,125) and the ability of oral tolerance to abrogate ongoing immunity extends to several experimental models of immunologically mediated disease, including EAE (7), collagen-induced arthritis (126), experimental autoimmune uveoretinitis (127), immune complex-mediated glomerulonephritis (128), insulin-dependent diabetes (129), and allograft rejection (130). The few studies conducted with model antigens in primed mice suggest that larger or more frequent doses may be required to induce oral tolerance in primed animals than in naive animals, and that this may only be possible for a limited period after systemic immunization. Higher doses of antigen were required to be fed to primed mice compared with naive mice in order to sufficiently suppress systemic delayed-type hypersensitivity (DTH) responses. Suppression of DTH responses was greatest when antigen was fed soon after immunization, and became less pronounced as the time interval between feeding and immunization increased over 7 days (124). As in naive animals, oral tolerance of established immune responses affects T cell immunity more than humoral responses and it has been reported that serum IgG antibody responses can be suppressed only by multiple feeds of high dose OVA given in the first 5 days after priming. In contrast DTH responses were suppressed by single feeds of high or medium doses of OVA given at the same time (124).

A recent study on the suppression of established murine chronic relapsing EAE by the oral administration of MBP has confirmed that oral tolerance in primed mice is induced better by high doses of antigen given sooner after immunisation and that multiple high doses were best. Thus, multiple oral doses of MBP given early after

immunisation were required to achieve suppression of clinical signs of disease (131). However, conflicting results were obtained using proteolipid protein peptide (PLP) orally to down-regulate ongoing EAE (132). Here neither feeding single nor multiple doses of PLP could down-regulate ongoing disease, inhibit the progression of relapsing EAE or reduce antigen-specific proliferation or cytokine (IL2, IL4 and IFNγ) production. Thus, the specific effects of feeding antigen to primed animals remain unclear and much remains to be learnt of the best protocols for inducing this form of oral tolerance, as well as of the mechanisms involved. To date there have been no formal studies in which wide ranges of single or multiple doses of antigen have been given at many different times after immunisation. In addition, the effects of such regimes in a full range of effector immune responses *in vivo* and *in vitro* have not been determined. Experiments of this nature are now feasible due to the great expansion in our knowledge of the equivalent events which occur in oral tolerance in naive animals.

1.6 Mechanisms of Oral Tolerance

1.6.1 Mechanisms of Peripheral Tolerance

Oral tolerance is one form of peripheral T cell tolerance in which mature lymphocytes in the peripheral tissues become unresponsive after contact with antigen. The mechanisms of peripheral tolerance to parenterally administered antigens has been investigated in great detail and can be organised generally as clonal ignorance, anergy, deletion and active regulation.

Naive T lymphocytes that come into contact with their specific antigen can effectively ignore the antigen thus rendering the animal phenotypically unresponsive. Evidence for this comes from transgenic models which show that peptides may be presented in the context of MHC Class I on cells which are unable to trigger any response from T cells with the appropriate TCR because they lack expression of the costimulatory or accessory molecules required to enhance T cell avidity (133-135). In many cases, extrathymically expressed antigens also appear to be ignored by CD4 T

cells and this may be because parenchymal tissues normally express relatively few MHC Class II molecules and therefore may not be surveyed efficiently by naive T cells (136,137). Although this prevents primary responses to tissue antigens, it is clear that the relevant T cells will remain present in the animals and will respond normally if the appropriate antigen is later presented by a professional APC or in the context of inflammation (137-140). This is unlikely to account for oral tolerance, as orally tolerised T cells do not respond normally when antigen is presented by professional APCs and in the context of inflammation due to systemic challenge with antigen in adjuvant.

An alternative means of T cell tolerance is direct T cell inactivation either by clonal deletion or functional anergy. Clonal deletion of self reactive T and B lymphocytes occurs predominantly in the thymus and bone marrow respectively and is required for central tolerance to self antigens (141). Clonal deletion of T cells may also occur extrathymically when peripheral tolerance is induced by exogenous superantigens (142) or after parenteral administration of conventional antigens to TCR transgenic animals (143). However, this is probably rare in normal animals, where clonal anergy is likely to be more frequent.

Anergy has been operationally defined as the state in which a lymphocyte may recognize its specific antigen but does not respond to subsequent stimulation by proliferation or generation of effector functions (144). Several investigators have suggested that induction of anergy in their systems is due at least in part to the lack of IL2 production by T cells upon TCR ligation.thus preventing autocrine IL2-mediated proliferation (145). Anergy can be reversed either by the addition of IL2 (146), or by resting the anergic cells in the absence of antigen (147). This unresponsive state is associated primarily with CD4 T cells and can occur under a number of circumstances, although one common feature is the presentation of antigen by APC that fail to prime T cells adequately. This can occur when antigen is presented by APC that do not provide costimulation, or when supraoptimal levels of antigen are used or when T cells are stimulated with altered peptide ligands (148). A number of costimulatory molecules are

important in the activation of T cells and avoidance of anergy. Although a key interaction of this kind is that between CD28 on T cells and B7-1 on APC, others include LFA-1/ICAM-1, VLA-4/VCAM-1, CD2/LFA-3, CD40L/CD40 or IL1. An alternative possibility is that distinctive costimulatory interactions are involved in the induction of anergy or activation. This is suggested by the recent report that the induction of tolerance by parenteral antigen specifically requires interaction between CTLA4 and B7, whereas immunity requires interaction between CD28 and B7 (149,150). It has been shown recently that CTLA4 may also be required for the induction of high dose oral tolerance (151).

The cardinal feature of T cell anergy is a lack of proliferation and production of IL2 and it has been shown that anergic T cells may have preserved production of certain cytokines, including IFNγ, IL3, IL10 and granulocyte macrophage colony stimulating factor (GMCSF) upon restimulation (152-155). The regulatory activity associated with some of these cytokines might explain why anergic cells from tolerised mice can act as suppressor T cells when adoptively transferred into athymic and SCID mice (152,156). This could also reflect the ability of $IL2R^+$ anergic T cells to compete with naive cells for available IL2 or for antigen-MHC complexes on APC (157).

Tolerance mediated by the regulation of one lymphocyte population by another is known as active suppression. This mechanism of tolerance has been known for some time and was initially demonstrated in adoptive transfer studies where lymphocytes from tolerised animals could transfer hyporesponsiveness to naive animals. This was often associated with CD8 T cells, although this idea has subsequently fallen into disrepute (158). Active suppression has been described in various forms of extrathymic tolerance (159) and is also believed to play an important role in the regulation of normal immune responses (160). Frequently the suppression is induced in an antigen-specific manner, but exerts its effects through a variety of antigen non-specific soluble factors (161,162), including glucocorticoids elicited during a stress response (163) and cytokines, such as IL6 (164), IL10 (165), IFNγ (166) and TGFβ (167). One possibility is that active suppression reflects cross-regulation of the T_H1

and T_H2 subsets of $CD4^+$ T helper cells. The T_H1 subset secretes IL2 and IFNγ production, promotes inflammation and assists B cell production of IgM and IgG2a, whereas T_H2 cells secrete IL4, IL5, IL6 and IL10 and promote B cell production of antibodies, especially IgE and IgG1. They are thus important in immune responses to allergens and helminth parasite infections (168). T_H1-dependent IFNγ downregulates production of lymphokines by T_H2 cells, which in turn can inhibit T_H1 cell activity via IL4 and IL10 production (169). Recently it has been suggested that there may be an additional susbset of regulatory T_H3 or T_R1 cells that can secrete inhibitory cytokines such as TGFβ and/or IL10 (170-172).

It is important to consider that these groups of mechanisms need not be mutually exclusive and that their relative importance may depend on the individual circumstances.

1.6.2 Mechanisms of Oral Tolerance

1.6.2.1 Introduction

As with peripheral tolerance, the mechanisms that have been implicated in oral tolerance can be divided into active modulation and direct inactivation of responding lymphocytes. Although these individual mechanisms may not be mutually exclusive, the applicability of oral tolerance in the treatment of autoimmune diseases may be critically dependent on which mechanism is triggered.

An active suppressor mechanism would imply that tolerance is mediated by the regulation of one lymphocyte subpopulation by another and that suppression of responses to an unrelated "bystander" antigen may be feasible if the regulatory mechanism was mediated by antigen non-specific factors such as cytokines. "Bystander suppression" occurs when an orally tolerised animal is challenged both with the original antigen and an unrelated antigen and it is believed to be mediated by antigen-non-specific cytokines, such as TGF-β, released by regulatory T cells (173,174). Bystander suppression is therefore the basis for most therapeutic uses of

oral tolerance, which employ antigens that are not normally the original antigen inducing the immunopathology. Although the presence of bystander suppression and other mechanisms of active regulation have not yet been examined directly in oral tolerance in primed mice it has been reported that TGF-β production is not enhanced in mice tolerised by feeding myelin basic protein after priming (131). A disadvatage of bystander suppression would be that this mechanism of tolerance might not be stable and could suppress the immune response to a harmful antigen at the site where active suppression was being induced. In contrast, antigen induced inactivation of potentially reactive lymphocytes due to deletion or functional anergy might imply a more stable, long-lasting tolerance that is less susceptible to modification by other immune responses, although anergy could be reversed by IL2 production released e.g. during inflammation or an infection. In addition, practical exploitation of this mechanism would require that the disease-inducing antigen was known. Interestingly, which mechanism that dominates in individual circumstances may be determined by the dose of antigen that is fed.

1.6.2.2 Direct Inactivation of Ag-specific Lymphocytes

Clonal deletion has not been described during oral tolerance induction in normal animals. Studies using TCR transgenic mice have shown that high doses of OVA or the immunodominant epitope of myelin basic protein can induce Ag-specific deletion of T cells in the spleen and gut-associated lymphoid tissue (GALT) of Ag-fed mice (175,176). However, 'unphysiologically' large doses of antigen were required to demonstrate such effects and it is difficult to detect functional tolerance *in vivo* in these fully transgenic animals. Circumstantial evidence that deletion may occur during oral tolerance under physiological conditions has been provided by the observation that orally tolerized lymphocytes die by apoptosis when cultured *in vitro* after an *in vivo* challenge (177). However, apoptosis of antigen specific lymphocytes has never been demonstrated *in vivo* in tolerised normal mice. Furthermore, the apoptosis *in vitro* only occurred if the animals had been challenged systemically with antigen in adjuvant. Oral

tolerance can also be induced normally in lpr mice arguing against a role for fas-dependent deletion in the intact animal (178). Overall, these results suggest that elimination of tolerant lymphocytes by classical mechanisms of clonal deletion may not normally occur after the induction of oral tolerance *in vivo*. Rather, the apoptosis of tolerised lymphocytes observed *in vitro* may reflect abnormal susceptibility of anergic cells to die when restimulated *in vivo* and removed to culture *in vitro*. However, it is possible that apoptosis in normal animals occurs at such a low frequency *in vivo* as to be undetectable.

Clonal anergy is therefore considered to be a more important mechanism of oral tolerance to peripheral antigens, especially when tolerance has been induced by feeding high doses of antigen. There are several experimental models of oral tolerance in which there was no evidence of active suppression and in which functional antigen-reactive lymphocytes appeared to be absent (58,89,90). Under these circumstances, the impaired ability of cells to respond to antigen *in vitro* was restored by a period of culture with exogenous IL2 indicating the continued presence of antigen-reactive T cells (89). More recently, it has been shown by using mice adoptively transferred with OVA-reactive transgenic T cells, that Ag-specific T cells persist after feeding tolerogenic doses of OVA, but are unresponsive to restimulation with Ag *in vitro* (114,179,180). This is direct evidence that anergy rather than deletion is one of the major mechanisms underlying oral tolerance. These findings have been extended by a recent report in which cytochrome c was fed to mice transgenic for the β chain of a cytochrome c-specific TCR. Using cyt c/I-E^k tetramer staining reagent to detect Ag-specific T cells, it was shown that multiple feedings of Cyt c decreased the number of antigen specific T cells in the periphery and reduced T cell responses (181). The results in this report are consistent with clonal anergy and perhaps some deletion. Interestingly, no evidence was found for skewed T cell cytokine production.

If anergy is the mechanism induced by oral administration of antigen then this makes it difficult to understand the functional tolerance which maintains the unresponsiveness throughout the animal, especially if naive cells repopulate from the

thymus. One possibility is that anergic cells might be able to mediate suppressor phenomena, as has been suggested by studies showing that anergic T cells may transfer tolerance into naive animals (156). In this study, in which they transferred orally tolerised $CD4^+$ T cells mixed with naive $CD4^+$ T cells to nude mice and investigated antibody production in response to immunisation, they found that the suppressive activity of the anergic $CD4^+$ T cells from tolerant mice might not be cytokine mediated but cell mediated. This was because suppressive cytokines, including IFNγ, TGFβ and IL10 from $CD4^+$ T cells were not observed. Their findings are consistent with the findings of Lombardi *et al* (182) who demonstrated that anergic T cells suppressed the proliferative response of normal T cells, and that suppression was not mediated by IL10 or TGFβ.

1.6.2.3 Active Suppression

There is substantial evidence that active suppressor mechanisms may play a role in oral tolerance (7,95). T cells with suppressive activity have been identified in the intestinal mucosa, mesenteric lymph nodes and spleen (11,125,173,183-185) and antigen fed mice, and many transfer experiments have demonstrated that systemic suppression can be transferred by orally tolerized spleen, MLN and Peyer's patch cells (118,186,187). Oral tolerance can be prevented with agents that were believed to be suppressor-cell specific toxins. These suppressor cells were believed to be induced in the Peyer's patches and migrate to the systemic immune system and were originally shown to be $CD8^+$ T cells (170,188-191). However, more recent studies have suggested that $CD8^+$ cells are not necessary for oral tolerance (57,59,192), as it can be induced in CD8-knockout mice (193) and in mice depleted of CD8 T cells using monoclonal antibodies (59,188). All of these studies have indicated that CD4 T cells rather than CD8 T cells are required for oral tolerance induction, even when the effector response is entirely CD8 dependent (192). In agreement, other recent work has shown that CD4 T cells can transfer oral tolerance *in vivo* (194). Nevertheless, some evidence persists that CD8 cells may play a role in oral tolerance, as it has been shown that there

may be a lack of local suppression in orally tolerant CD8-deficient mice, despite full tolerance in the periphery (193).

T cells expressing the γδ TCR have more recently been suggested as playing a role in mucosally induced systemic tolerance (195,196). *In vivo* treatment with antibodies specific for the γδ TCR inhibited induction of oral tolerance in OVA-fed mice and γδ TCR knock-out mice have defects in oral tolerance (197,198). Conversely, unconfirmed work has shown that γδ T cells from the gut epithelium may abrogate oral tolerance (199). Thus, more work is required to define the regulatory role of γδ T cells in oral tolerance and to examine if these cells are intraepithelial lymphocytes or other, systemic populations of γδ T cells.

As noted above, recent evidence suggests that one of the primary mechanisms of active suppression may be via the secretion of suppressive cytokines such as IL4, IL10, and transforming growth factor β (TGFβ) following Ag-specific triggering (200-205). Thus it has been proposed that the induction of oral tolerance may reflect a preferential activation of T helper 2 (T_H2) cells with down-regulation of T_H1-dependent DTH and IFNγ responses by T_H2 cytokines such as IL4, IL5 and IL10. This idea is compatible with the fact that it is easier to induce and maintain oral tolerance of CMI responses in comparison with humoral responses. Indeed, a recent report has shown that treatment with an anti-IL4 monoclonal antibody blocks suppression of collagen-induced arthritis in mice induced by oral administration of type II collagen. However, it must be noted that T_H2-type responses *in vivo* (e.g. IL4-dependent IgE production) can also be suppressed by feeding antigen. Furthermore, oral tolerance can still be induced in IL4 knockout mice (57,206) and a concomitant helminth-induced mucosal T_H2 response prevents oral tolerance of T_H2, but not T_H1, responses to OVA (207).

IL10 appeared to be an attractive candidate as a mediator of oral tolerance since it is a T_H2 cytokine (208) which suppresses T_H1 cell activity via downregulation of macrophage IL12 production (203). Also, its absence in IL10 knock-out mice allows the development of intestinal pathology due to hyperreactivity to components of the

normal gut flora (209). Initial reports suggested that the production of IL10 was enhanced in oral tolerance and some reports showed that IL10-producing T cell clones could be isolated from animals tolerised by feeding MBP (170). Indeed IL10 has recently been shown to be a growth factor for regulatory T cells, termed Tr1 cells, that prevent colitis and may thus be involved in oral tolerance (171,172). However, other groups have found marked suppression of IL10 in OVA fed mice (57), and normal tolerance occurs in mice depleted of IL10 (204), indicating that this cytokine is not a central mediator of oral tolerance.

TGFβ is the mediator receiving most current attention and is abundantly produced in the normal intestine by cells of both haematopoietic and epithelial origin. It is important in regulating epithelial homeostasis, IgA switching and may also be involved in the homing of cells to high endothelial venules (210,211). It has also been recently demonstrated that antigen-specific TGFβ production is preserved in orally tolerised TCR transgenic animals (175), although the source of this cytokine following the clonal deletion of antigen-specific T cells that occurs in these animals is unclear. The prevention of EAE by oral administration of MBP is associated with upregulation of TGFβ in the brain (212), and TGFβ-secreting T cell clones can be produced from animals tolerised in this way (213). Furthermore, protection from EAE can be transferred with $CD8^+$ T cells or clones that produce TGFβ, and bystander suppressor effects exerted by these cells *in vitro* can be prevented with anti-TGFβ. Oral tolerance can be enhanced by anti-IL12 antibodies and this is associated with inhibition of T_H1 responses and high TGFβ production (214,215). Another more recent study has shown that TGFβ induced by oral tolerance ameliorates experimental tracheal eosinophilia (216). Although these findings are consistent with an important role for TGFβ, the biological significance of the levels of TGFβ measured *in vitro* is uncertain and very few studies have addressed directly the role of this cytokine in oral tolerance *in vivo* using appropiate depleting antibodies. A recent report on the induction of oral tolerance in TGFβ1 Null mice has shown that mice fed high doses of OVA still exhibited highly significant suppression compared with controls, showing that

suppression via TGFβ is not the only mechanism of oral tolerance in high dose fed mice (217). However, a weaker, but still significant, suppression was observed in lymphocytes from the majority of TGFβ1 Null mice fed low doses of OVA suggesting that suppression via TGFβ may be a more important mechanism of oral tolerance in low dose fed mice.

It has been reported that TGFβ may be produced by both CD4 and CD8 GALT-derived regulatory T cells (170,189,190,200). Recently it has been suggested that there may be a unique subset of CD4 helper T cells that primarily produce TGFβ or IL10. These have been termed T_H3 or Tr1 cells (170-172,189,190). To generate the regulatory T_H3 cells, MLN of mice that had been fed MBP and immunised with MBP/CFA were cultured *in vitro* with MBP and the CD4 T cells from these cultures were cloned. The cytokine profile of each of the clones after activation with MBP or anti-CD3 was determined and most of the clones produced mainly TGFβ and these clones were termed T_H3 cells (170). Transfer of these T_H3 cells to mice prior to immunization with MBP suppressed EAE, as measured by disease incidence, day of onset, maximum disease score and fatality. An analogous population of *in vitro* generated antigen-specific IL10-dependent regulatory cells (Tr1) that also produce TGFβ has recently been shown to be capable of down-regulating a murine model of inflammatory bowel disease, further highlighting a possible role for this cytokine in mucosal immune regulation (171,172). To generate Tr1 cells, naive OVA-TCR Tg CD4 cells from DO11.10 transgenic mice were repeatedly stimulated *in vitro* with splenic APCs and OVA peptide in the presence of IL10. Then they transferred OVA-specific Tr1 cells into SCID mice and observed that they were able to prevent IBD induced by pathogenic $CD4^+CD45RB^{hi}$ splenic T cells only upon stimulation *in vivo* by feeding the mice with OVA.

Priming of antigen-specific IFNγ secretion in Peyer's patches and peripheral tissues has been demonstrated in mice transgenic for a myelin basic protein (MBP)-specific TCR or OVA-specific TCR (214,215), suggesting that priming of T_H1 responses may occur before oral tolerance is established. Indeed, nasal tolerance

appears to depend on IFNγ (218,219). This is because adoptive transfer of small numbers of IFNγ-producing γδ T cells from nasally tolerised mice to recipients that were subsequently immunised with OVA selectively suppressed TH2-dependent IgE antibody production without affecting parallel IgG responses (218). Further, nasally induced tolerance to OVA in rats involves active suppression of IgE responses by IFNγ-producing $CD8^+$ T cells (219). However, studies using mice depleted of IFNγ have produced conflicting results, as although it has been reported that IFNγ KO mice have a defect in oral tolerance (220), others find normal oral tolerance in IFNγR KO mice (221). In addition, most workers find that the production of IFNγ is highly susceptible to the suppressive effects of feeding antigen (57,90).

1.6.2.4 Influence of Ag Dose on Mechanisms of Oral Tolerance

It is important to note once again that it is likely that many of the mechanisms may not be mutually exclusive. However, whether active regulation or direct T cell inactivation predominates as a mechanism of oral tolerance may depend on the circumstances of antigen exposure and, in particular, may be influenced by the dose and frequency of antigen administration. Clonal anergy or deletion of antigen-specific T cells seems to occur in oral tolerance induced by high doses (ie greater than 25mg) of antigen in the context of high affinity T cells (175,214). In contrast, feeding lower doses of antigen on multiple occasions is associated with active regulatory mechanisms and bystander suppression. However, it is difficult to extrapolate doses from antigen to antigen and animal to animal thus making it hard to make firm conclusions or predictions as to the mechanism of oral tolerance induced by a certain dose of antigen.

Two previously mentioned recent studies which highlight the influence that the dose of antigen has on the mechanisms of oral tolerance and the difficulty in making firm conclusions as to the mechanism of oral tolerance induced by a certain dose of antigen. For example, the first of these studies used cyt c tetramer staining reagents and concluded that multiple low doses of cyt c fed to mice down-regulates the systemic immune response which can be correlated with a reduction of antigen-specific T cells

via anergy and/or deletion and not with immune deviation (181). The second study, in TGFβ KO mice, revealed that active suppression, as mediated by TGFβ, is not the mechanism of tolerance induced by feeding a high dose of antigen. The same study also concluded that while feeding a low dose of antigen may result in some active suppression via TGFβ, it is not the exclusive mechanism (217).

1.7 Aims of this Study

The clinical application of oral tolerance will be to treat established disease, but most existing studies of oral tolerance have concentrated on the ability of feeding antigen to prevent subsequent systemic immune responses to specific antigen in previously naive animals. Therefore the aims of my studies were to define feeding regimes for inducing optimal oral tolerance in primed mice, to establish the effects of feeding antigen to primed mice on a variety of parameters of systemic immunity and to investigate the mechanisms of oral tolerance in primed mice. I decided to use a model antigen in my studies as many previous studies have used disease models which are complex. In addition, this allowed me to exploit experimental systems already developed in the laboratory for the induction and assessment of oral tolerance to OVA in naive mice.

In Chapter 3 I describe the experiments designed to establish optimal protocols for inducing tolerance in primed mice fed antigen by using antigen-specific *in vivo* and *in vitro* assays to investigate the scope of responses influenced by the tolerance. The longevity of these effects is examined in Chapter 4 since oral tolerance is currently under evaluation as a potential immunotherapy. In an attempt to understand more fully the mechanisms of oral tolerance that are induced in mice fed after priming I employed several new strategies including the use of knockout mice, TCR-specific transgenic mice and a DC growth factor. In Chapter 5 I investigated the role of the cytokines, IL4 and IL12, by examining tolerance in knockout mice with targetted lesions in the appropriate genes in order to investigate the roles of TH2 cells and TGFβ respectively in oral tolerance in primed mice. In Chapter 6, a novel dendritic cell inducer, the growth

factor Flt3L, was used to examine if it was possible to increase the scope of oral tolerance by expanding the numbers of dendritic cells *in vivo*. The work described in Chapter 7 then employed mice transgenic for the T cell receptor specific for the MHC Class II restricted immunodminant peptide of OVA to analyse directly the effects of feeding antigen on primed T cells at the clonal level. Finally, in Chapter 8, I explored why oral tolerance was harder to induce in primed mice compared with naive mice, by investigating the influence of persistence of antigen and the susceptibility of memory T cells to oral tolerance.

Chapter 2 Materials and Methods

2.1 Mice

BALB/c (H-2^d) mice were obtained from Harlan Olac (Bicester, Oxon, U.K.) or were bred in house (Central Research Facility, CRF, University of Glasgow) and maintained in the CRF. IL4$^{-/-}$ (129Sv x C57Bl/6)F_2 (H-2^b) mice (222) were obtained originally from Dr. H. Bluethmann, F. Hoffman-La Roche AG, Basel, Switzerland and maintained in CRF. p40 IL12 deficient (IL12$^{-/-}$) BALB/c mice (223) were obtained from Dr. J. Magram, Roche Pharmaceuticals, Piscataway, NJ and maintained in CRF. Mice transgenic for the OVA323-339 and I-A^d-specific DO11.10 TCR on a BALB/c genetic background (224) were obtained from Dr. Nils Lycke, Goteborg, Sweden and maintained in the CRF.

Mice were specified pathogen free and were maintained under standard animal house conditions with free access to both water and standard rodent pellets, containing no ovalbumin, until use at 6-8 weeks of age. In most experiments, female mice were used.

2.2 Antigens and Mitogens

Ovalbumin (OVA, Fraction V), lipopolysaccharide (LPS, from *Salmonella enteritidis*), cholera toxin (CT) and concanavalin A (Con A) were obtained from Sigma, while purified protein derivative (PPD) from *M. tuberculosis* was obtained from Central Veterinary Laboratory, New Haw, Addlestone, Surrey, U.K. and prepared according to the manufacturer's instructions.

Heat-aggregated OVA (HAO) was prepared by heating a 2% (w/v) solution of OVA in saline (Baxter Healthcare Ltd. Thetford, Norfolk) 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 in a glass bijoux for 20 minutes to produce a colloidal suspension (50).

2.3 Oral Administration of OVA

Mice were fasted for 18 hours prior to being fed OVA 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.

2.4 Systemic Immunisation Procedures

Systemic immune 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).

In most 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, U.K.). In other experiments, s.c. immunisations were performed by injection of a total volume of 0.2ml distributed between three sites on the back under light anaesthetic.

2.5 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 20 minutes at 450g and stored at -20°C until use.

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

OVA-specific DTH responses were assessed as decribed in detail elsewhere (51). 20 days after immunisation with OVA/CFA, mice were anaesthetised with halothane and the thickness of the unimmunised rear footpad measured using skinfold calipers (0-10mm in 0.1mm; Kroeplinn Langenmessstecknik, 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.

2.7 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, diluted 1:400 in PBS/0.05% Tween 20/1%FCS (Gibco Life Technologies, Paisley, U.K.), were added to the plates. 100µl aliquots of serum samples diluted 1:400 in PBS/Tween 20, were added in triplicate 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, 120µl/well of phosphatase substrate (1mg/ml in 10% diethanolamine, DEA, dissolved in distilled water; both from Sigma) was added and 5-10 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.

2.8 Measurement of Antigen-specific Serum IgG Isotype Antibodies

Enhanced protein binding, 96-well ELISA plates (Immulon-4) were coated overnight at 4°C with 50µl 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 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), diluted 1:16000, or biotinylated rat anti-murine IgG2a (both AMS Biotechnology, Witney, Oxon, U.K.) diluted 1:500 in PBS/Tween 20 was added at 75µl/well and incubated at room temperature for 1 hour. The plates were then washed three times and 75µl/well extravidin-peroxidase (Sigma) diluted 1:1000 in PBS/Tween 20 was added. 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.

2.9 Preparation of Lymphoid Cells

Single-cell suspensions of popliteal lymph nodes (PLN) were prepared in RPMI-1640 by rubbing gently over Nitex mesh (gauge 100µm, Cadisch & Sons, London, U.K.) using a syringe plunger and passed through Nitex mesh to remove any clumps. After washing the cells twice in RPMI 1640 by centrifugation at 4°C for 8 minutes at 450g and resuspending in 10ml RPMI 1640, viable cells were counted by

phase contrast microscopy (Nikon Labophot microscope, x40 objective) using a haemocytometer (Neubauer). Cells were finally resuspended in complete medium (RPMI 1640 containing 10% FCS, 100U/ml penicillin, 100µg/ml streptomycin, 50µg/ml fungizone, 2mM L-glutamine (all Gibco BRL), 25mM Hepes (Sigma) and 50µM 2-mercaptoethanol (Sigma)).

2.10 Measurement of T Cell Proliferation *in vitro*

200µl aliquots of lymphoid cells resuspended at 10^6/ml in complete medium 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 to be optimal (1mg/ml, 50µg/ml and 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 (Heraeus Instruments, U.K.) 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).

2.11 Measurement of Cytokine Production *in vitro*

Lymphoid cells resuspended at 10^7/ml in complete medium were added in 400µl 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 two 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 then washed six times and 100µl/well extravidin-peroxidase (Sigma) diluted 1:1000 in PBS/10% FCS was added. After further incubation for 1 hour at 37°C, the plates were washed eight 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).

2.12 Phenotypic Analysis of Lymphocytes by Flow Cytometry

10^6 lymphoid cells freshly prepared in a single cell suspension were resuspended in plastic conical tubes (Falcon, Cowley Oxford, U.K.) in 50µl staining buffer (SB, Appendix 1) and stained with the antibodies shown in Table 3. Biotinylated antibodies were detected using FITC-SAV or PE-SAV and all staining reactions were performed for 20 minutes on ice. Between steps, cells were washed once in 2ml FACS Buffer (Appendix 1) by centrifugation for 5 minutes at 1200rpm. After staining, cells were washed twice in FACS Buffer 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.

2.13 Adoptive Transfer of OVA specific Transgenic T Cells

BALB/c mice were adoptively transferred with DO11.10 TCR transgenic T cells as described by Kearney *et al* (143) A single cell suspension of TCR transgenic lymph node and spleen cells from DO11.10 mice was made as described above, the percentage of KJ1-26$^+$ cells present calculated by flow cytometry and mice were injected with 3 x 10^6 KJ1-26$^+$CD4$^+$ cells intravenously in a volume of 0.3ml saline. Recipient mice in any given experiment were age- and sex- matched.

2.14 Statistical Analysis

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

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

Table 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	R4-6A2	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}	1
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: Recombinant Murine Cytokine Standards used in Sandwich ELISAs

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

Table 3: Primary Antibodies used In Flow Cytometric Analysis

Specificity	Clone	Isotype
PE-anti-murine CD4 (L3T4)	GK1.5	Rat IgG2a
biotinylated-anti-murine KJ1-26	KJ1-26	Mouse IgG2a

Anti-CD4 was obtained from Pharmingen and anti-KJ1-26 was obtained from Marc Jenkins, Minneapolis, U.S.A. and both were used at 1:25 dilutions in staining buffer.

Appendix 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

0.1M Carbonate Buffer

3.2g Na_2CO_3

5.9g $NaHCO_3$

Add to 1l ddH_20 and pH to 9.3

0.05M Carbonate Buffer

1.6g Na_2CO_3

2.95g $NaHCO_3$

Add to 1l ddH_20 and pH to 9.3

Staining Buffer

PBS containing:

10%normal goat serum (Sigma),

10μg/ml purified anti-mouse CD16/32 (FcγIII/II Receptor) monoclonal antibody (clone 2.4G2, rat IgG2b,κ) (Pharmingen),

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

FACS buffer

PBS containing:

10% foetal bovine serum (GibcoBRL),

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

Chapter3 Oral Tolerance in Primed Mice

3.1 Introduction

Despite the success of oral tolerance in several models of autoimmune disease, most experimental studies on the induction of oral tolerance have used naive animals and little is known about the immunoregulatory mechanisms induced when antigen is fed to primed animals. The limited work that has been done found that feeding OVA to systemically primed mice suppressed subsequent DTH responses better than humoral immunity and indicated that tolerance was induced better by high doses of antigen given soon after immunisation (124,125). Also, recent work on oral tolerance in established experimental acute encephalomyelitits (EAE) concluded that the frequency of feeding, rather than the dose of antigen was the most important factor in determining the efficacy of the tolerising regime (131). However, the mechanisms have not been explored in any detail and only a limited scope of responses has been examined. The aim of my thesis was to apply recent insights into the mechanisms of oral tolerance in naive mice to investigate the equivalent phenomena in primed animals.

The experiments described in this chapter were designed to establish a model for inducing oral tolerance to OVA in mice with an established systemic immune response to OVA. Thus I examined a variety of antigen doses administered at different times after priming with OVA/CFA and investigated the scope of responses influenced by the tolerance.

3.2 Experimental Protocol

BALB/c mice were immunised s.c. with OVA/CFA on d0 and oral tolerance was induced by feeding mice OVA at various times thereafter. As controls, separate groups were fed 25mg OVA 10 days before immunisation, in a standard oral tolerance protocol. Control mice were fed saline. Systemic immune responsiveness was assessed by measuring antigen specific proliferation and production of IFNγ and IL4 and 5 in the draining popliteal lymph nodes 14 days after the original immunisation and

by assessing serum antibody levels and OVA-specific DTH responses 20 days after immunisation.

3.3 Results

3.3.1 Effects of Feeding OVA Before or After Priming on Subsequent Effector Functions

3.3.1.1 *In Vivo* Responses

As expected from previous studies, the OVA-specific DTH responses of mice fed 25mg OVA 10 days prior to priming were significantly lower than those of immunised controls. Mice fed the same dose of OVA 7 days after priming also had DTH responses lower than those of controls, but this was not statistically significant (Fig 3.1).

The OVA-specific total IgG serum antibody levels of mice fed 25mg OVA before or after priming were not significantly lower than those of immunised controls (Fig 3.2a). OVA-specific IgG serotype levels, IgG1 and IgG2a, were also measured but no significant tolerance was found in either of the OVA fed groups (Fig 3.2b and c respectively).

3.3.1.2 *In Vitro* Responses

Cell-mediated immunity *in vitro* was measured by OVA-specific proliferation and cytokine production of PLN cells.

The OVA specific proliferative responses of PLN cells taken from mice fed OVA before immunisation were significantly lower than those of control mice (Fig 3.3). These mice also had suppressed production of IFNγ, IL4 and IL5 (Fig 3.4a,b and c). These results confirm previous studies in the lab and demonstrate that this regime of oral tolerance affects both T_H1 and T_H2 responses in naive animals. IFNγ production

was also significantly tolerised in mice fed OVA after immunisation, but IL4 or IL5 production was not (Fig 3.4a, b and c).

The results of this preliminary experiment indicated that it might be possible to induce oral tolerance in previously primed mice but that this was not as profound as that found when antigen is fed to naive mice and affects a narrower range of systemic responses. I therefore went on to examine if I could improve this tolerance by modifying the dose of fed antigen or the time of feeding.

3.3.2 Effect of Feeding Time on Oral Tolerance in Primed Mice

In the first experiments, I examined whether primed mice could be tolerised more readily if the interval between immunisation and feeding was altered. Therefore, I fed 25mg OVA 2, 4 or 8 days after immunisation on d0 with OVA/CFA.

3.3.2.1 *In Vivo* Responses

The OVA-specific DTH responses of mice fed 25mg OVA 2 days after priming were significantly lower than those of immunised controls. Significant OVA-specific DTH tolerance was not found in mice fed 25mg OVA 4 or 8 days after priming, although these responses were both somewhat lower than those of controls (Fig 3.5). There appeared to be a high variability in these groups, with some mice being profoundly tolerant and others showing normal responses (data not shown).

The OVA-specific total IgG antibody levels of mice fed 25mg OVA 2, 4 or 8 days after priming were not significantly lower than those of immunised controls (Fig 3.6a). OVA-specific IgG1 and IgG2a antibody responses were also not reduced in mice fed OVA at any time after immunisation, except the IgG1 response of mice fed on d2 (Fig 3.6b and Fig 3.6c respectively).

3.3.2.2 *In Vitro* Responses

OVA specific proliferative responses of PLN cells taken from mice fed OVA 2 or 4 days after immunisation were significantly lower than those of control mice, but,

mice fed 8 days after priming no longer had significant tolerance of their proliferative responses (Fig 3.7). OVA specific IFNγ and IL5 production by PLN cells taken from mice fed OVA 2 or 4 days after immunisation was also significantly lower than that of control mice but mice fed 8 days after priming only had significantly reduced IL5 production (Fig 3.8). OVA specific IL4 production was measured but no tolerance was ever found and therefore the data is not shown subsequently.

Thus, oral tolerance of both *in vivo* and *in vitro* responses is induced more readily when antigen is fed soon after priming.

3.3.3 The Role of Antigen Dose and Feeding Frequency in Oral Tolerance in Primed Mice.

Previous studies in which antigen was fed after priming suggested that oral tolerance was more profound when higher doses of antigen or multiple feeds of antigen were used. I therefore examined a number of protocols of tolerance induction in which I fed a variety of single doses of OVA (2, 25, 200, 400mg) 7 days after priming, or in which I administered five feeds of 5mg or 25mg starting 7 days after priming.

3.3.3.1.1 *In Vivo* Responses

In the first study, the effects of feeding 2-200mg OVA 7 days after priming were compared with 25mg fed 10 days before as a control. In a separate experiment, I examined a single feed of 400mg given on d+7.

All groups of OVA fed mice had significantly suppressed DTH responses compared with controls with the exception of those fed 2mg 7 days after priming. This tolerance was greatest in mice fed before immunisation, but there was a dose dependent increase in tolerance in mice fed increasing doses of OVA after priming, with 400mg having almost as much effect as 25mg before priming (Fig 3.9a and b).

A similar pattern was seen when antibody responses were examined. Total IgG antibody levels as well as OVA-specific IgG1 and IgG2a antibodies were significantly suppressed in mice fed 25mg OVA 10 days before priming (Fig3.10a, Fig3.11a and

Fig 3.12a respectively) and mice fed 400mg after priming had significantly suppressed total IgG antibody levels (Fig3.10b, Fig3.11b and Fig 3.12b). Consistent with the previous results in this chapter, no OVA-specific antibody tolerance was found with any other feeding regime, although there was some evidence that total IgG responses were decreasing at higher doses of OVA (Fig3.10, Fig3.11 and Fig 3.12).

3.3.3.1.2 *In Vitro* Responses

All doses of fed OVA given before or after priming significantly suppressed subsequent OVA specific proliferative responses and there was little evidence of a clear dose response in the mice fed 2-400mg OVA after priming (Fig 3.13a,b). As expected, OVA specific IFNγ and IL5 production were significantly reduced in mice fed OVA 10 days before immunisation (Fig 3.14a and Fig 3.15a). OVA specific IFNγ production was also significantly reduced in all groups of mice fed OVA after immunisation, with the exception of those fed 25mg 7 days after priming. OVA specific IL5 production was also significantly lower than that of control mice in all these groups except for mice fed 400mg (Fig 3.15b).

Thus, increasing the dose of fed antigen given after immunisation enhances the degree and scope of oral tolerance, although there is not always a clear dose response and certain aspects of the immune response, such as humoral immunity, remain relatively resistant. I therefore proceeded to investigate whether more frequent feeding of antigen would increase tolerance.

3.3.3.2.1 *In Vivo* Responses

In the first experiment I administered five feeds of 5mg starting 7 days after priming and in the second experiment I administered five feeds of 25mg starting 7 days after priming.

In both experiments, OVA fed mice had significantly suppressed DTH responses compared with controls, although this appeared to be more marked in mice fed 5 x 25mg OVA. Consistent with the previous results in this chapter, no OVA-

specific antibody tolerance was found with either of the feeding regimes (Fig3.17, Fig3.18 and Fig 3.19).

3.3.3.2.2 *In Vitro* Responses

Both doses of OVA given after priming significantly suppressed subsequent OVA specific proliferative responses and again this was more marked when mice were fed 5 x 25mg rather than 5mg (Fig 3.20a,b). OVA specific IFNγ and IL5 production was also significantly reduced in both groups of mice fed OVA after immunisation (Fig 3.21 and 3.22). Mice fed 5mg appeared to have more suppressed IFNγ responses when compared with mice fed 25mg, however, mice fed 25mg appeared to have more suppressed IL5 responses when compared with mice fed 5mg.

Thus, multiple feeding of antigen given after immunisation does not enhance the degree and scope of oral tolerance induced when compared to feeding a single dose of antigen, although multiple feeding of high doses of antigen did however, seem to cause slightly better suppression, on the whole, than that of low doses.

3.3.4 Time Course of OVA-Specific IgG Production in Mice Primed with OVA/CFA.

To investigate whether the reduction of tolerance in mice fed 7 days after priming compared with feeding earlier after priming coincided with the appearance of OVA-specific antibody in the serum I immunised mice with OVA/CFA and assessed the serum levels of Total OVA-specific IgG (Figure 3.23). Here it is shown that antibody does appear in the serum 7 days after priming.

3.4 Conclusions

Here I have confirmed that it is possible to induce tolerance by feeding antigen to primed mice, with DTH responses, proliferation and IFNγ production being readily tolerised. However, the degree and scope of tolerance were less than that found in equivalent dose fed before immunisation. For example, TH2 cytokine production and

antibody responses were not as tolerisable when antigen is fed after priming compared with antigen fed before priming. In contrast to previous reports, I also found that only a brief time window was available after priming when mice were susceptible to induction of tolerance, which in my hands, was in order of a week. In an attempt to enhance tolerance, I fed higher single doses of antigen and increased frequency of feeding. Generally this did result in more intense and wider tolerance, although this was still less than in mice fed before priming. Together, these results illustrate the difficulty in tolerising a primed immune system and in subsequent chapters, I went on to examine the reasons why this should be the case and what the mechanisms responsible for the tolerance might be.

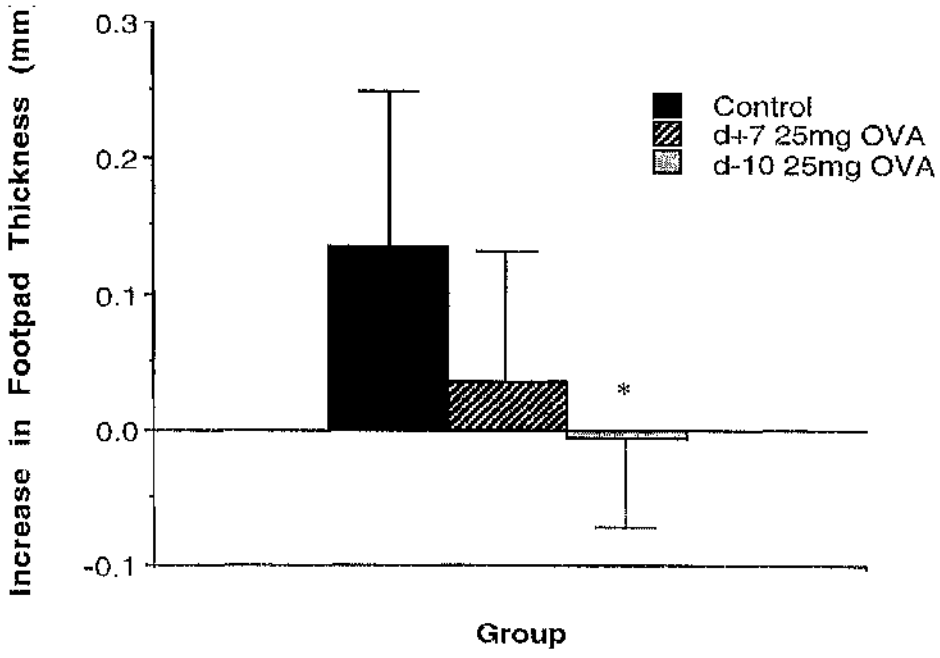
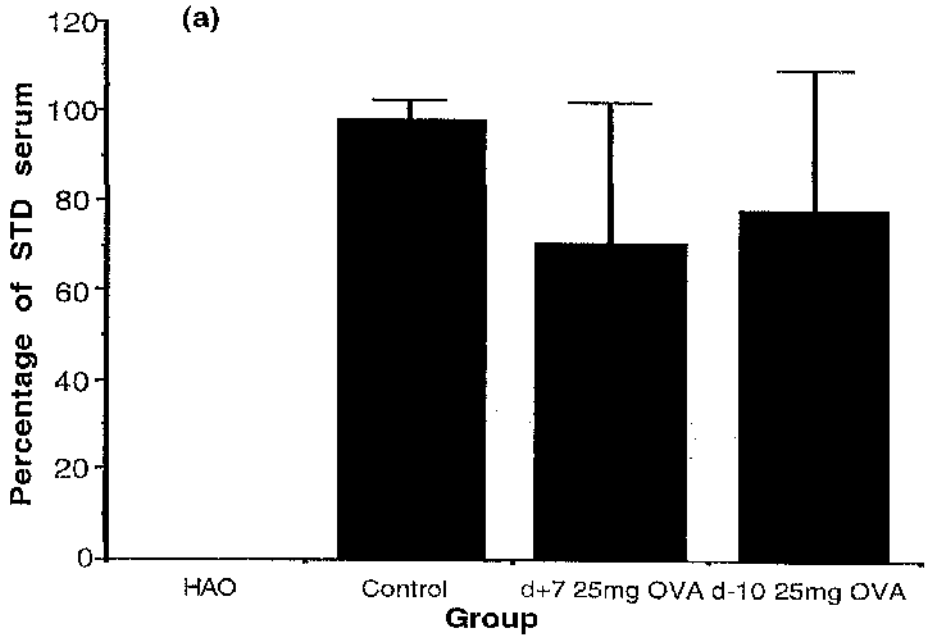
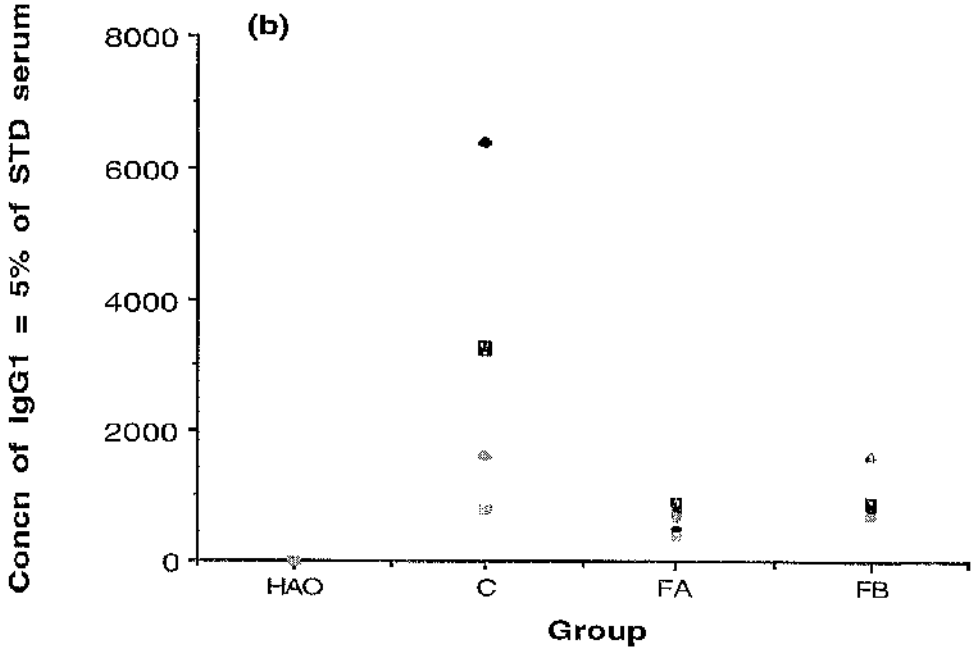


Figure 3.1 Induction of Oral Tolerance by Feeding Antigen Before or After Immunisation.

Systemic DTH responses in mice given a single feed of 25mg OVA 10 days before immunisation or 7 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. The results shown are mean specific increments in footpad thickness ± 1 SD for 5 mice per group. (*$p<0.05$ versus controls)


(a)
Percentage of STD serum
120
100
80
60
40
20
0
HAO
Control
d+7 25mg OVA
d-10 25mg OVA
Group


(b)
Concn of IgG1 = 5% of STD serum
8000
6000
4000
2000
0
HAO
C
FA
FB
Group

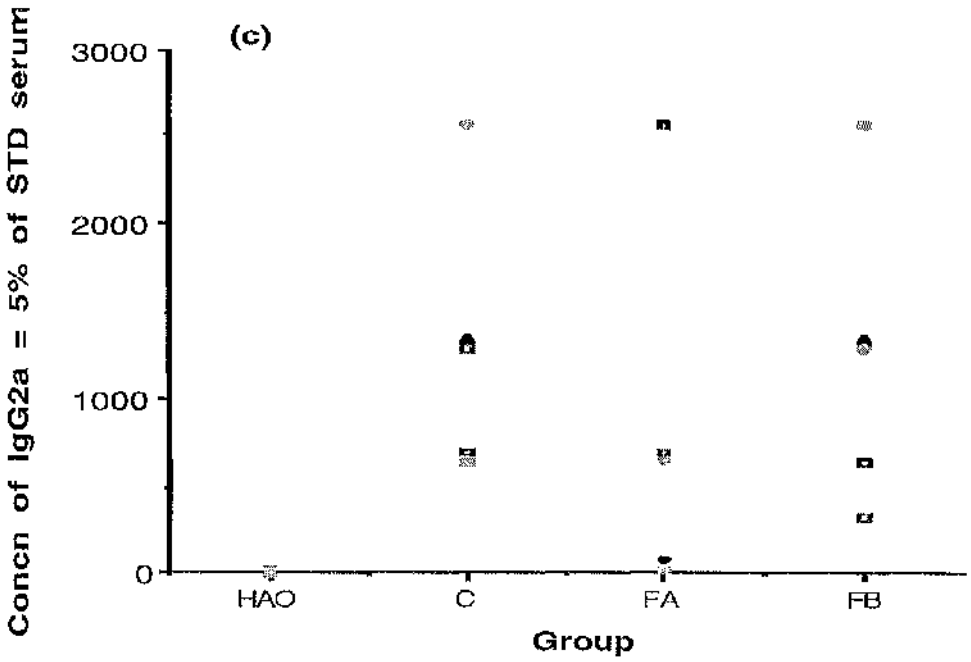


Figure 3.2 Induction of Oral Tolerance by Feeding Antigen Before or After Immunisation.

(a) OVA-specific total serum IgG antibody responses in mice given a single feed of 25mg OVA 10 days before immunisation or 7 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. The results shown are mean % of purified IgG standard ± 1 SD for individual sera from 5 mice per group. **(b)** OVA-specific serum IgG1 and **(c)** IgG2a antibody responses in control and OVA fed mice. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group.

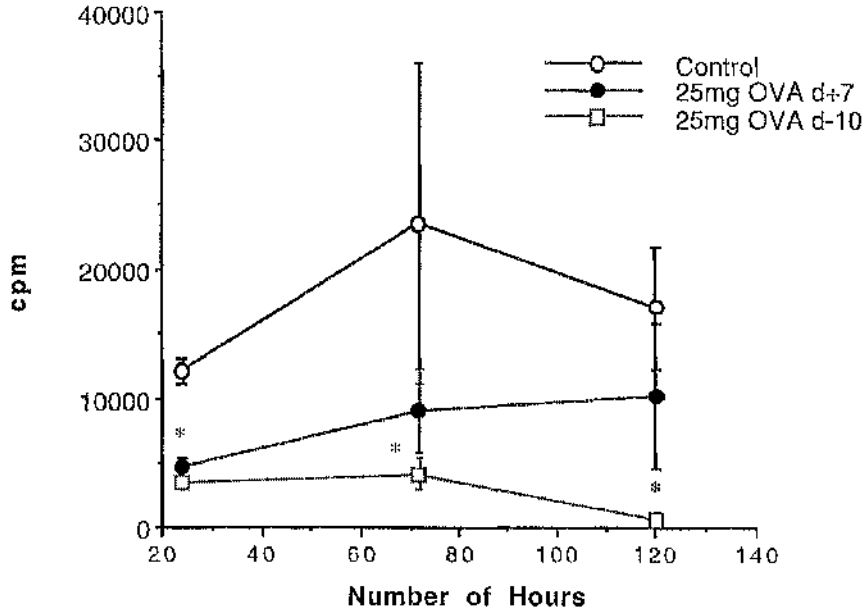
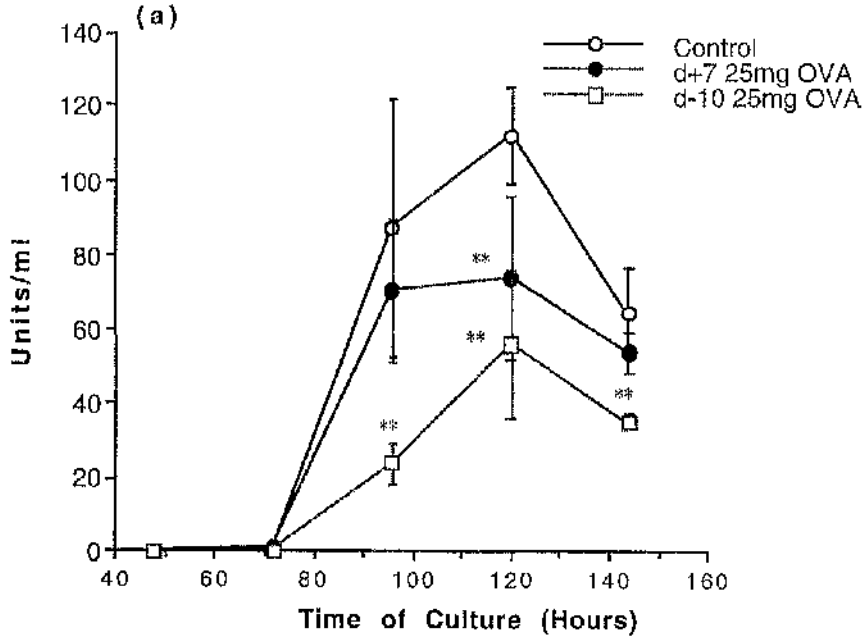
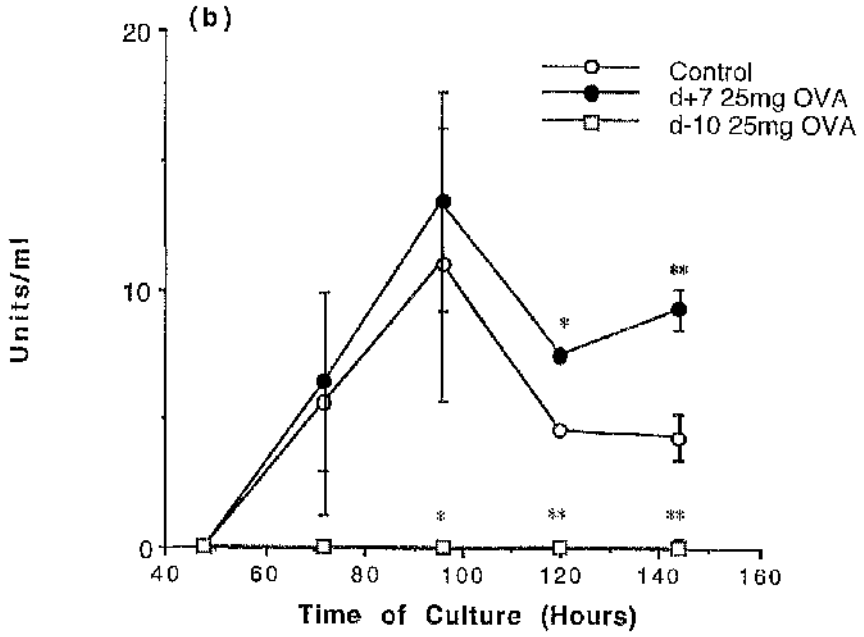


Figure 3.3 Induction of Oral Tolerance by Feeding Antigen Before or After Immunisation.

OVA-specific proliferative responses in draining lymph nodes of mice given a single feed of 25mg OVA 10 days before immunisation or 7 days after subcutaneous immunisation with OVA/CFA, and saline fed controls. The results shown are mean uptake of ^{3}H-TdR ± 1 SD for quadruplicate cultures of lymph node cells pooled from 3 mice per group. (*$p<0.005$ versus controls)


(a)
Control
d+7 25mg OVA
d-10 25mg OVA
Units/ml
140
120
100
80
60
40
20
0
40
60
80
100
120
140
160
Time of Culture (Hours)
**
**
**


(b)
Control
d+7 25mg OVA
d-10 25mg OVA
Units/ml
20
10
0
40
60
80
100
120
140
160
Time of Culture (Hours)
*
**
*
**
**

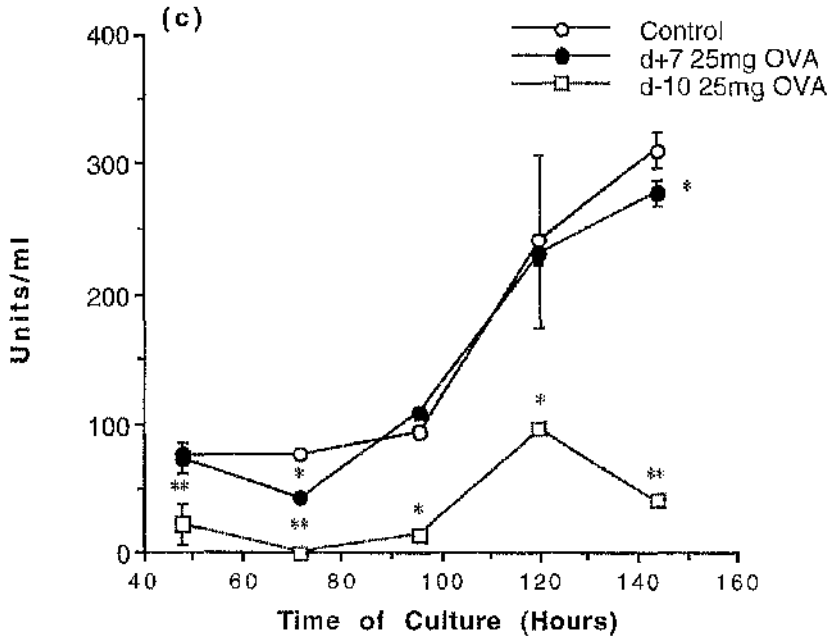


Figure 3.4 Induction of Oral Tolerance by Feeding Antigen Before or After Immunisation.

Antigen specific IFN-γ **(a)**, IL4 **(b)** and IL5 **(c)** production by draining popliteal lymph node cells of mice given a single feed of 25mg OVA 10 days before immunisation or 7 days after subcutaneous immunisation with OVA/CFA, and saline fed controls. The results shown are mean cytokine levels (U/ml) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 2-4 days. There was little or no production of cytokines from cells cultured in the absence of antigen. (*$p<0.01$ versus controls, **$p<0.005$ versus controls)

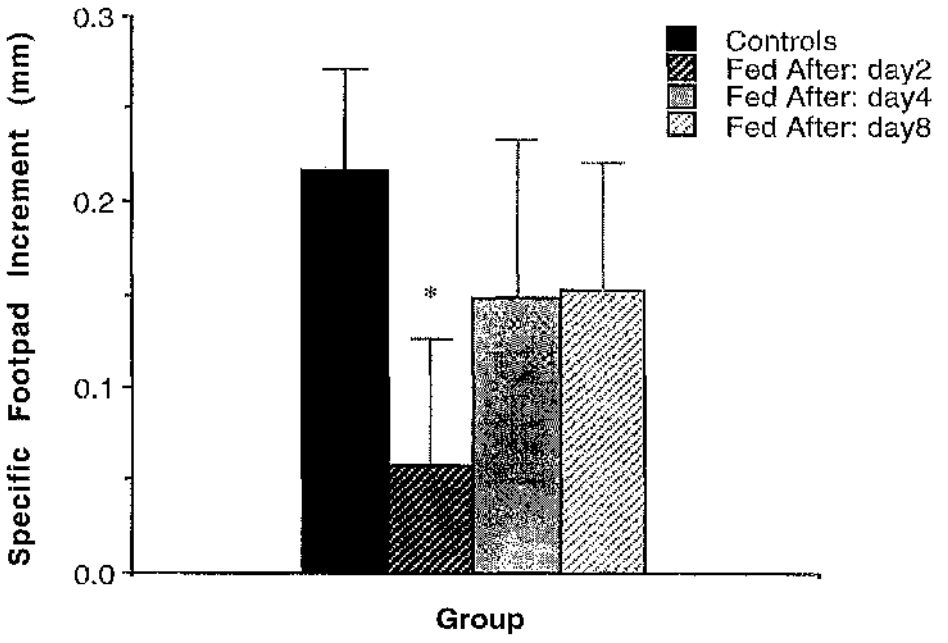
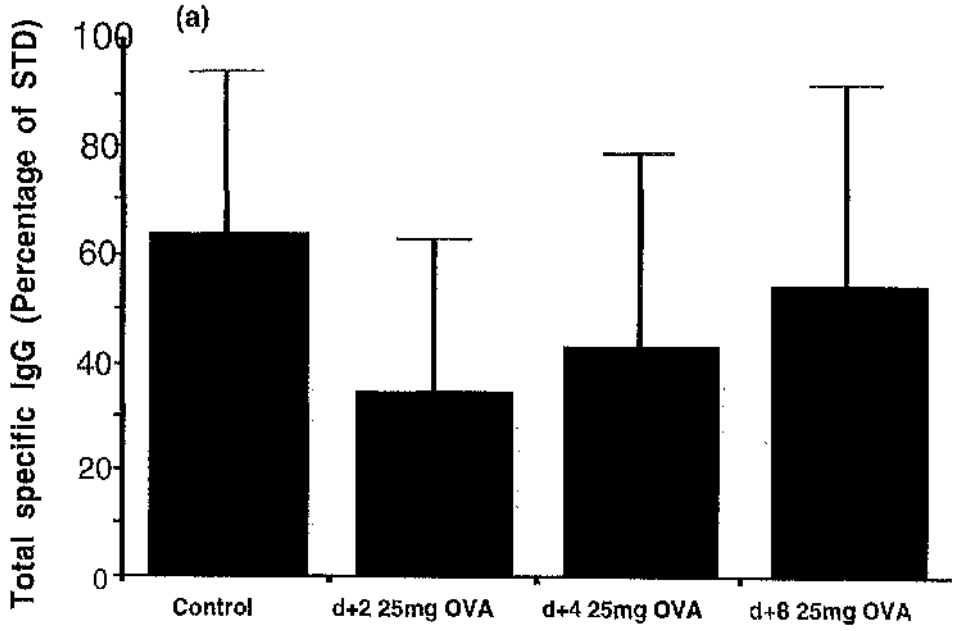
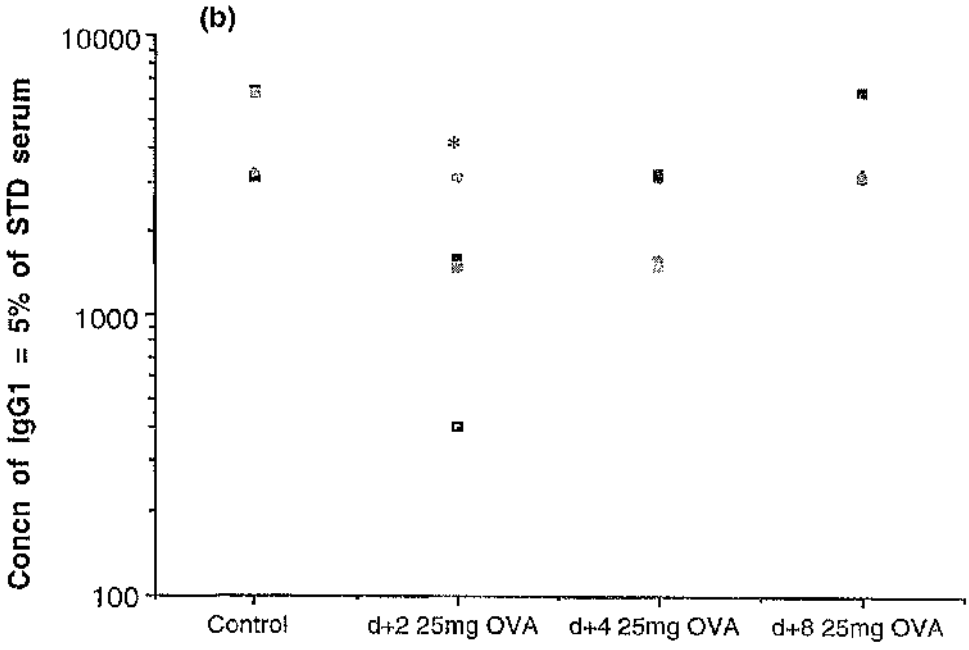


Figure 3.5 Effects of Feeding Antigen at Different Times After Immunisation on Systemic Immune Responses.

Systemic DTH responses in mice given a single feed of 25mg OVA 2, 4 or 8 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. The results shown are mean specific increments in footpad thickness ± 1 SD for 5 mice per group. (*$p<0.005$ versus controls)


(a)
Total specific IgG (Percentage of STD)
100
80
60
40
20
0
Control
d+2 25mg OVA
d+4 25mg OVA
d+8 25mg OVA


(b)
Concn of IgG1 = 5% of STD serum
10000
1000
100
Control
d+2 25mg OVA
d+4 25mg OVA
d+8 25mg OVA

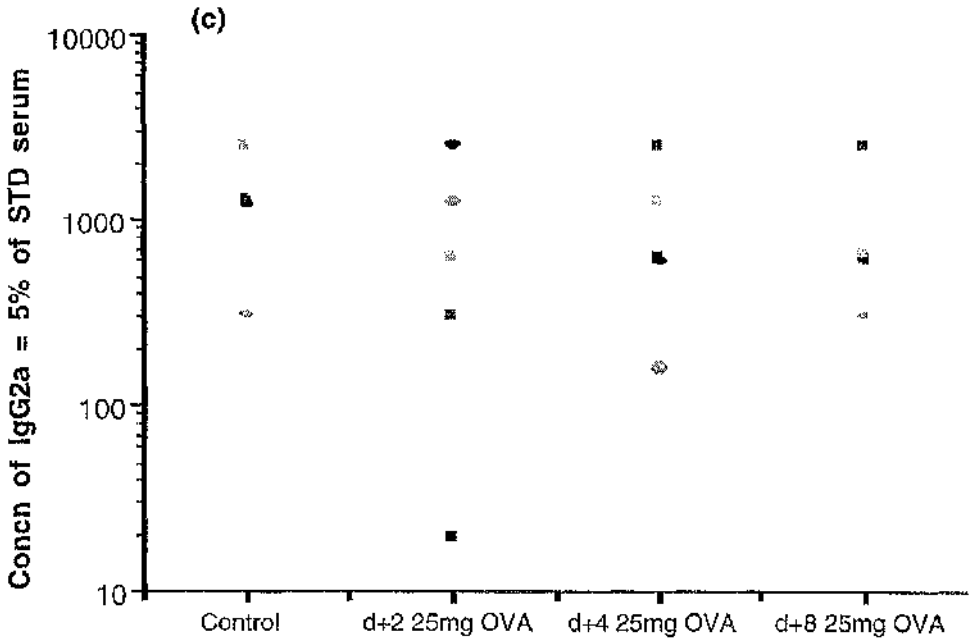


Figure 3.6 Effects of Feeding Antigen at Different Times After Immunisation on Systemic Immune Responses.

(a) OVA-specific total serum IgG antibody responses in mice given a single feed of 25mg OVA 2, 4 or 8 days after subcutaneous immunisation with OVA/CFA, and saline fed controls measured 21 days after immunisation. The results shown are mean % of purified IgG standard ± 1 SD for individual sera from 5 mice per group. **(b)** OVA-specific serum IgG1 and **(c)** IgG2a antibody responses in control and OVA fed mice. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group (*$p<0.05$ versus controls)

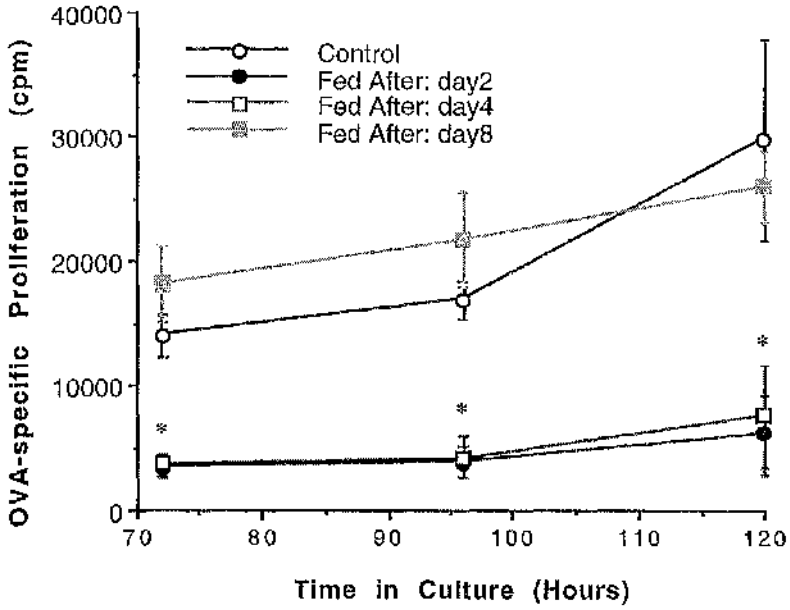


Figure 3.7 Effects of Feeding Antigen at Different Times After Immunisation on Systemic Immune Responses.

OVA-specific proliferative responses in draining lymph nodes of mice given a single feed of 25mg OVA 2, 4 or 8 days after subcutaneous immunisation with OVA/CFA, and saline fed controls. The results shown are mean uptake of ^{3}H-TdR ± 1 SD for quadruplicate cultures of lymph node cells pooled from 3 mice per group. (*$p<0.005$ versus controls)

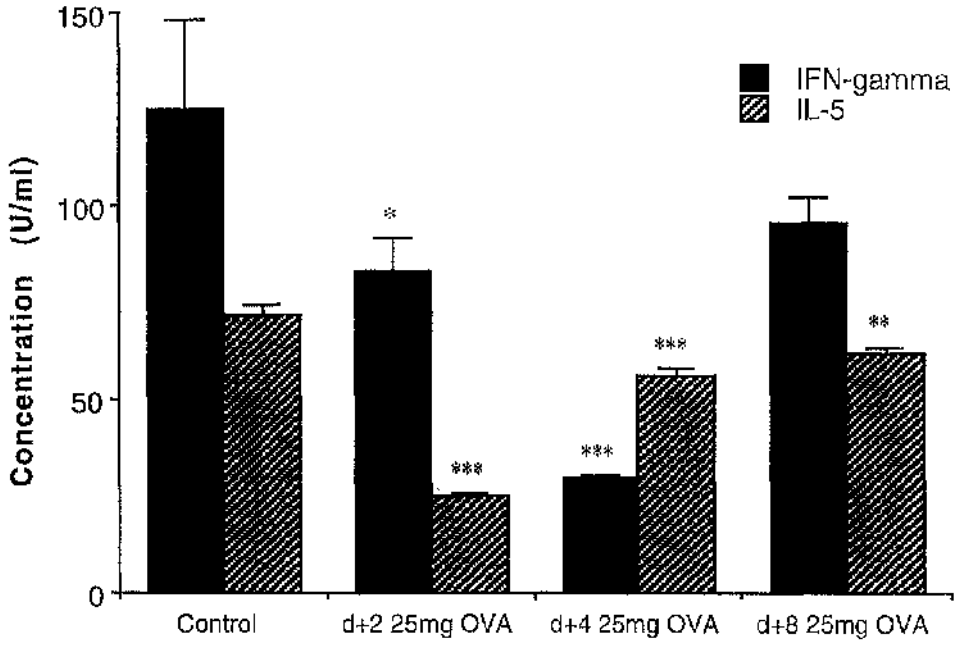


Figure 3.8 Effects of Feeding Antigen at Different Times After Immunisation on Systemic Immune Responses.

Antigen specific IFN-γ and IL5 production by draining popliteal lymph node cells of mice given a single feed of 25mg OVA 2, 4 or 8 days after subcutaneous immunisation with OVA/CFA, and saline fed controls. The results shown are mean cytokine levels (U/ml) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 2-4 days. There was little or no production of cytokines from cells cultured in the absence of antigen. (*$p<0.05$ versus controls, **$p<0.01$ versus controls, ***$p<0.005$ versus controls)

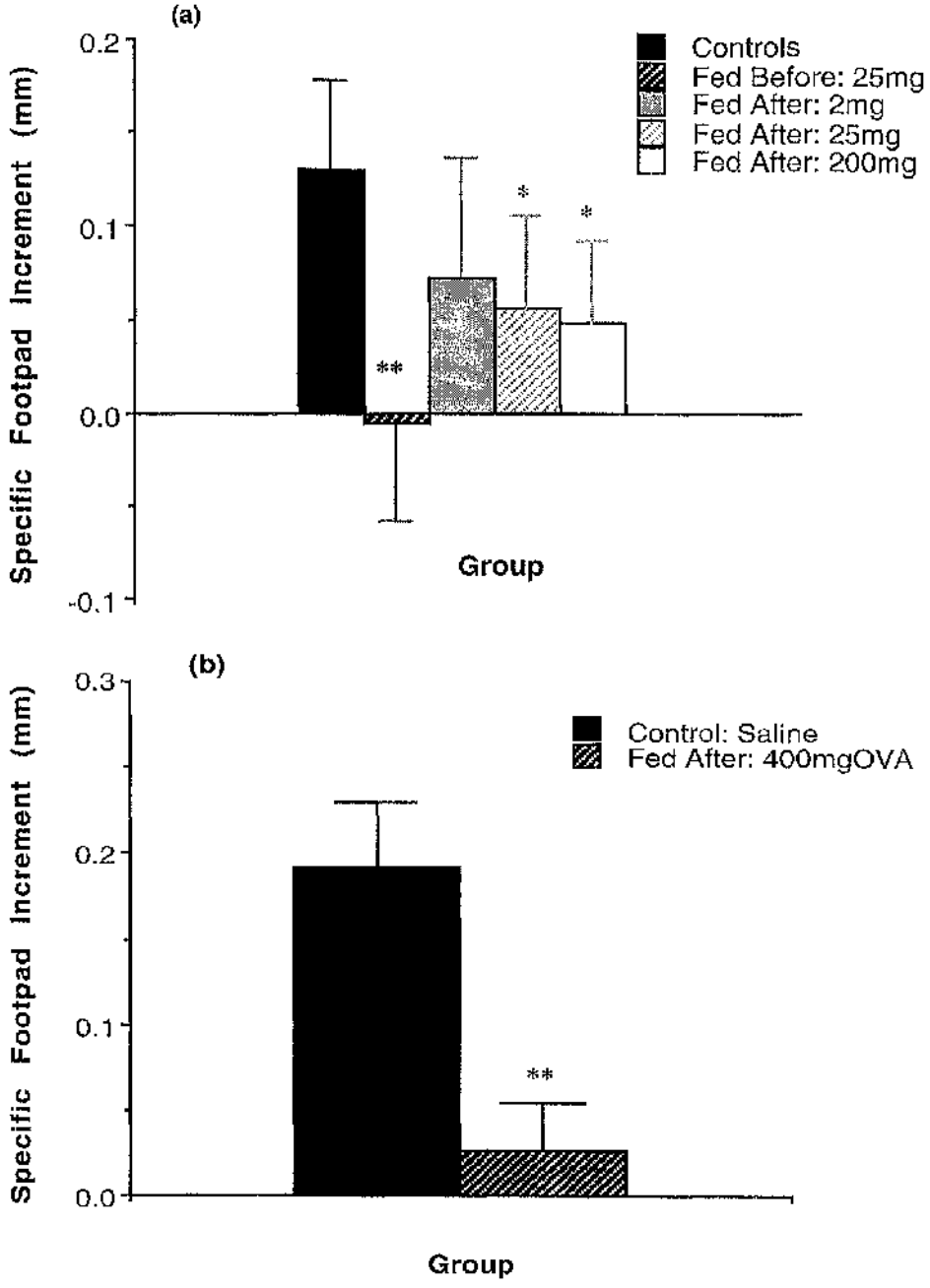


Figure 3.9 Effects of Feeding Different Doses of Antigen After Immunisation on Systemic DTH Responses.

Systemic DTH responses in mice given a single feed of 2, 25 or 200mg OVA (**a**) or 400mg OVA (**b**) 7 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. The results shown are mean specific increments in footpad thickness ± 1 SD for 5 mice per group. (*$p<0.05$ versus controls, **$p<0.005$ versus controls)

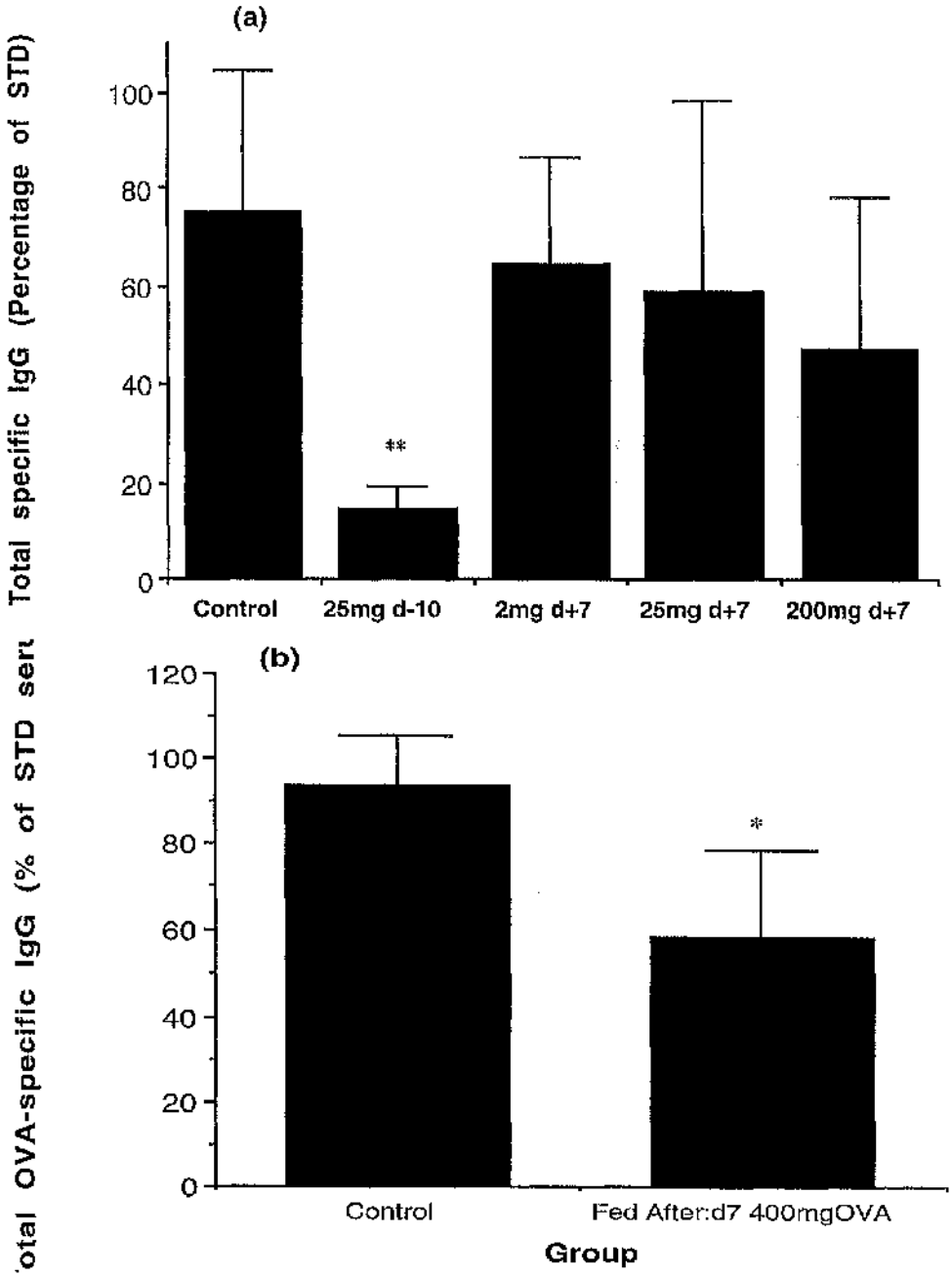


Figure 3.10 Effects of Feeding Different Doses of Antigen After Immunisation on Total OVA-Specific IgG Production.

Total primary OVA-specific serum IgG responses in mice given a single feed of 2, 25 or 200mg OVA (**a**) or 400mg OVA (**b**) 7 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. The results shown are mean % of purified IgG standard ± 1 SD for individual sera from 5 mice per group. (*$p<0.02$ versus controls, **$p<0.005$ versus controls)

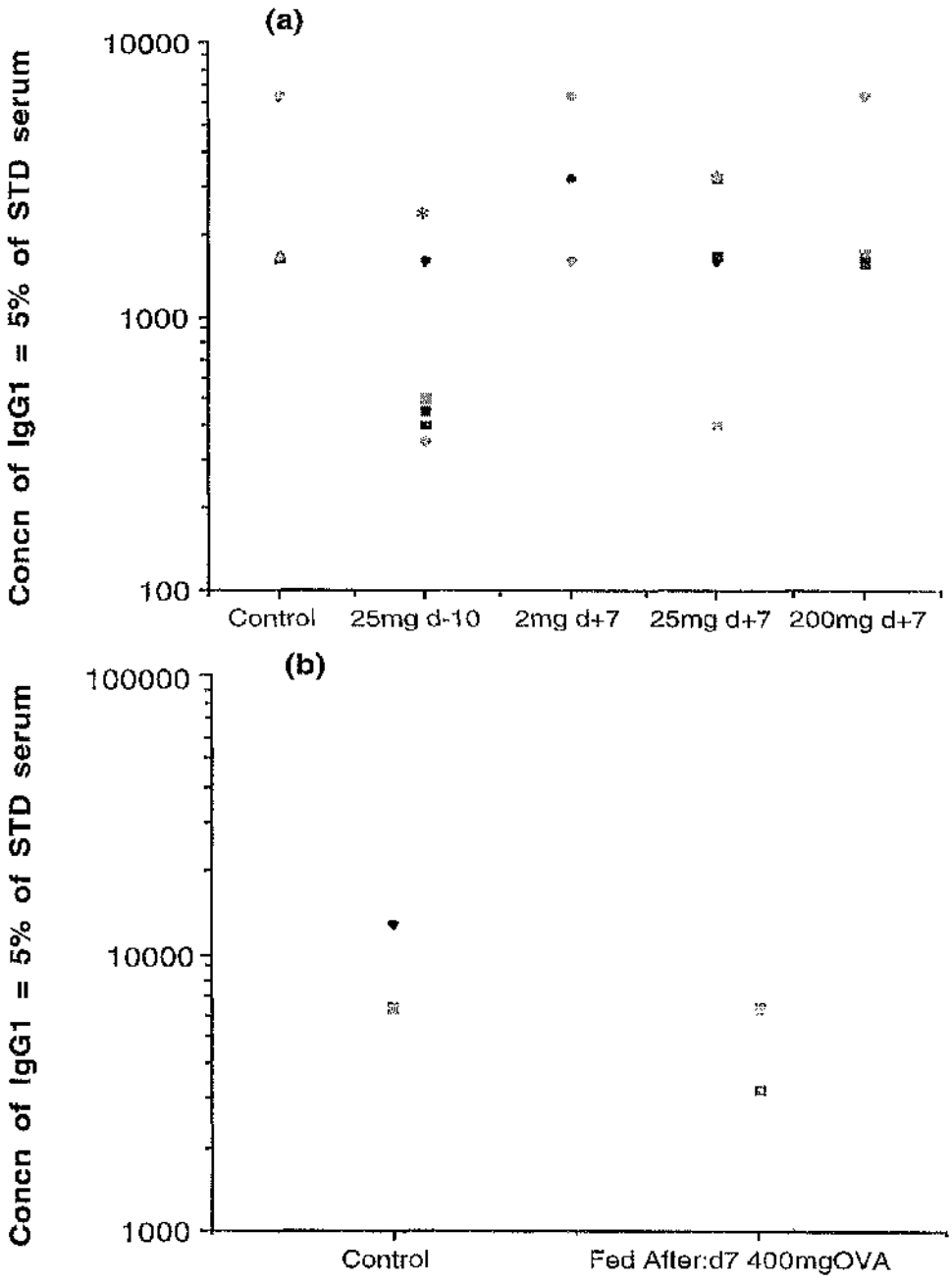


Figure 3.11 Effects of Feeding Different Doses of Antigen After Immunisation on OVA-Specific IgG1 Production.

OVA-specific serum IgG1 responses in mice given a single feed of 2, 25 or 200mg OVA (**a**) or 400mg OVA (**b**) 7 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group and the reciprocal dilutions were done in duplicate. (*$p<0.05$ versus controls)

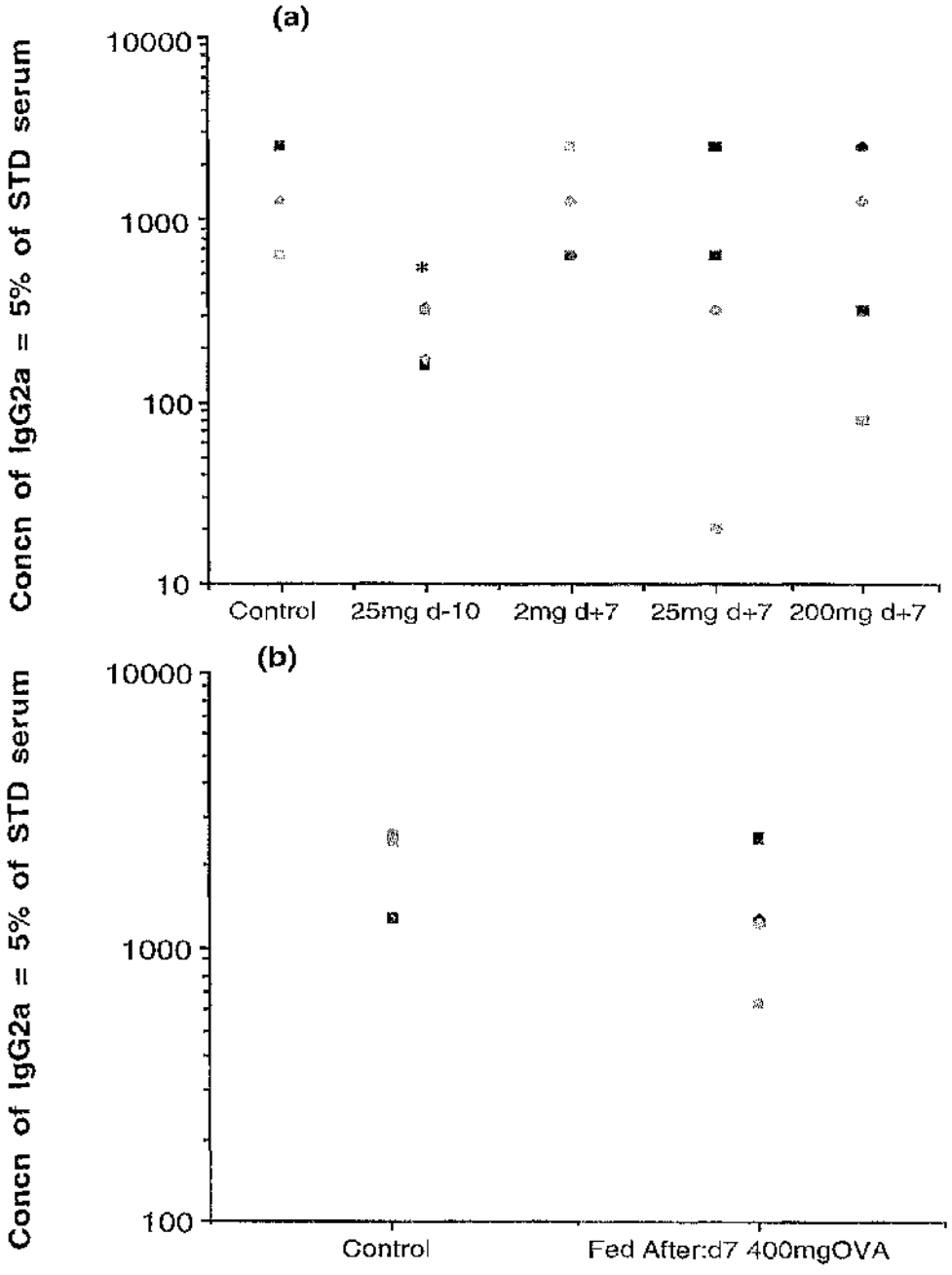


Figure 3.12 Effects of Feeding Different Doses of Antigen After Immunisation on OVA-Specific IgG2a Production.

OVA-specific serum IgG2a responses in mice given a single feed of 2, 25 or 200mg OVA **(a)** or 400mg OVA **(b)** 7 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group and the reciprocal dilutions were done in duplicate. (*$p<0.05$ versus controls)

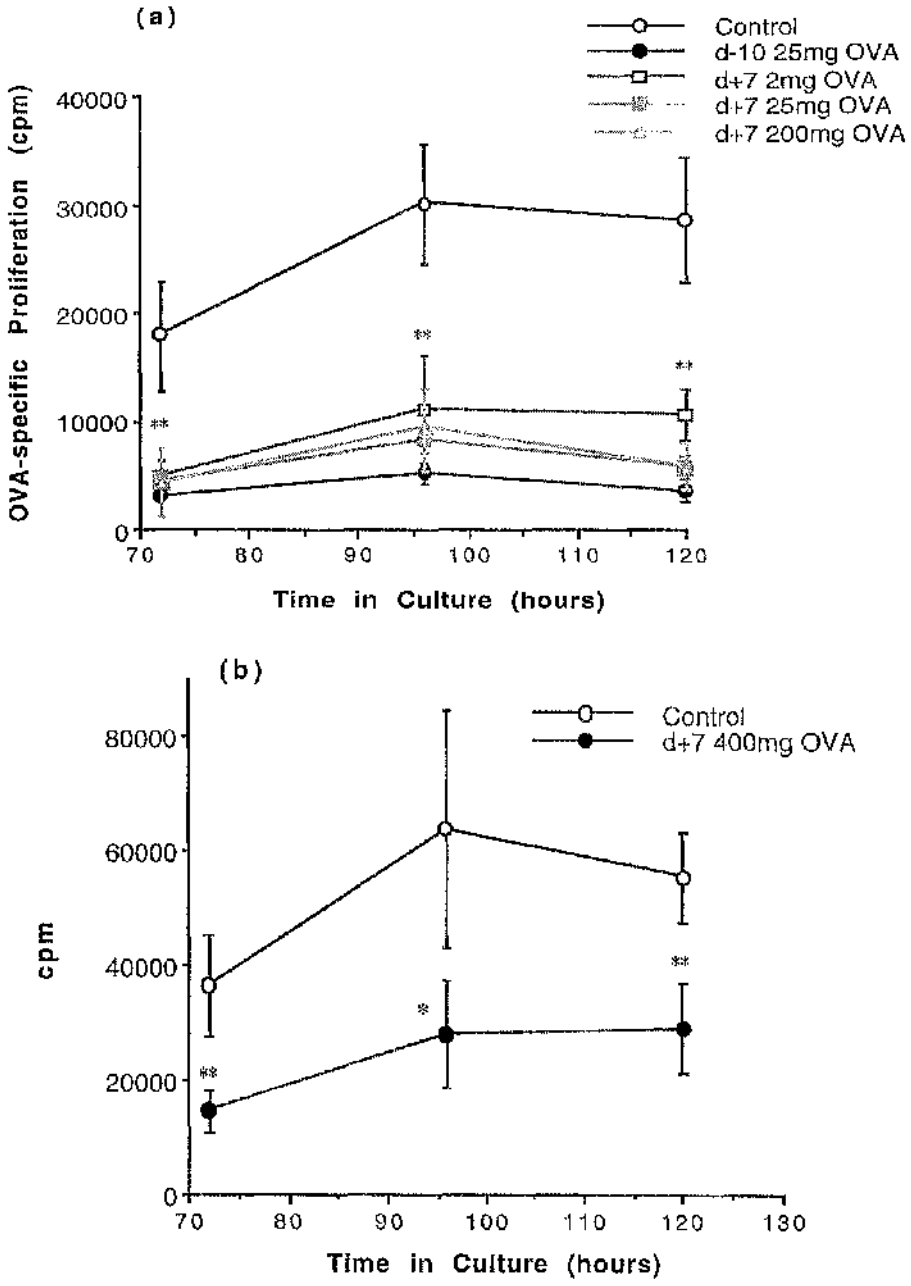


Figure 3.13 Effects of Feeding Different Doses of Antigen After Immunisation on OVA-Specific Proliferation.

OVA-specific proliferative responses in draining lymph nodes of mice given a single feed of 2, 25 or 200mg OVA **(a)** or 400mg OVA **(b)** 7 days after subcutaneous immunisation with OVA/CFA, and saline fed controls. The results shown are mean uptake of ^{3}H-TdR ± 1 SD for the last 24 hours of culture in quadruplicate cultures from lymph node cells pooled from 3 mice per group. (*$p<0.02$ versus controls, **$p<0.005$ versus controls)

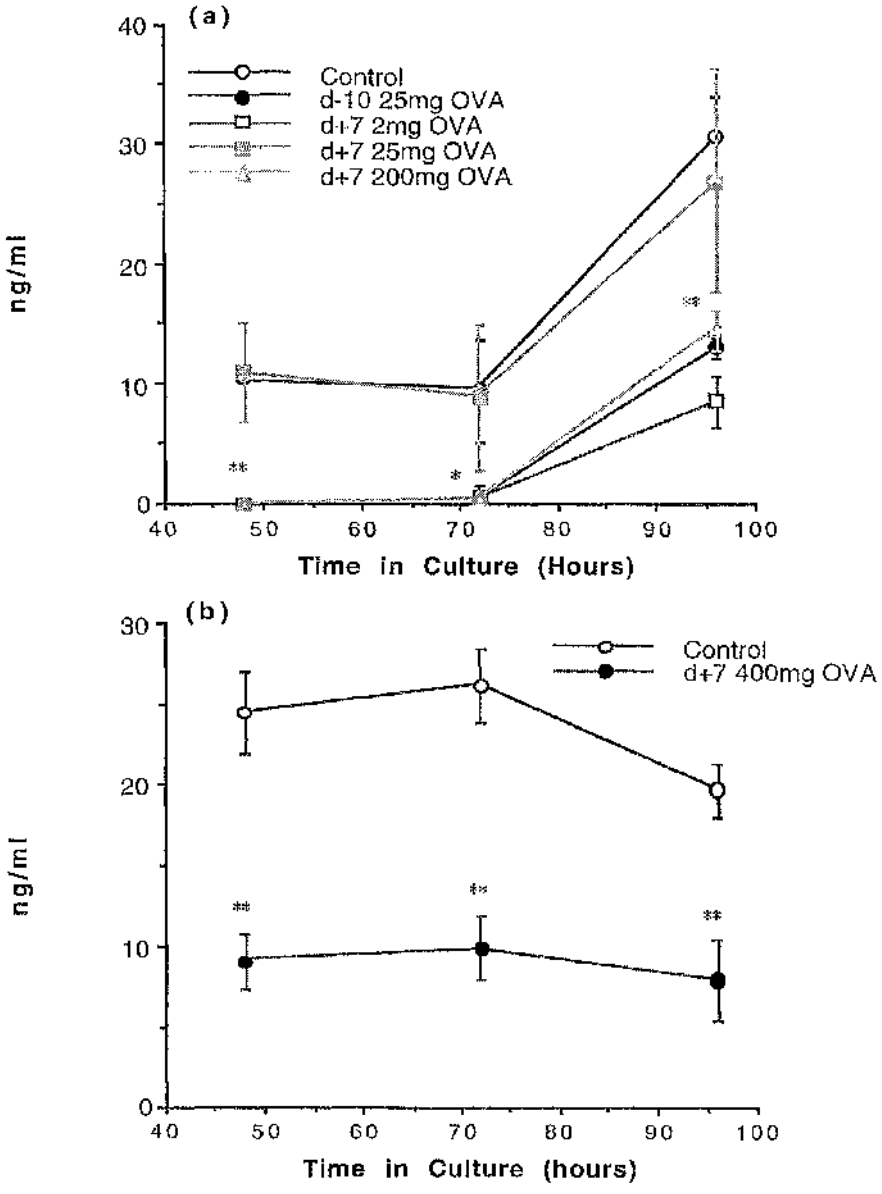


Figure 3.14 Effects of Feeding Different Doses of Antigen After Immunisation on OVA-Specific IFNγ Production.

Antigen specific IFNγ production by draining lymph nodes of mice given a single feed of 2, 25 or 200mg OVA (**a**) or 400mg OVA (**b**) 7 days after subcutaneous immunisation with OVA/CFA, and saline fed controls. The results shown are mean cytokine levels (ng/ml) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 2-4 days. There was little or no production of IFNγ from cells cultured in the absence of antigen. (*$p<0.05$ versus controls, **$p<0.005$ versus controls)

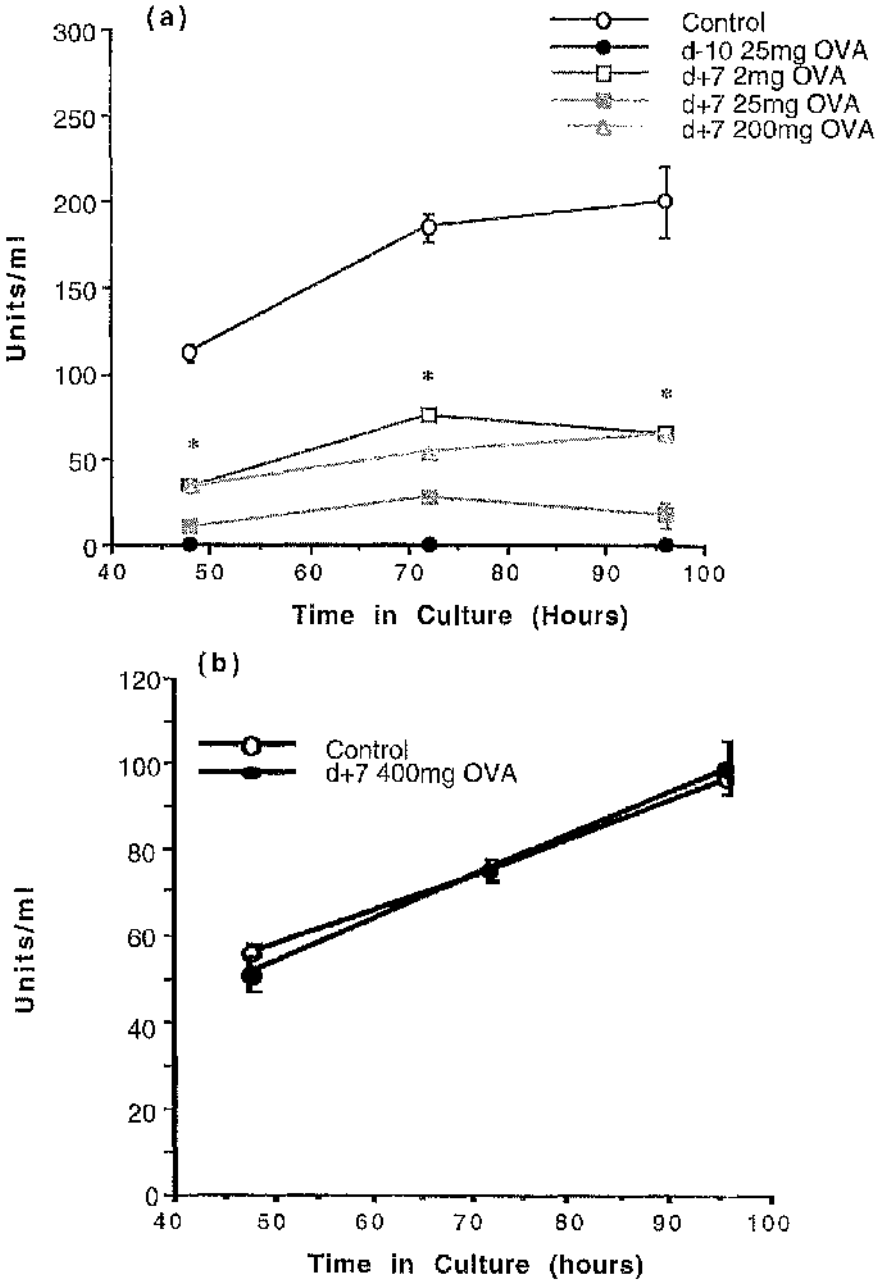


Figure 3.15 Effects of Feeding Different Doses of Antigen After Immunisation on OVA-Specific IL5 Production.

Antigen specific IL5 production by draining lymph nodes of mice given a single feed of 2, 25 or 200mg OVA **(a)** or 400mg OVA **(b)** 7 days after subcutaneous immunisation with OVA/CFA, and saline fed controls. The results shown are mean cytokine levels (U/ml) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 2-4 days. There was little or no production of IL5 from cells cultured in the absence of antigen. (*$p<0.001$ versus controls)

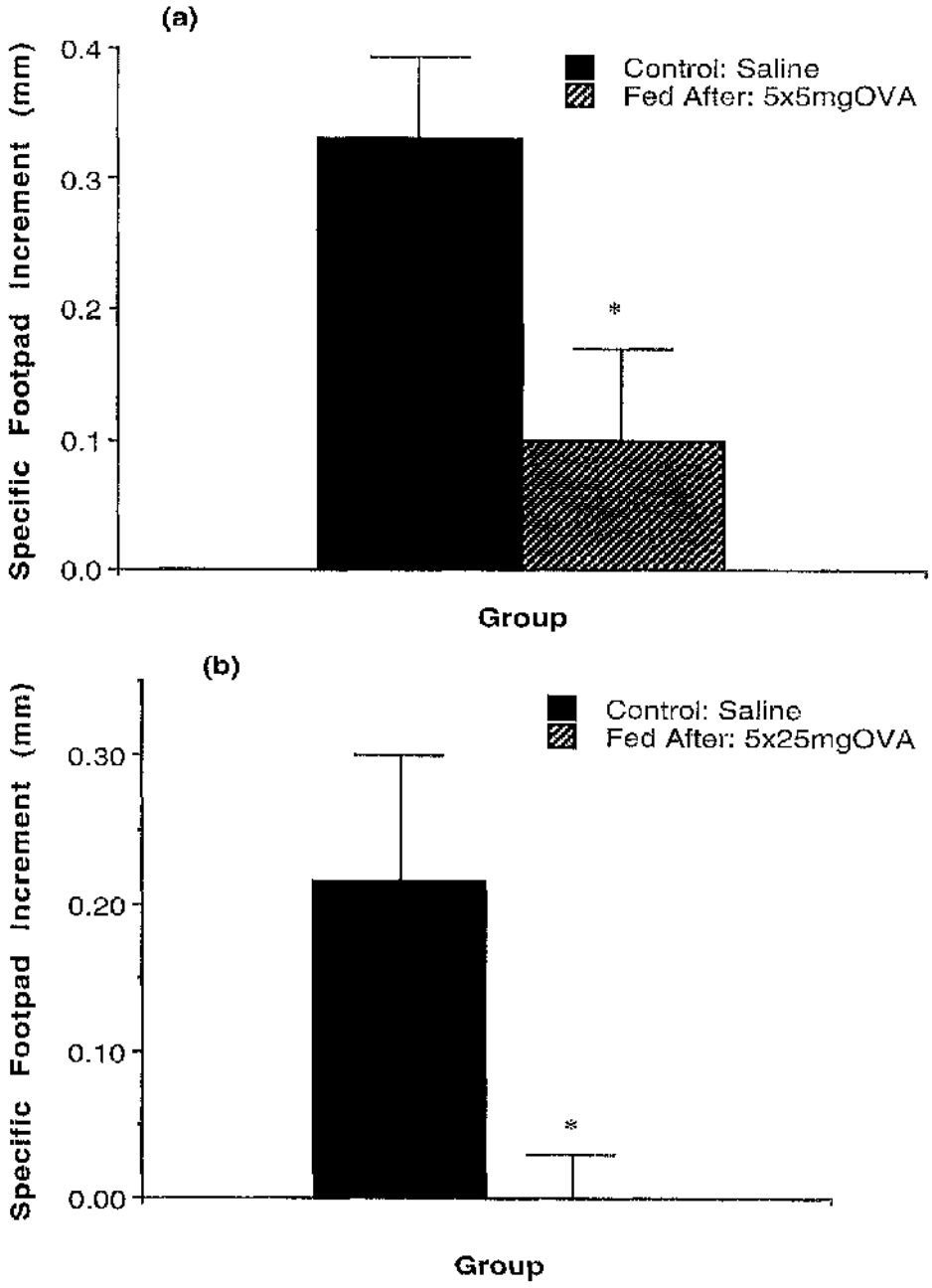


Figure 3.16 Effects of Different Antigen Feeding Regimes on Systemic DTH Responses in Primed Mice.

Systemic DTH responses in mice given 5 single feeds of 5mg OVA (**a**) or 25mg OVA (**b**) starting 7 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. The results shown are mean specific increments in footpad thickness ± 1 SD for 5 mice per group. (*$p<0.005$ versus controls)

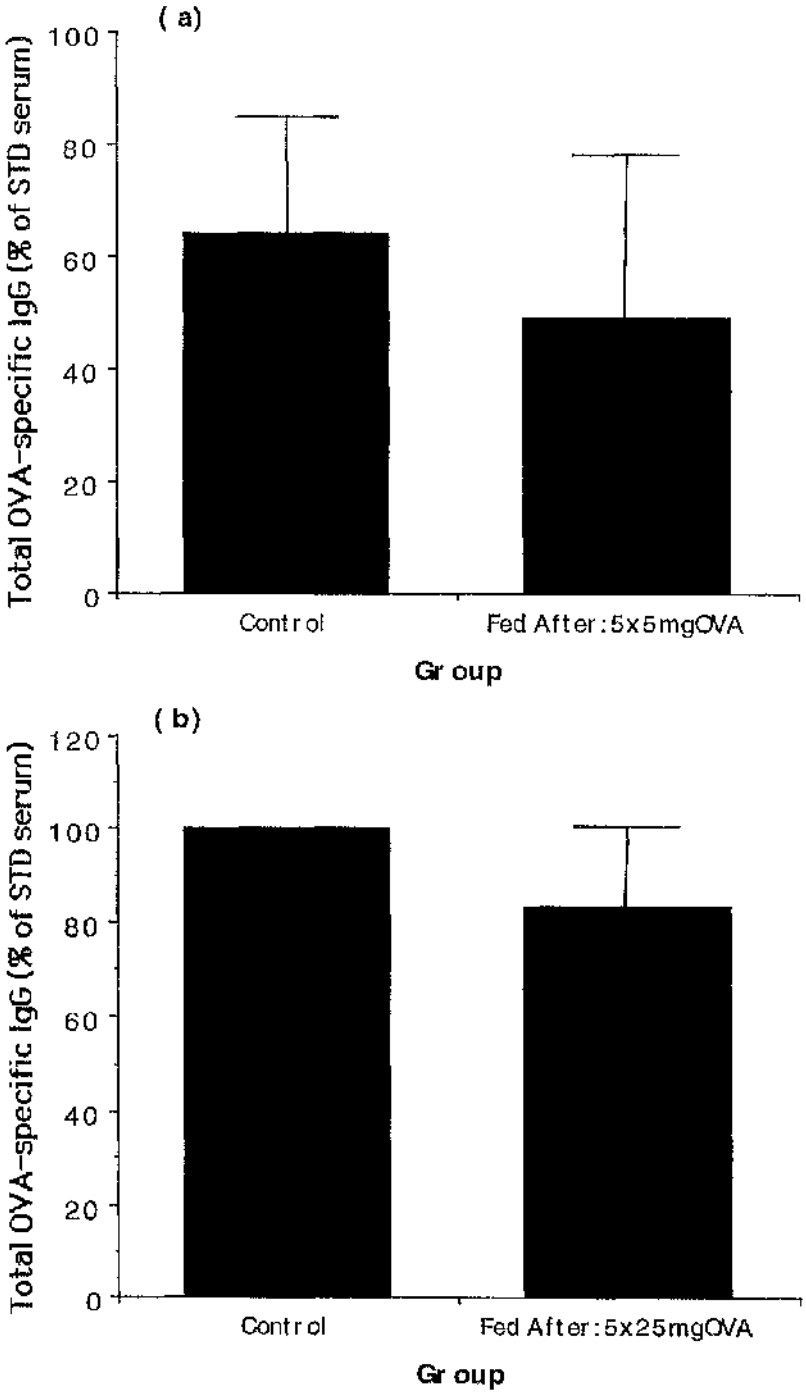


Figure 3.17 Effects of Different Antigen Feeding Regimes on Total OVA-Specific IgG Production in Primed Mice.

Total primary OVA-specific serum IgG responses in mice given 5 single feeds of 5mg OVA **(a)** or 25mg OVA **(b)** starting 7 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. The results shown are mean % of purified IgG standard ± 1 SD for individual sera from 5 mice per group.

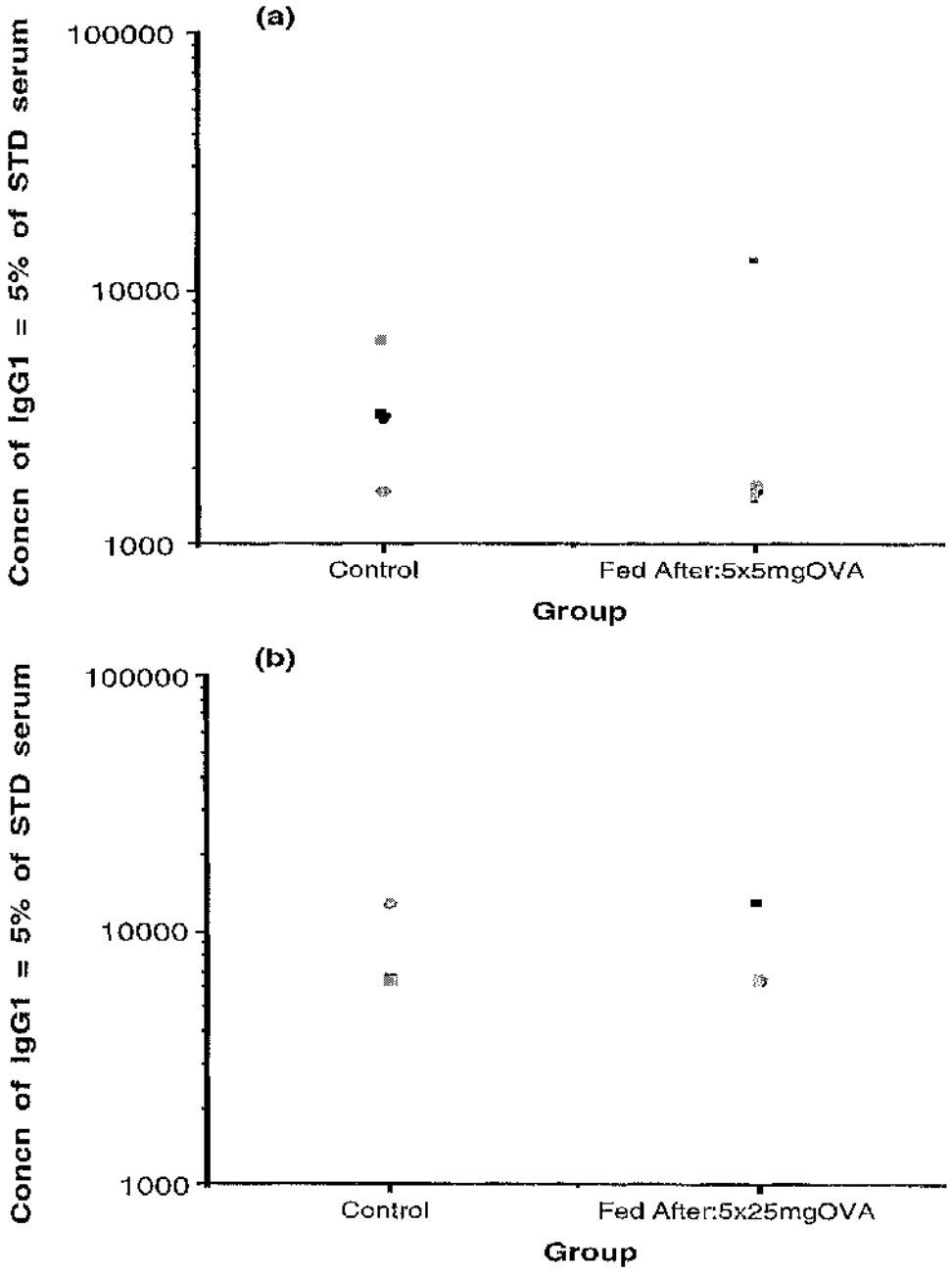


Figure 3.18 Effects of Different Antigen Feeding Regimes on OVA-Specific IgG1 Production in Primed Mice.

OVA-specific serum IgG1 responses in mice given 5 single feeds of 5mg OVA **(a)** or 25mg OVA **(b)** starting 7 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group and the reciprocal dilutions were done in duplicate.

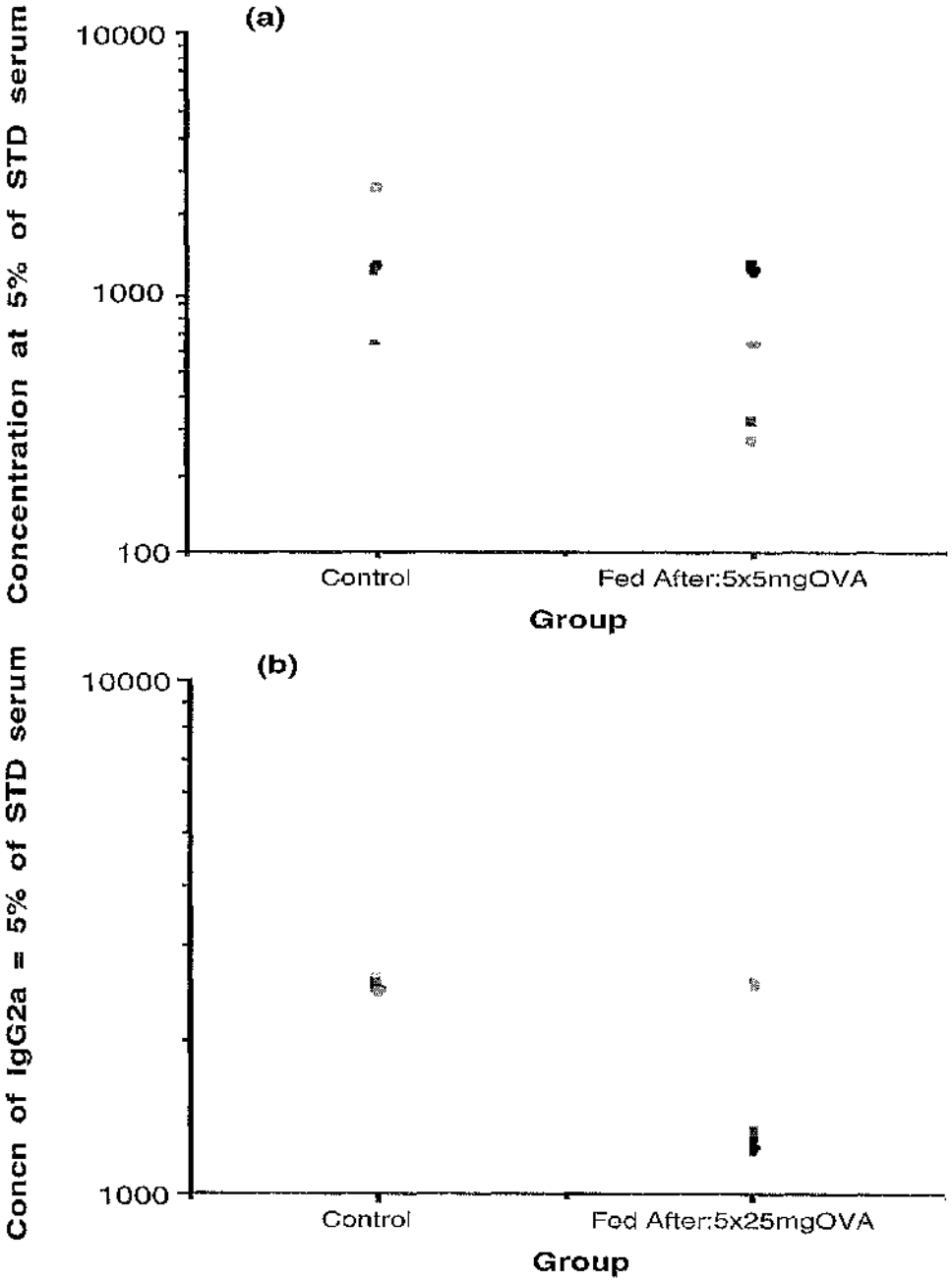


Figure 3.19 Effects of Different Antigen Feeding Regimes on OVA-Specific IgG2a Production in Primed Mice.

OVA-specific serum IgG2a responses in mice given 5 single feeds of 5mg OVA **(a)** or 25mg OVA **(b)** starting 7 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group and the reciprocal dilutions were done in duplicate.

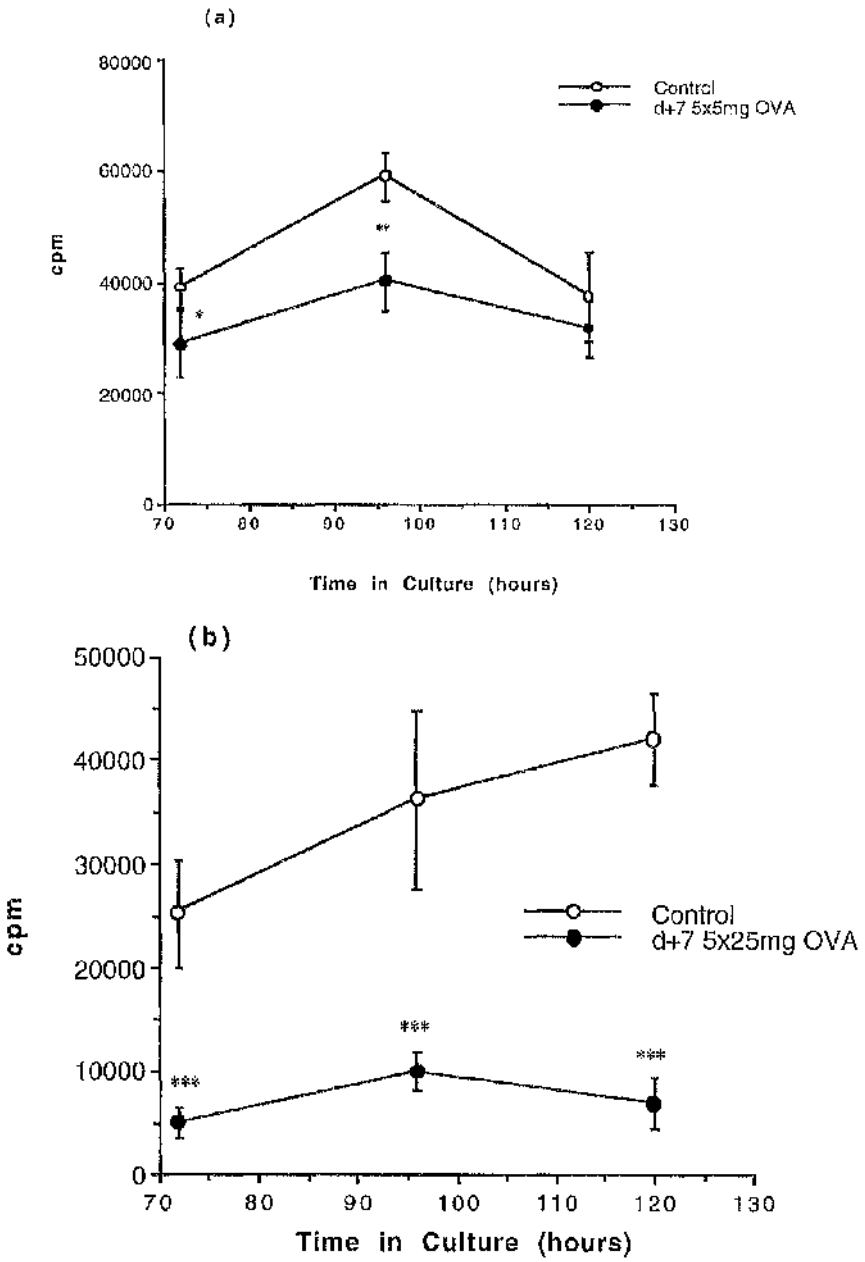


Figure 3.20 Effects of Different Antigen Feeding Regimes on OVA-Specific Proliferation in Primed Mice.

OVA-specific proliferative responses in draining lymph nodes of mice given 5 single feeds of 5mg OVA **(a)** or 25mg OVA **(b)** starting 7 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls. The results shown are mean uptake of ^{3}H-TdR ± 1 SD for the last 24 hours of culture in quadruplicate cultures from lymph node cells pooled from 3 mice per group. (*$p<0.02$ versus controls, **$p<0.005$ versus controls, ***$p<0.001$ versus controls)

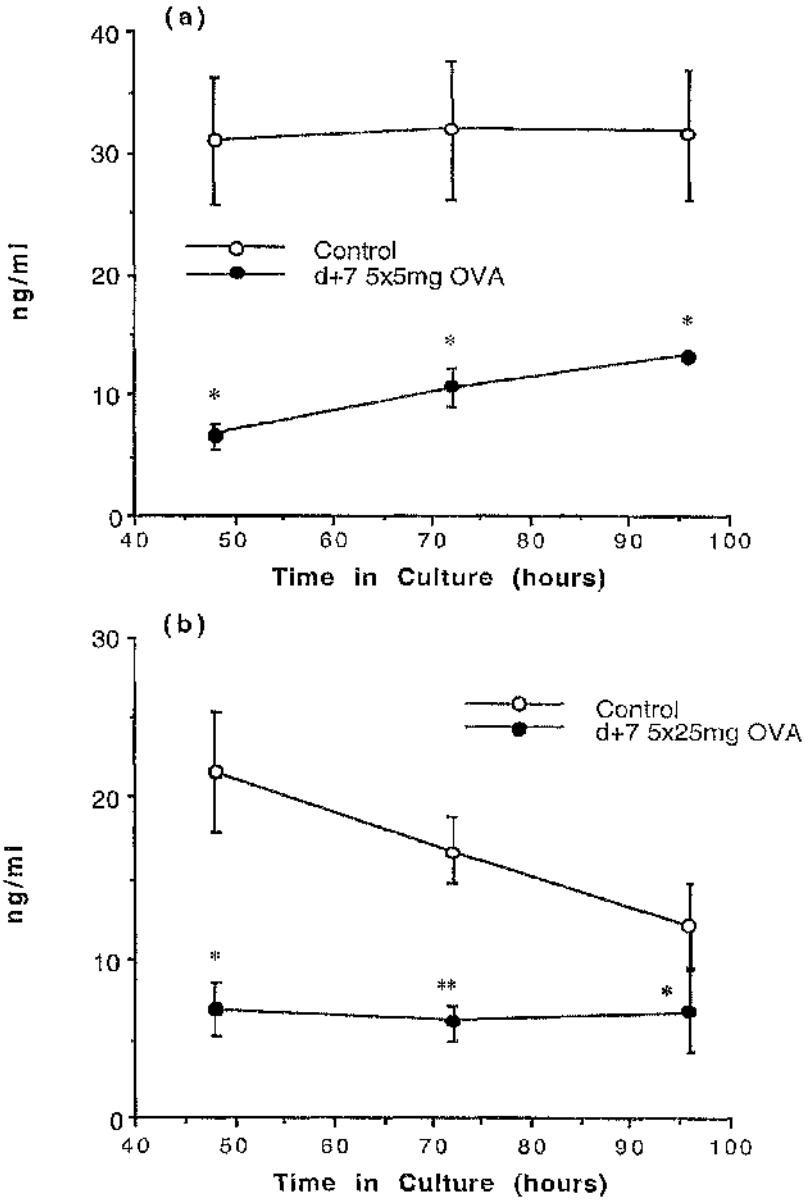


Figure 3.21 Effects of Different Antigen Feeding Regimes on OVA-Specific IFNγ Production in Primed Mice.

Antigen specific IFNγ production by draining lymph nodes of mice given 5 single feeds of 5mg OVA **(a)** or 25mg OVA **(b)** starting 7 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls. The results shown are mean cytokine levels (ng/ml) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 2-4 days. There was little or no production of IFNγ from cells cultured in the absence of antigen. (*$p<0.005$ versus controls, **$p<0.001$ versus controls)

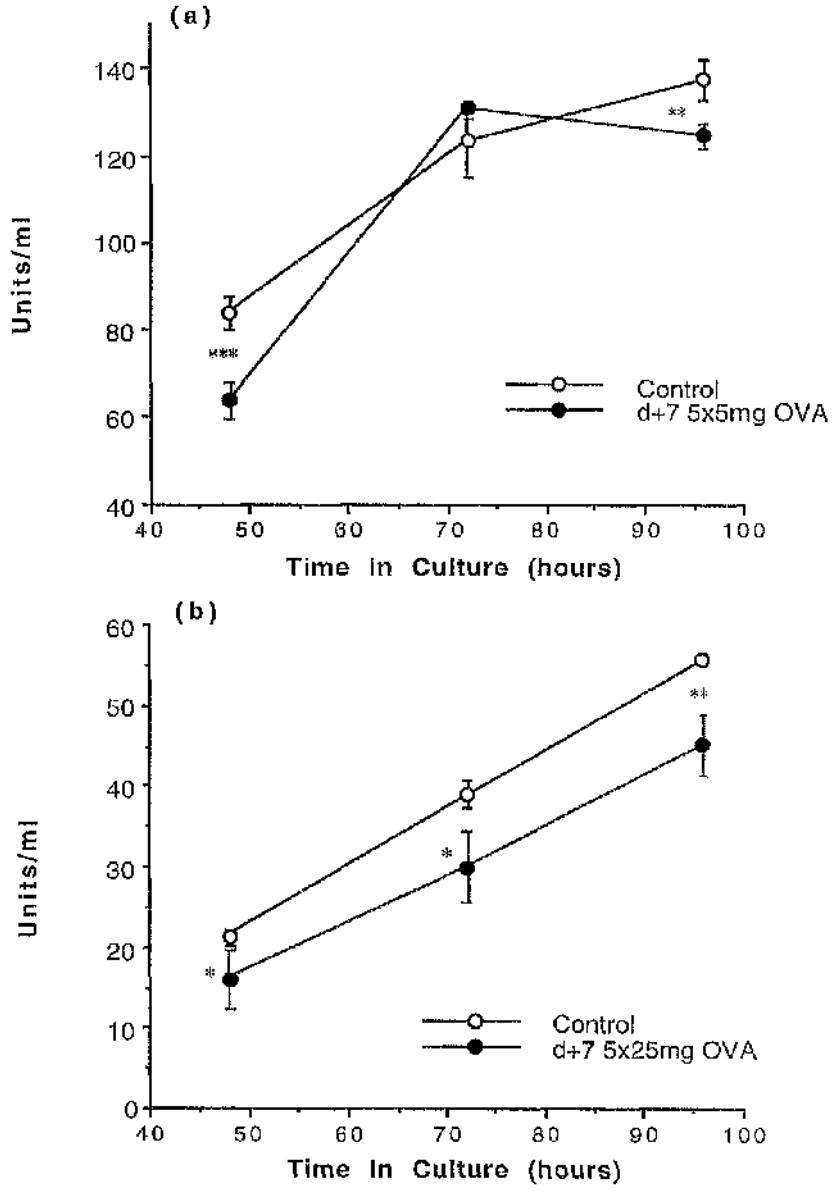


Figure 3.22 Effects of Different Antigen Feeding Regimes on OVA-Specific IL5 Production in Primed Mice.

Antigen specific IL5 production by draining lymph nodes of mice given 5 single feeds of 5mg OVA (**a**) or 25mg OVA (b) starting 7 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls. The results shown are mean cytokine levels (U/ml) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 2-4 days. There was little or no production of IL5 from cells cultured in the absence of antigen. (*$p<0.05$ versus controls, **$p<0.02$ versus controls, ***$p<0.01$ versus controls)

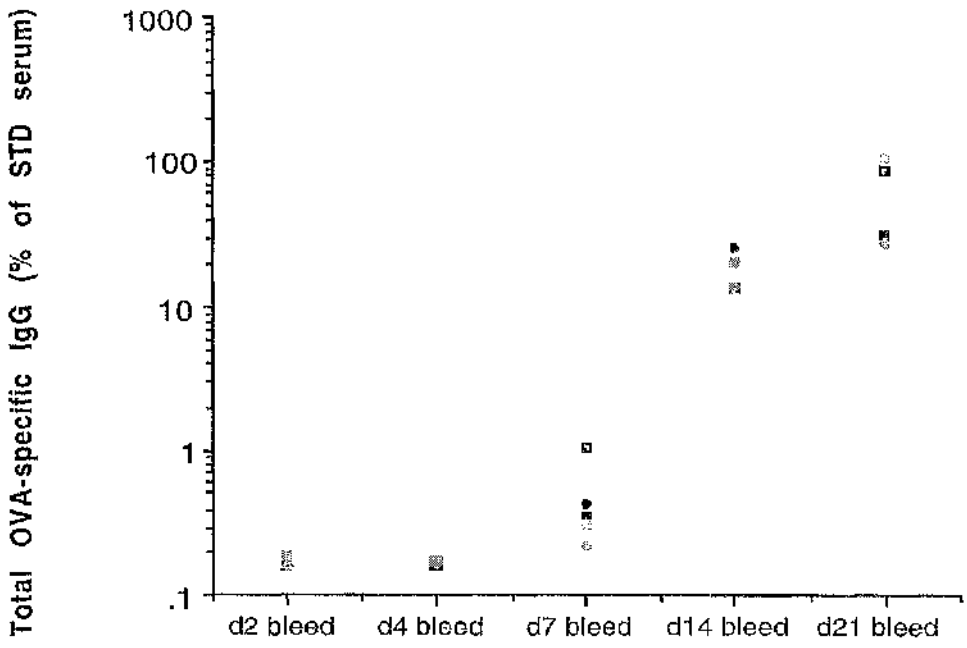


Figure 3.23 Time Course of OVA-Specific IgG Production in Mice Primed with OVA/CFA.

Total primary OVA-specific serum IgG responses in mice over 3 weeks after subcutaneous immunisation with OVA/CFA. The results shown are mean % of purified IgG standard for individual sera from 5 mice per time point.

Chapter4 Longevity of Oral Tolerance In Mice Fed Antigen After Systemic Priming

4.1 Introduction

The experiments in the previous chapter show that feeding after systemic priming results in tolerance of some *in vivo* and *in vitro* immune responses. If oral tolerance is to be used as a therapy, the antigen specific suppressive effects will need to be long lasting. Previous studies in naive mice found that several parameters of the systemic immune responses remained tolerant for up to 17 months after feeding (178) and, in this chapter, I investigated how long tolerance persisted after a single feed of 25mg OVA given 7 days after priming.

4.2 Experimental Protocol

BALB/c mice were immunised s.c. with OVA/CFA on d0 and oral tolerance was induced by feeding mice 25mg OVA on d+7. Control mice were fed saline after immunisation. 7 days after feeding, PLN were taken and Ag specific proliferation and cytokine production was assessed. 14 days after feeding, mice were bled for assessment of serum antibody levels. Three separate groups of mice were then used. The first group were challenged immediately with HAO in the opposite footpad for measurement of DTH responses. Two further groups were given a secondary immunisation 6 and 12 months later with OVA/CFA in the base of the tail and their PLN taken 7 days later for *in vitro* assessment. Serum antibodies and systemic DTH were measured 14 days after the secondary immunisation.

4.3 Results

4.3.1 Persistence of Oral Tolerance *in vivo*

OVA-specific DTH responses were significantly reduced in mice fed OVA when examined 14 days after feeding confirming the results in Chapter 3 (Fig 4.1a).

However, by 6 and 12 months after immunisation, there was no tolerance of specific DTH responses in OVA fed mice (Fig4.1b+c).

At the first time point examined, the serum levels of total OVA-specific IgG, IgG1 and IgG2a antibodies were not significantly reduced by feeding 25mg OVA after immunisation, confirming my previous results (Figs 4.2a, 4.3a, and 4.4a). As might be expected from this finding, none of the antibody responses were tolerant in mice examined 6 months (Figs 4.2b, 4.3b, and 4.4b) or 12 months (Figs 4.2c, 4.3c, and 4.4c) after feeding.

4.3.2 Persistence of Oral Tolerance *in vitro*

Cell-mediated immunity *in vitro* was measured by OVA-specific proliferation and cytokine production of PLN cells.

4.3.2.1 Proliferative Responses

As expected from my previous findings, OVA-specific proliferation was significantly tolerised in the OVA fed mice examined 14 days after feeding (Fig 4.5a). By 6 months, this tolerance was no longer present (Fig 4.5b) and at 12 months, the proliferative response of the OVA fed group was significantly increased compared with unfed controls (Fig 4.5c). This priming of the proliferative response in OVA fed mice was confirmed by the fact that OVA-specific proliferation was still present in PLN cells taken from mice in which DTH responses had been measured, whereas responses in immunised control mice had disappeared by this time (Fig 4.5d). Thus proliferative tolerance is lost by six months after feeding.

4.3.2.2 Cytokine Production

In contrast to my previous findings, OVA-specific IFNγ production was not significantly reduced in mice examined 14 days after feeding compared with controls

(Fig. 4.6a). However, at 6 months and 12 months after feeding, IFNγ production was significantly suppressed in OVA fed mice.

OVA-specific IL5 production was significantly primed in OVA fed mice examined at 14 days (Fig. 4.7a). However, IL5 production was significantly reduced in mice examined at 6 months (Fig. 4.7b) before returning to control levels at 12 months.

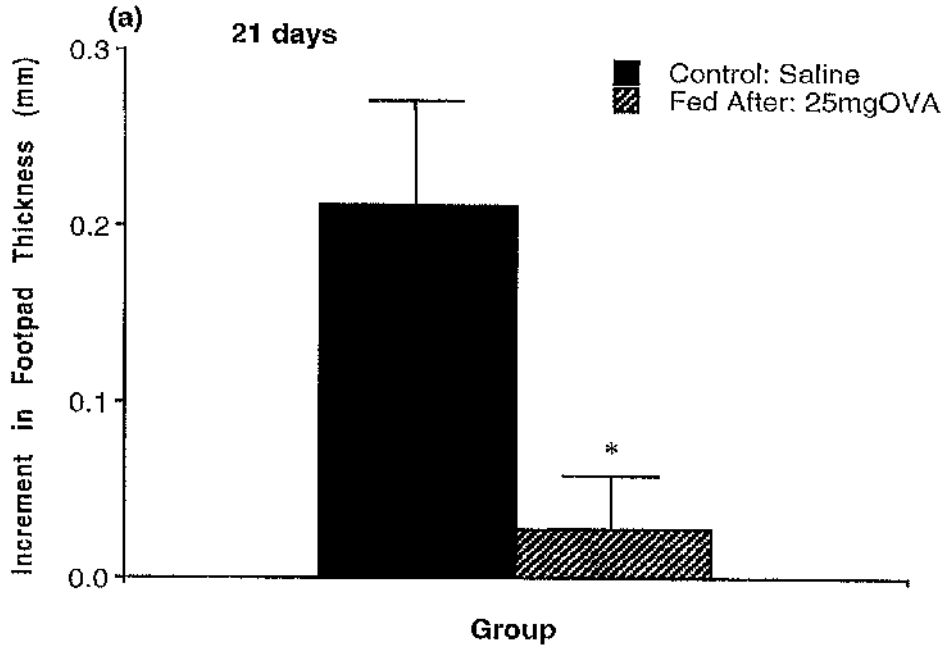
4.4 Conclusions

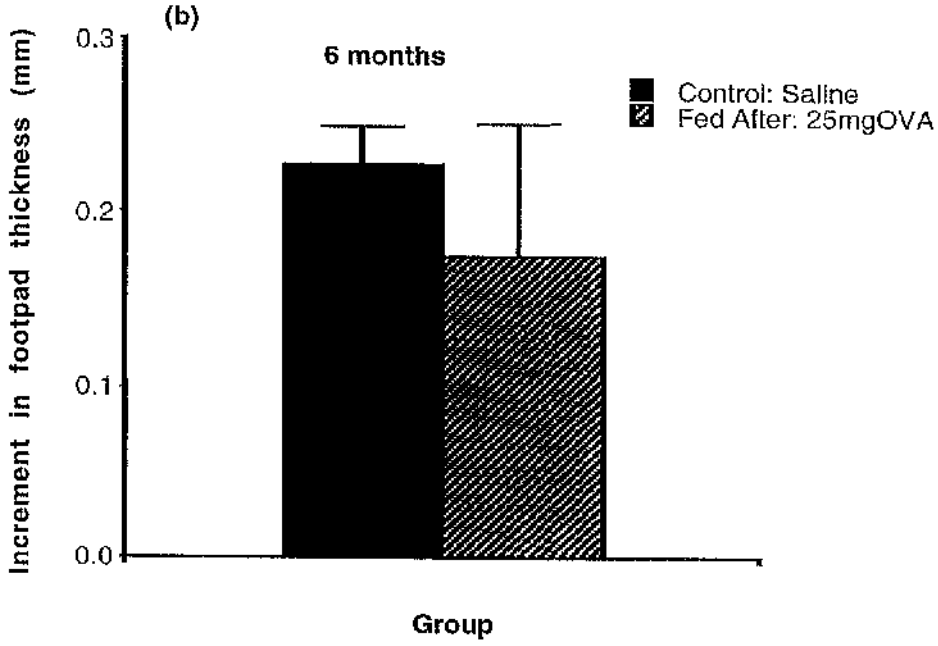
The results presented in this chapter indicate that the oral tolerance induced when antigen is fed after immunisation is not as long lasting as that found in naive mice, where several parameters remained tolerised for up to 17 months.*In vivo* tolerance was only found early after feeding, when antigen specific DTH responses were suppressed with controls. DTH responses were normal at later time points and antibody responses were not tolerised at any time after feeding, confirming my initial findings in Chapter 3.

Some aspects of the *in vitro* response showed longer lasting tolerance however. Thus IFNγ production remained tolerant in OVA fed mice for 12 months after feeding, although the interpretation of these findings is complicated by the fact this function was not inhibited at the earliest time as I would have expected. The reason for this discrepancy is unknown and time did not permit me to repeat the experiment. Similarly, the experiment in the current chapter indicated that IL5 production was primed early after feeding, whereas it had been unaffected in my first experiments. This underlines the possible resistance of T_H2 responses to feeding antigen but it should be noted that IL5 production was tolerised at 6 months, before returning to normal at 12 months. OVA-specific proliferation was found to be tolerised at the first time point but then returned to control levels by 6 month time point. Surprisingly, the proliferative response was enhanced in the group fed OVA at the 12 month time point.

Overall, these results suggest that only some aspects of the established immune response can be tolerised for any length of time by a single feed of antigen. It seems that as in other forms of oral tolerance, IFNγ production is particularly sensitive to

feeding in primed mice, although my results suggest that other aspects such as IL5 might also be tolerised at different times. Thus oral tolerance is a dynamic phenomenon but there is surprisingly little correlation between those aspects which can be tolerised at different times. Although this is consistent with previous results in the lab in naive mice, it emphasises the unpredictability of tolerance in primed animals.


(a)
21 days
Control: Saline
Fed After: 25mgOVA
Increment in Footpad Thickness (mm)
0.3
0.2
0.1
0.0
*
Group


(b)
6 months
Control: Saline
Fed After: 25mgOVA
Increment in footpad thickness (mm)
0.3
0.2
0.1
0.0
Group

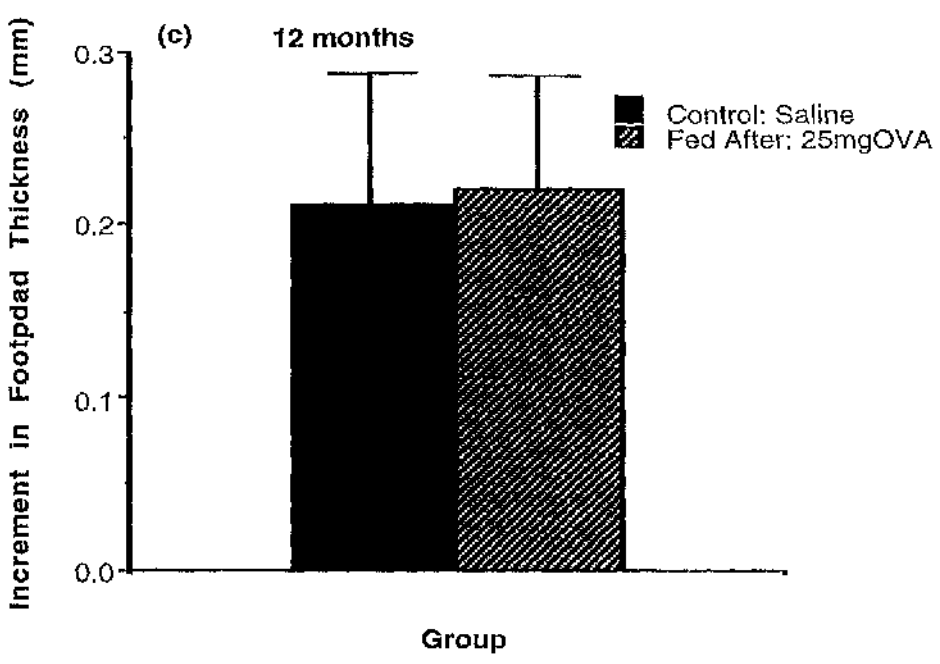
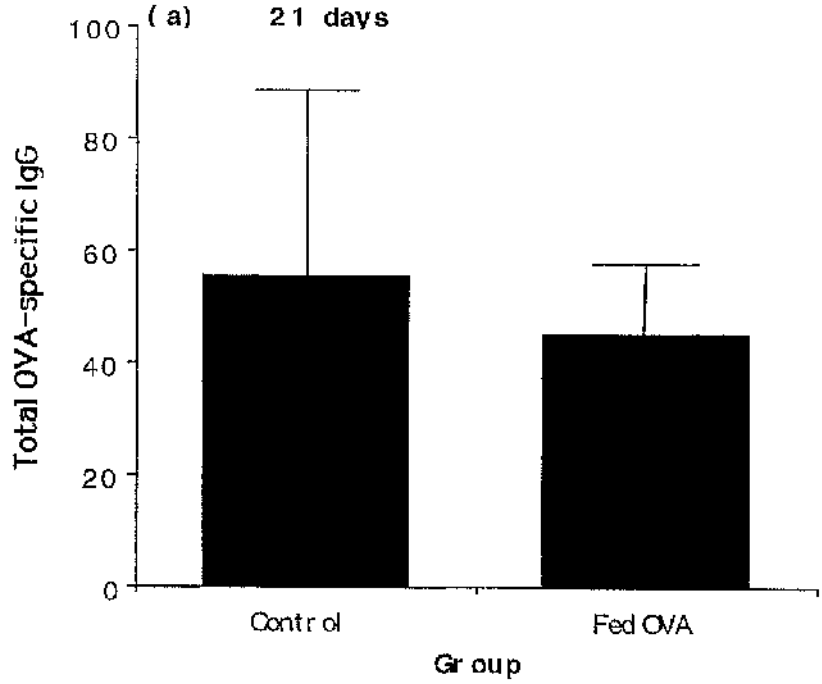
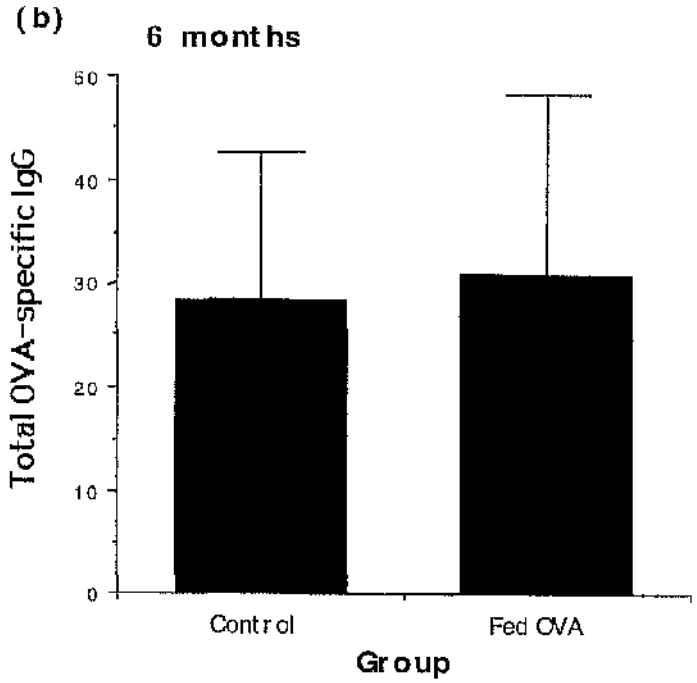


Figure 4.1 Time Course of Oral Tolerance in Primed Mice.

Systemice DTII responses in mice given a single feed of 25mg OVA 7 days after primary immunisation with OVA/CFA assessed 21 days, 6 months or 12 months after primary immunisation. Mice examined at 6 or 12 months received a secondary immunisation 21 days before DTH response was assessed. The results shown are mean specific increments in footpad thickness ± 1 SD for 5 mice per group. (*$p<0.001$ versus controls)


(a)
21 days
100
80
60
40
20
0
Total OVA-specific IgG
Control
Fed OVA
Group


(b)
6 months
50
40
30
20
10
0
Total OVA-specific IgG
Control
Fed OVA
Group

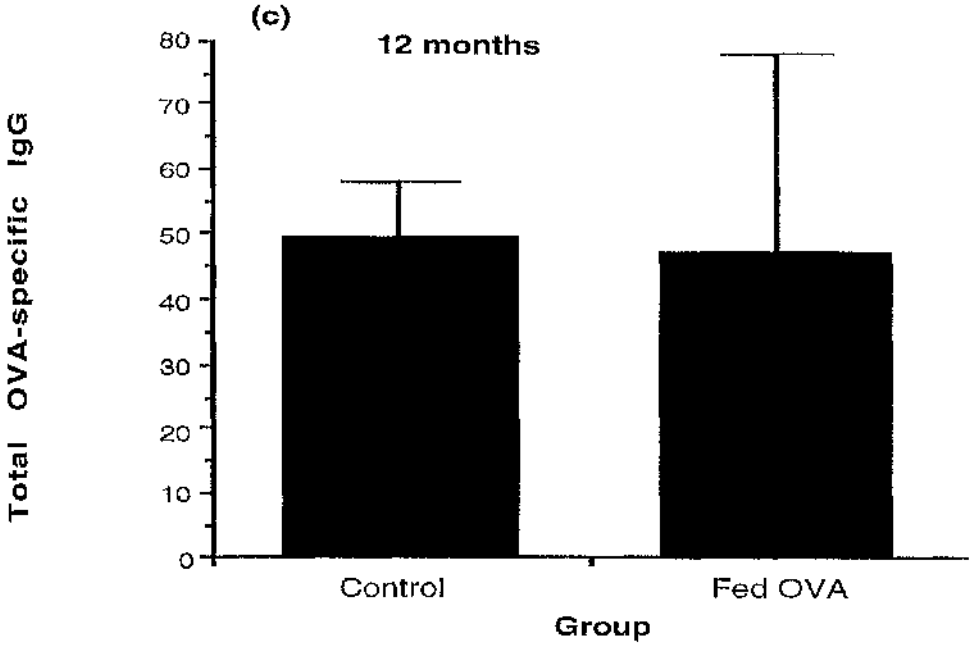
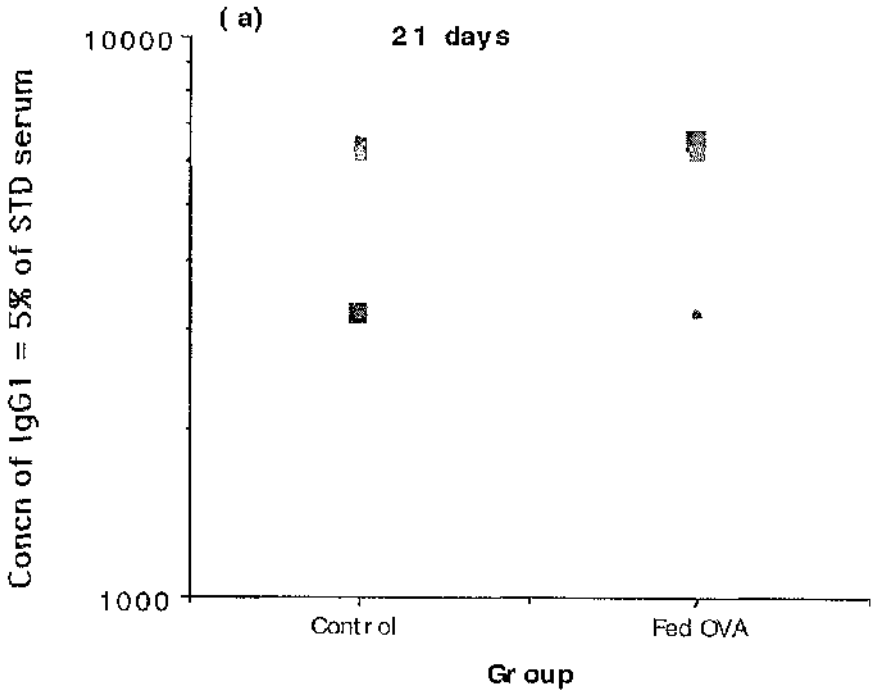
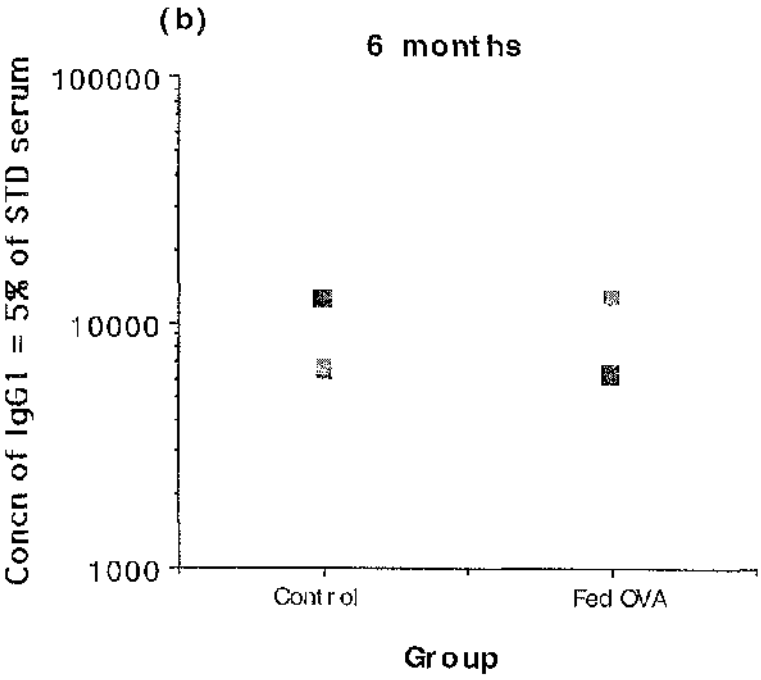


Figure 4.2 Time Course of Oral Tolerance in Primed Mice.

Total primary OVA-specific serum IgG responses in mice given a single feed of 25mg OVA 7 days after primary immunisation with OVA/CFA assessed 21 days, 6 months or 12 months after primary immunisation. Mice examined at 6 or 12 months received a secondary immunisation 21 days before serum IgG response was assessed. The results shown are mean % of purified IgG standard ± 1 SD for individual sera from 5 mice per group.


(a)
21 days
10000
1000
Concn of IgG1 = 5% of STD serum
Control
Fed OVA
Group


(b)
6 months
100000
10000
1000
Concn of IgG1 = 5% of STD serum
Control
Fed OVA
Group

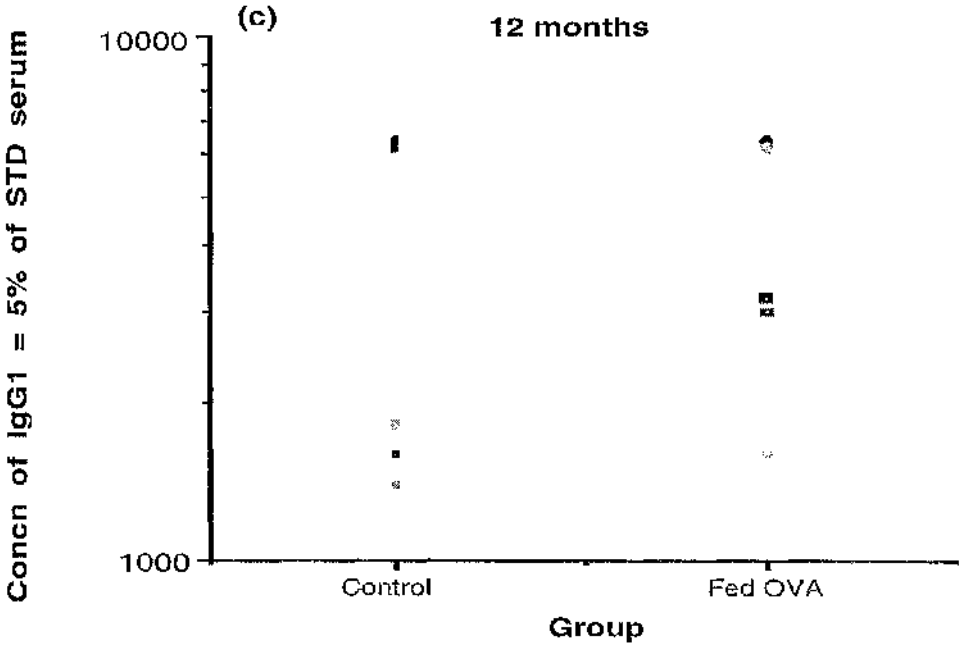
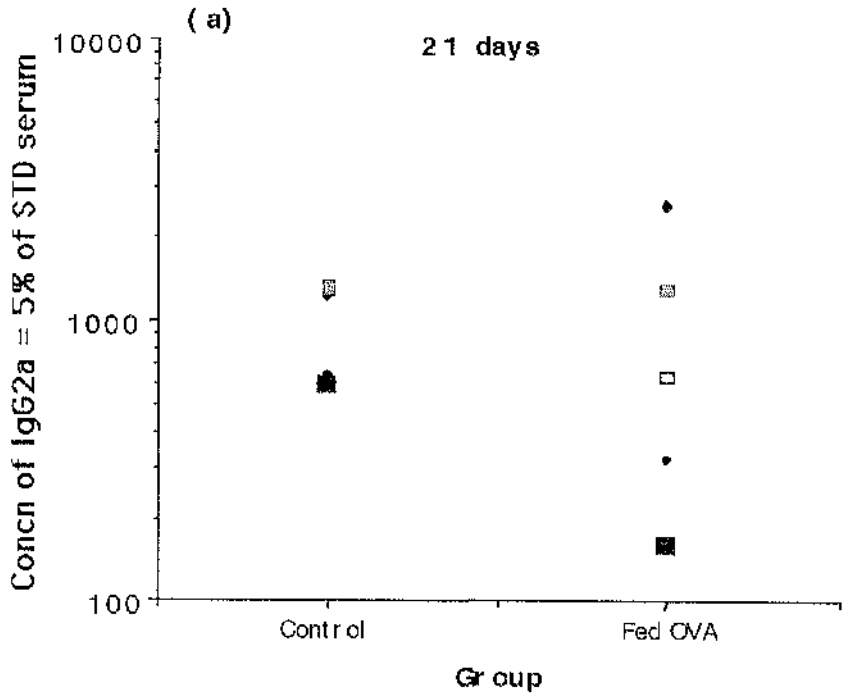
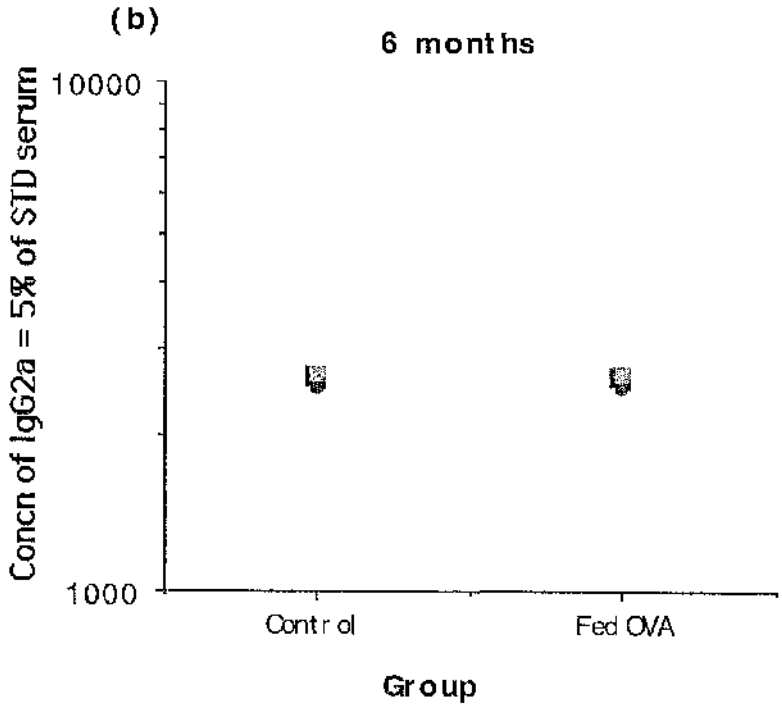


Figure 4.3 Time Course of Oral Tolerance in Primed Mice.

OVA-specific serum IgG1 responses in mice given a single feed of 25mg OVA 7 days after primary immunisation with OVA/CFA assessed 21 days, 6 months or 12 months after primary immunisation. Mice examined at 6 or 12 months received a secondary immunisation 21 days before the serum IgG1 response was assessed. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group and the reciprocal dilutions were done in duplicate.


(a)
21 days
10000
1000
100
Concn of IgG2a = 5% of STD serum
Control
Fed OVA
Group


(b)
6 months
10000
1000
Concn of IgG2a = 5% of STD serum
Control
Fed OVA
Group

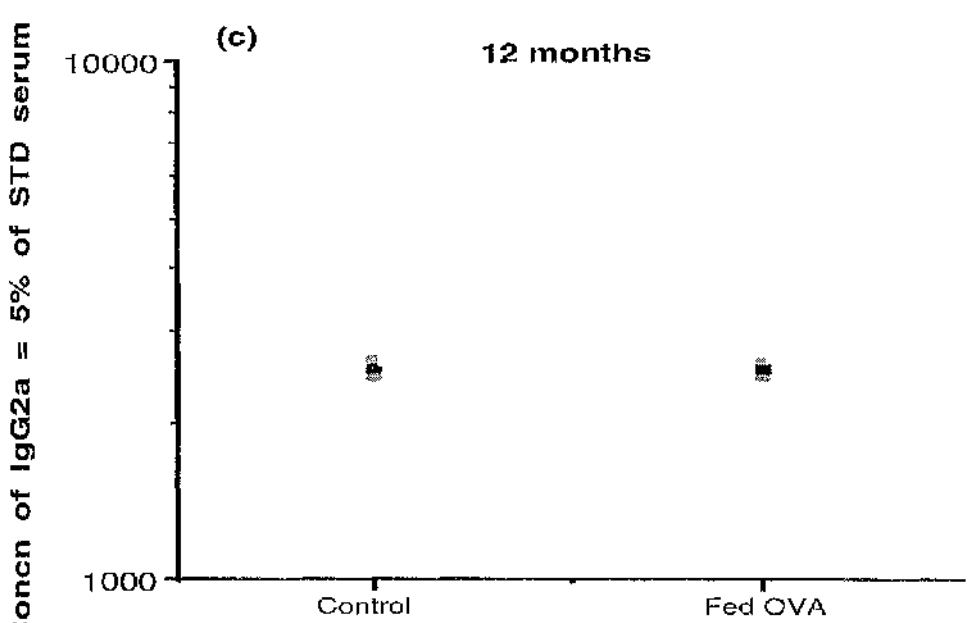
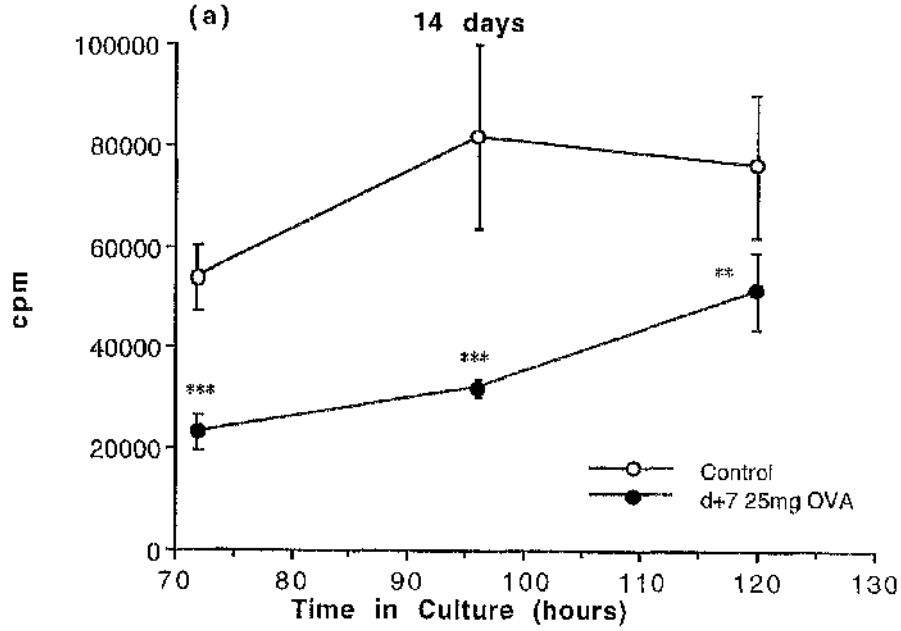
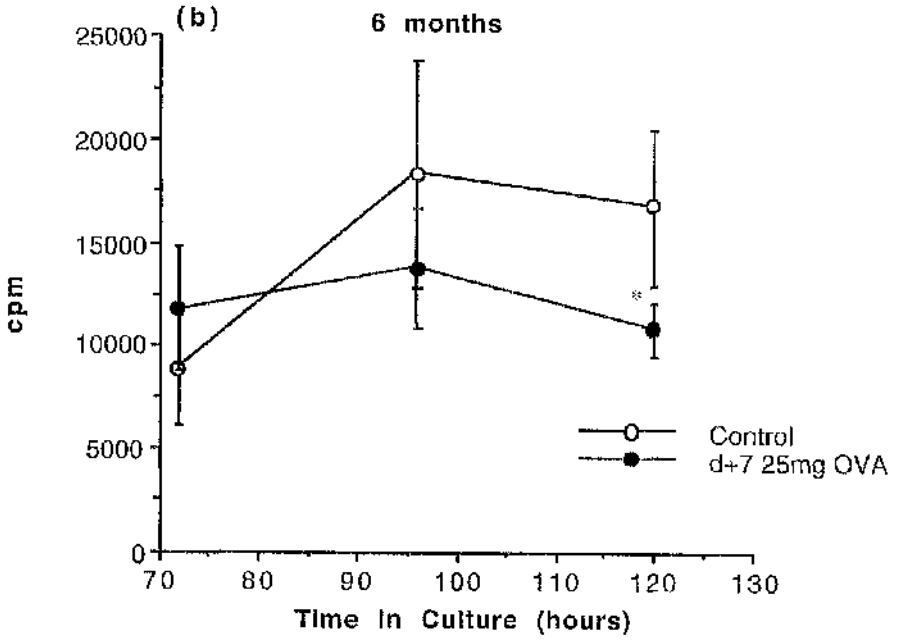


Figure 4.4 Time Course of Oral Tolerance in Primed Mice.

OVA-specific serum IgG2a responses in mice given a single feed of 25mg OVA 7 days after primary immunisation with OVA/CFA assessed 21 days, 6 months or 12 months after primary immunisation. Mice examined at 6 or 12 months received a secondary immunisation 21 days before the serum IgG2a response was assessed. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group and the reciprocal dilutions were done in duplicate.


(a)
14 days
100000
80000
60000
40000
20000
0
cpm
***
***
**
Control
d+7 25mg OVA
70
80
90
100
110
120
130
Time in Culture (hours)


(b)
6 months
25000
20000
15000
10000
5000
0
cpm
*
Control
d+7 25mg OVA
70
80
90
100
110
120
130
Time in Culture (hours)

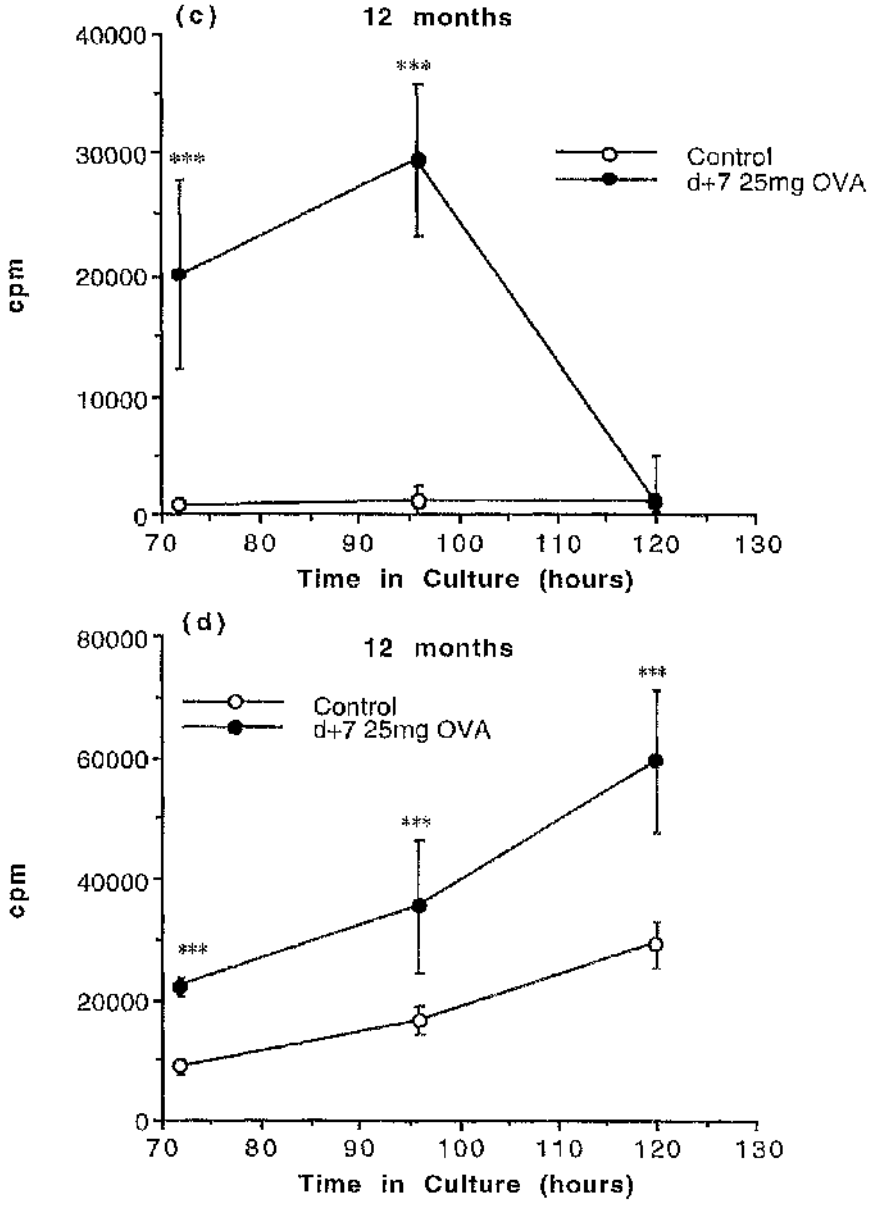
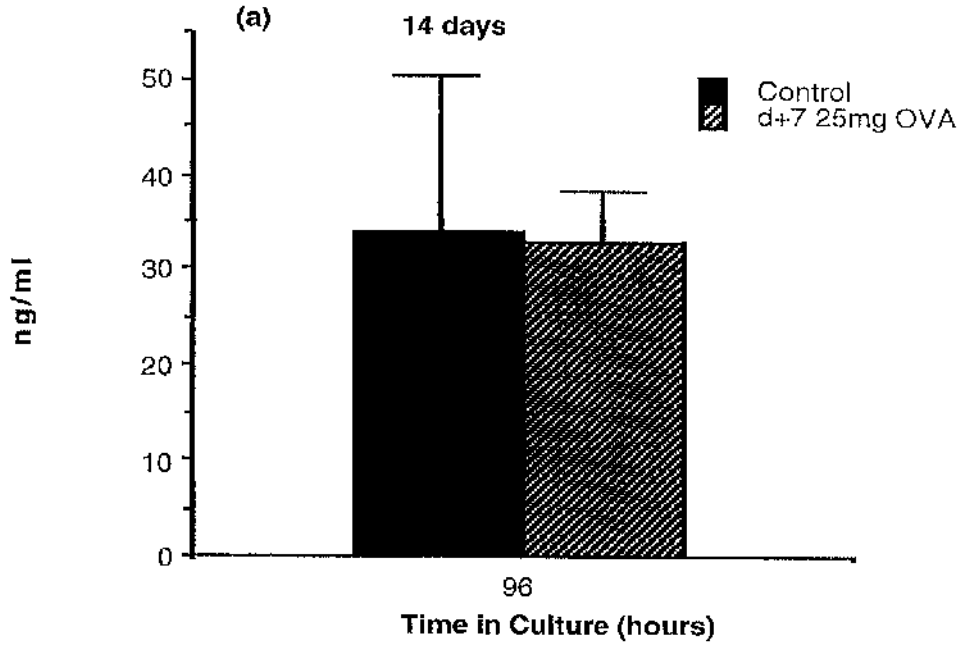
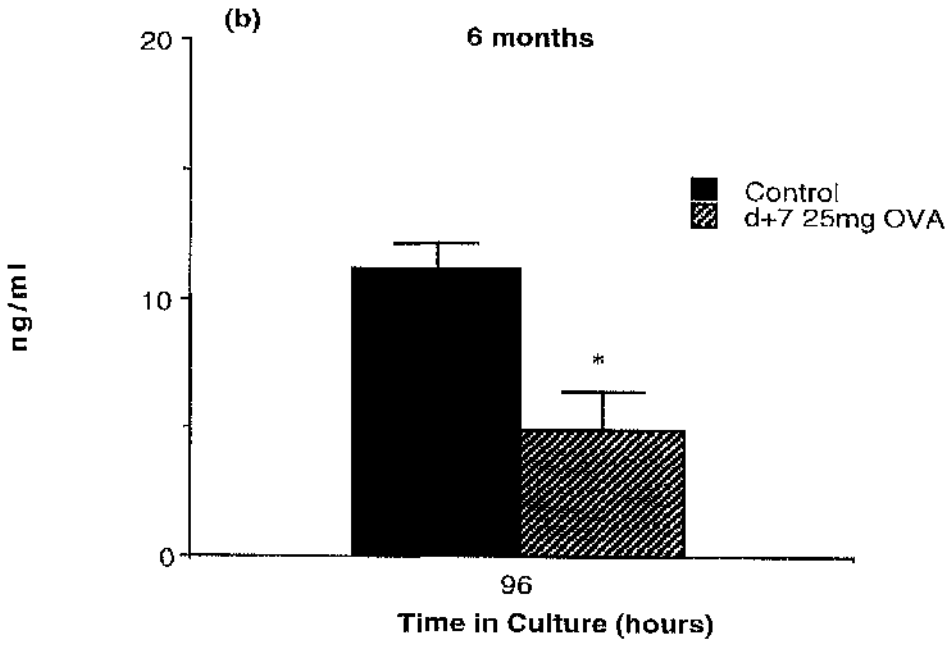


Figure 4.5 Time Course of Oral Tolerance in Primed Mice.

OVA-specific proliferative responses in mice given a single feed of 25mg OVA 7 days after primary immunisation with OVA/CFA assessed 14 days, 6 months or 12 months after primary immunisation. Mice examined at 6 or 12 months received a secondary immunisation 14 days before proliferation response was assessed. The results shown are mean uptake of ^{3}H-TdR ± 1 SD for the last 24 hours of culture in quadruplicate cultures from lymph node cells pooled from 3 mice per group. (*p<0.05 versus controls, **p<0.02 versus controls, ***p<0.005 versus controls)


(a)
14 days
Control
d+7 25mg OVA
50
40
30
20
10
0
ng/ml
96
Time in Culture (hours)


(b)
6 months
Control
d+7 25mg OVA
20
10
0
ng/ml
*
96
Time in Culture (hours)

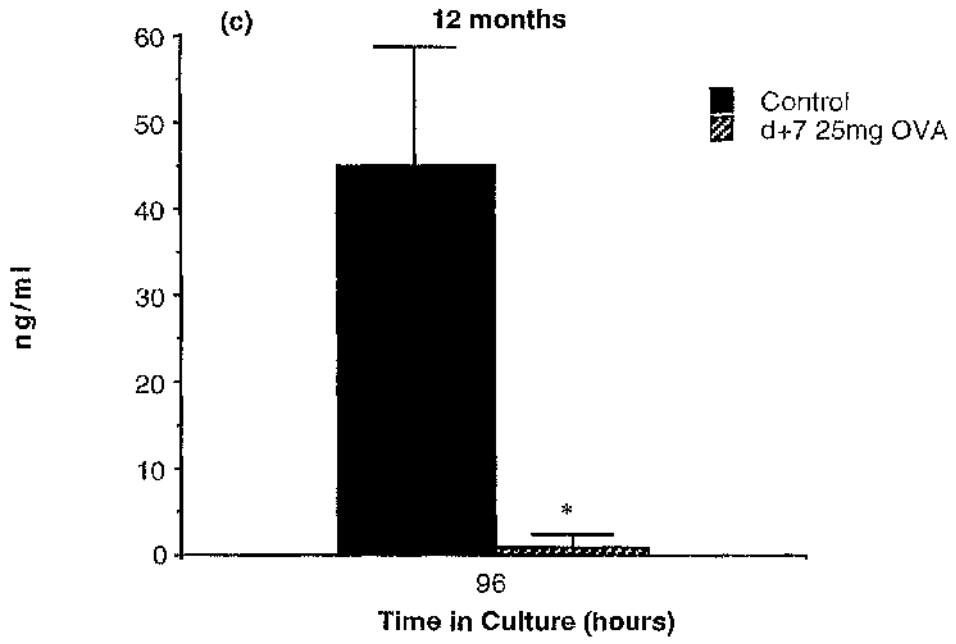
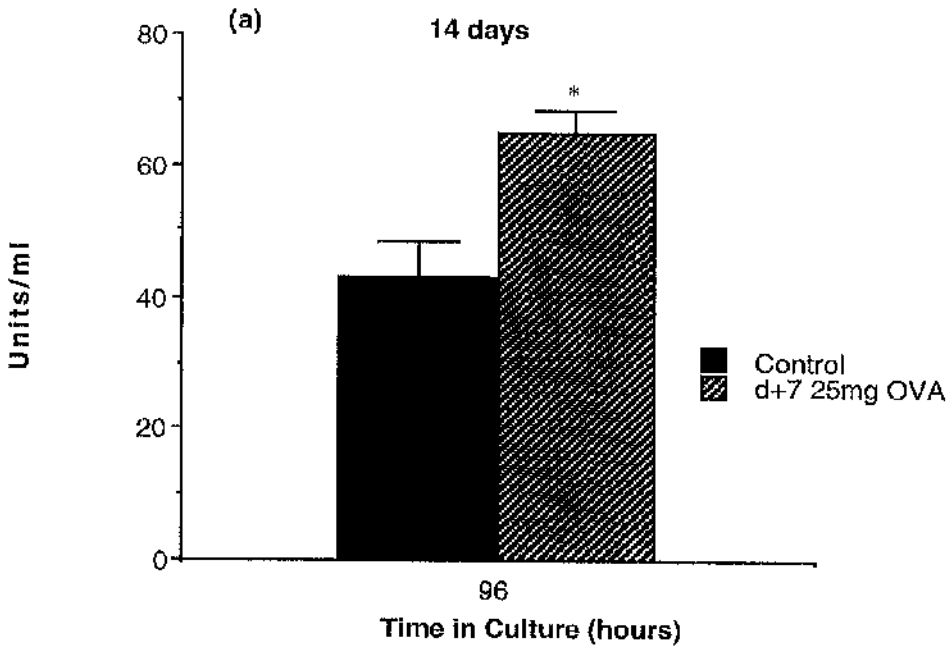
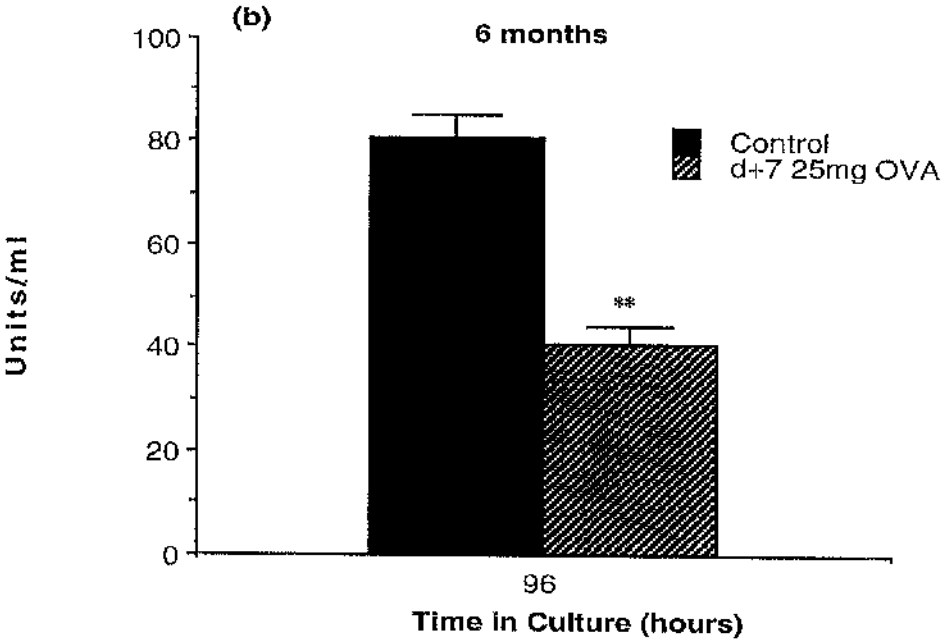


Figure 4.6 Time Course of Oral Tolerance in Primed Mice.

Antigen specific IFNγ responses in mice given a single feed of 25mg OVA 7 days after primary immunisation with OVA/CFA assessed 14 days, 6 months or 12 months after primary immunisation. Mice examined at 6 or 12 months received a secondary immunisation 14 days before IFNγ response was assessed. The results shown are mean cytokine levels (U/ml) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 2-4 days. There was little or no production of IFN-γ from cells cultured in the absence of antigen. (*$p<0.05$ versus controls)


(a)
14 days
80
60
40
20
0
*
Units/ml
Control
d+7 25mg OVA
96
Time in Culture (hours)


(b)
6 months
100
80
60
40
20
0
**
Units/ml
Control
d+7 25mg OVA
96
Time in Culture (hours)

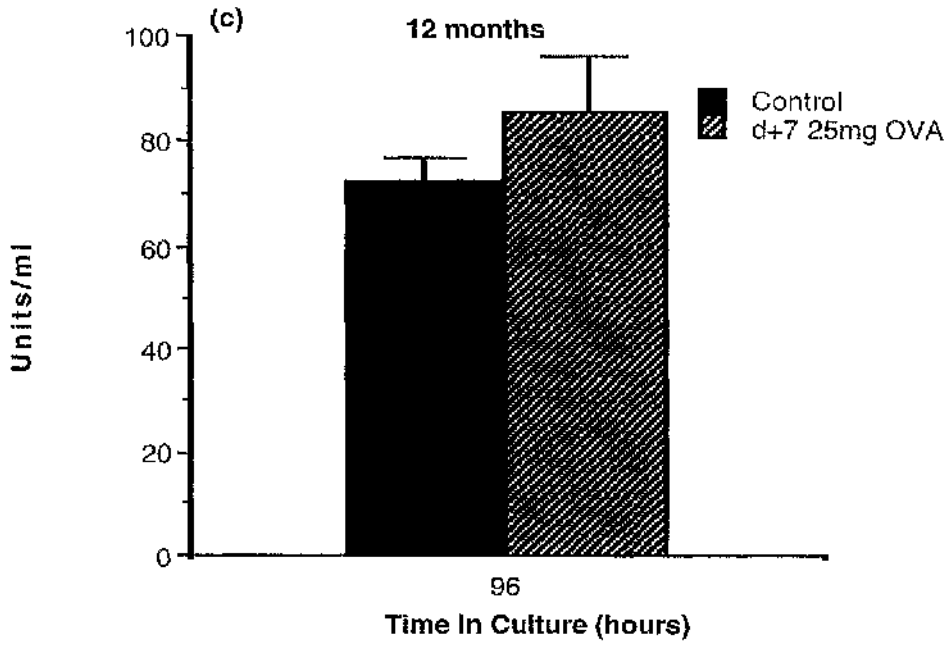


Figure 4.7 Time Course of Oral Tolerance in Primed Mice.

Antigen specific IL5 responses in mice given a single feed of 25mg OVA 7 days after primary immunisation with OVA/CFA assessed 14 days, 6 months or 12 months after primary immunisation. Mice examined at 6 or 12 months received a secondary immunisation 21 days before IL5 response was assessed. The results shown are mean cytokine levels (U/ml) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 2-4 days. There was little or no production of IL-5 from cells cultured in the absence of antigen. (*$p<0.05$ versus controls, **$p<0.001$ versus controls)

Chapter5 The Role of IL4 and IL12 in Oral Tolerance in Primed Mice

5.1 Introduction

The experiments in chapters 3 and 4 showed that feeding antigen after systemic priming results in *in vivo* and *in vitro* tolerance of several aspects of the systemic immune response. In these experiments it was impossible to tolerise antibody responses and in some cases, IL5 production was more difficult to tolerise than IFNγ. This contrasts with the findings in naive mice from our laboratory (57) and could suggest that T_H2 responses are unusually resistant to oral tolerance induction in primed mice. Some workers have reported that T_H2 dependent responses may also be relatively preserved in orally tolerised naive mice and have suggested that IL4 dependent T_H2 cells may act as active suppressor cells under their conditions (188,200). Thus, I decided to investigate whether T_H2 cells were necessary for oral tolerance in primed mice by examining the effects of feeding OVA to $IL4^{-/-}$ mice which had been immunised with OVA/CFA.

A further active regulatory mechanism which has been implicated in oral tolerance is the release of TGFβ by a discrete subset of T cells. This is proposed to operate primarily when oral tolerance has been induced when multiple low doses of antigen are administered to mice (170,190,200,205) and it has been suggested that TGFβ and IFNγ play opposing roles in the regulation of mucosal immune responses (214,215). This idea is supported by the fact that IFNγ dependent gut inflammation caused by TNBS or $CD45RB^{hi}CD4^{+}$ T cells in scid mice can be prevented by TGFβ-secreting T cells (171,172) and by the ability of anti-IL12 to enhance oral tolerance. I therefore decided to investigate if I could exploit this proposed regulatory axis to improve oral tolerance in primed mice, by feeding antigen to mice lacking IL12 ($IL12^{-/-}$). As these mice have deficient production of IFNγ, it would be predicted that this would allow the development of enhanced TGFβ responses and might increase susceptibility to oral tolerance compared with wild type mice. I therefore examined oral

tolerance in naive IL12$^{-/-}$ mice using protocols which might be likely to involve TGFβ-dependent mechanisms.

5.2 Experimental Protocol

IL4$^{-/-}$ mice were challenged s.c. with OVA/CFA on d0 and oral tolerance was induced by feeding mice either 25mg OVA 10 days prior to immunisation or by feeding 2, 25 or 200mg OVA 7 days after immunisation. Control IL4$^{-/-}$ mice were fed saline after immunisation. p40 IL12$^{-/-}$ BALB/c mice and wild type BALB/c mice were challenged s.c. with OVA/CFA on d0 and oral tolerance was induced by feeding mice either a single dose of 2mg OVA 10 days prior to immunisation, or five feeds of 1mg OVA starting 10 days prior to immunisation or by feeding 25mg OVA 7 days after immunisation. In all experiments, OVA specific proliferation and cytokine production was assessed 14 days after immunisation, while antibody was assessed 20 days after immunisation. On the same day, mice were given HAO in the other rear footpad and 24 hours later OVA-specific DTH responses were measured.

5.3 Results

5.3.1 Effects of Feeding OVA Before or After Priming on Subsequent Effector Functions in IL4$^{-/-}$ mice

5.3.1.1 *In vivo* Responses

As I found previously in BALB/c mice (Chapter 3), OVA-specific DTH responses of IL4$^{-/-}$ mice fed OVA before or after priming were significantly lower than those of immunised controls and there was no difference between the different doses of OVA fed after priming (Fig 5.1).

OVA-specific total IgG and IgG2a antibody levels were reduced by feeding 25mg OVA before immunisation of IL4$^{-/-}$ mice but not by any of the doses of OVA fed after priming, except in mice fed 200mg OVA, where there was a significant

reduction in total IgG production (Figs 5.2 and 5.3). As expected, IL4$^{-/-}$ mice did not make any IgG1 antibodies (data not shown). Thus, primed IL4$^{-/-}$ mice show similar patterns of oral tolerance *in vivo* to that found in normal animals.

5.3.1.2 *In vitro* Responses

PLN cells from IL4$^{-/-}$ mice fed 25mg OVA before immunisation had significant tolerance of OVA-specific proliferation and there was dose dependent suppression of these responses in IL4$^{-/-}$ mice fed 2-200mg OVA after systemic immunisation (Fig 5.4), again confirming my findings in normal BALB/c mice.

As expected, PLN cells from unfed IL4$^{-/-}$ mice made substantial levels of OVA-specific IFNγ when restimulated *in vitro*, but produced only low amounts of IL5. As I found in BALB/c mice, IL4$^{-/-}$ mice fed before immunisation had suppressed IFNγ and IL5 responses (Fig 5.5 and 5.6 respectively). However, in primed IL4$^{-/-}$ mice, significant suppression of IFNγ responses was found only after feeding 200mg OVA and not with lower doses (Fig 5.5). Furthermore, in primed IL4$^{-/-}$ mice, significant suppression of the already low IL5 responses was found only after feeding 200mg OVA, but not with lower doses (Fig 5.6). Indeed, mice fed 2 and 25mg OVA had increased IL5 production compared with saline fed controls. Thus the defective T_H2 cytokine production in IL4$^{-/-}$ mice is relatively resistant to induction of oral tolerance in primed animals.

Together these results suggest that the pattern of tolerance in primed IL4$^{-/-}$ mice is generally similar to that in normal animals, although some small differences may exist.

5.3.2 Effects of Feeding OVA Before or After Priming on Subsequent Effector Functions in IL12$^{-/-}$ mice

5.3.2.1 *In vivo* Responses

Wild type BALB/c mice fed either a single low dose of 2mg OVA or 5x1mg OVA before priming had significant tolerance of OVA-specific DTH responses, as did mice fed 25mg OVA after priming, confirming my previous results in Chapter 3 (Fig 5.7). Saline fed IL12$^{-/-}$ mice made good DTH responses and these were tolerised in mice fed 2mg OVA once or 5x1mg OVA before priming. In contrast, IL12$^{-/-}$ mice fed 25mg OVA after priming did not have significant tolerance of DTH responses compared with saline fed controls, although their responses were somewhat lower (Fig 5.7).

Wild type BALB/c mice fed either a single low dose of 2mg OVA or 5x1mg OVA before priming had significant tolerance of OVA-specific total IgG responses, but mice fed 25mg OVA after priming did not (Fig 5.8). Saline fed IL12$^{-/-}$ mice made good OVA-specific total IgG responses and these were tolerised in mice fed 2mg OVA once or 5x1mg OVA before priming. In contrast, IL12$^{-/-}$ mice fed 25mg OVA after priming did not have significant tolerance of OVA-specific total IgG responses compared with saline fed controls (Fig 5.8).

Wild type BALB/c mice fed 5x1mg OVA before priming had significant tolerance of OVA-specific IgG1 responses, but mice fed either a single low dose of 2mg OVA before priming or 25mg OVA after priming did not (Fig 5.9). Saline fed IL12$^{-/-}$ mice made good OVA-specific IgG1 responses and these were tolerised in mice fed 2mg OVA once or 5x1mg OVA before priming, as well as in mice fed 25mg OVA after priming (Fig 5.9).

Wild type BALB/c mice fed 5x1mg OVA before priming had significant tolerance of OVA-specificIgG2a responses, but mice fed either a single low dose of 2mg OVA before priming or 25mg OVA after priming did not (Fig 5.10). Saline fed IL12$^{-/-}$ mice made good OVA-specific IgG2a responses and these were tolerised in

mice fed 2mg OVA. In contrast, IL12$^{-/-}$ mice fed 5x1mg OVA before priming or 25mg OVA after priming did not have significant tolerance of OVA-specific IgG2a responses compared with saline fed controls (Fig 5.10).

5.3.2.2 *In vitro* Responses

Wild type BALB/c mice fed either a single low dose of 2mg OVA or 5x1mg OVA before priming had significant tolerance of OVA-specific proliferative responses. However, in contrast to my previous results, mice fed 25mg OVA after priming did not have significant tolerance (Fig 5.11). Saline fed IL12$^{-/-}$ mice made good OVA-specific proliferation responses and these were tolerised in mice fed 5x1mg OVA before priming and in mice fed 25mg OVA after priming. In contrast, IL12$^{-/-}$ mice fed a single low dose of 2mg OVA before priming did not have significant tolerance of OVA-specific proliferation responses compared with saline fed controls (Fig 5.11).

In contrast to my previous results, wild type mice did not have tolerance of IFNγ production after any regime of feeding OVA before or after priming (Fig 5.12). However, IL5 production was markedly suppressed in mice fed 2mg OVA once or 5x1mg OVA before immmunisation and was also significantly reduced in mice fed after priming (Fig 5.13).

As expected, IL12$^{-/-}$ mice made no OVA-specific IFNγ (Fig 5.12), but had higher IL5 levels than wild type mice (Fig 5.13). These IL5 levels were very markedly suppressed in mice fed before immunisation and were also significantly tolerant in mice fed after priming.

Together, these findings indicate that oral tolerance in naive and primed IL12$^{-/-}$ mice, despite some individual discrepancies, is generally similar to that in equivalent wild type mice.

5.4 Conclusions

The results presented in this chapter confirm previous findings from our laboratory that feeding OVA to naive IL4$^{-/-}$ mice results in oral tolerance. I have also

extended these studies by showing that primed IL4$^{-/-}$ mice develop a pattern of oral tolerance similar to that found in normal animals, with suppression of DTH *in vivo* and antigen-specific proliferation *in vitro*, as well as some suppression of IFNγ and IL5 production *in vitro*, but no effects on serum antibodies. Thus the partial effects of oral tolerance in primed mice do not depend on the presence of IL4 dependent T_H2 cells, but could reflect another mechanism such as clonal anergy/deletion or an alternative active suppressor mechanism.

An alternative active suppressor mechanism that I studied in this chapter was TGFβ. To do this, I made use of the suggestion that TGFβ production is downregulated by IL12 and IFNγ and examined the induction of tolerance in IL12$^{-/-}$ mice. Certain aspects of tolerance were enhanced in IL12$^{-/-}$ mice compared with wild type mice, namely IgG1 antibody levels. However, other components of tolerance such as total OVA-specific IgG antibody levels were normal and tolerance of OVA-specific DTH levels were reduced, and no clear pattern emerged. Therefore, it is probably true to conclude that the absence of IL12 has no overall effect on the induction of tolerance induced either before or after immunisation, with no reprodcible enhancement of tolerance in primed animals. However, these experiments need to be repeated.

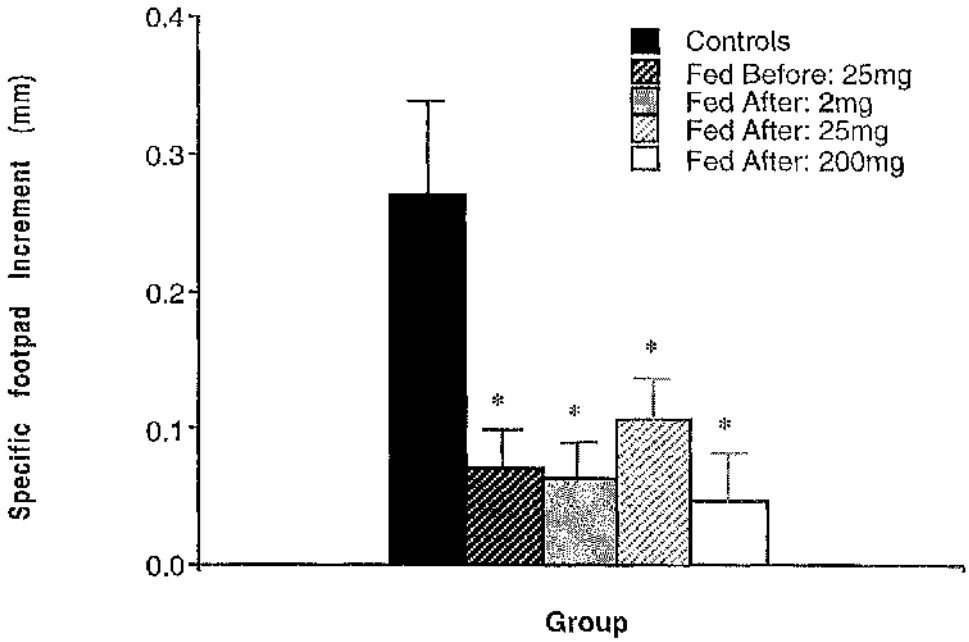


Figure 5.1 Induction of Oral Tolerance by Feeding Ovalbumin Before or After Immunisation in IL4$^{-/-}$ Mice.

OVA-specific DTH responses in mice given a single feed of 25mg OVA 10 days prior to immunisation or of 2-200mg OVA 7 days after immunisation with OVA/CFA. The results shown are mean specific increments in footpad thickness ± 1 SD for 5 mice per group. (*$p<0.002$ versus controls)

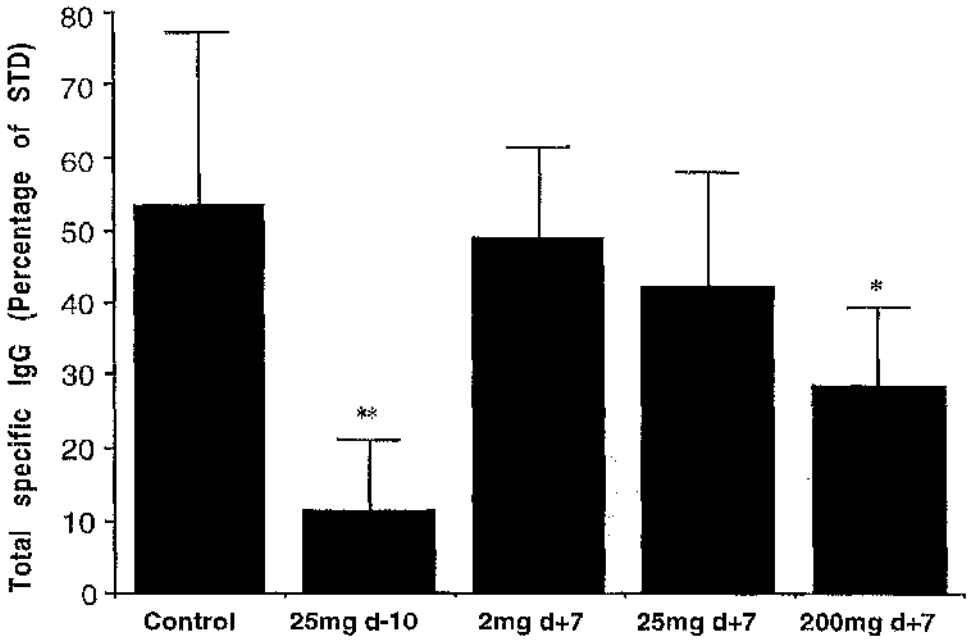


Figure 5.2 Induction of Oral Tolerance by Feeding Ovalbumin Before or After Immunisation in IL4$^{-/-}$ Mice.

Total serum IgG antibody levels in mice given a single feed of 25mg OVA 10 days prior to immunisation or of 2-200mg OVA 7 days after immunisation with OVA/CFA. The results shown are mean % of purified IgG standard ± 1 SD for individual sera from 5 mice per group. (*$p<0.05$ versus controls, **$p<0.02$ versus controls)

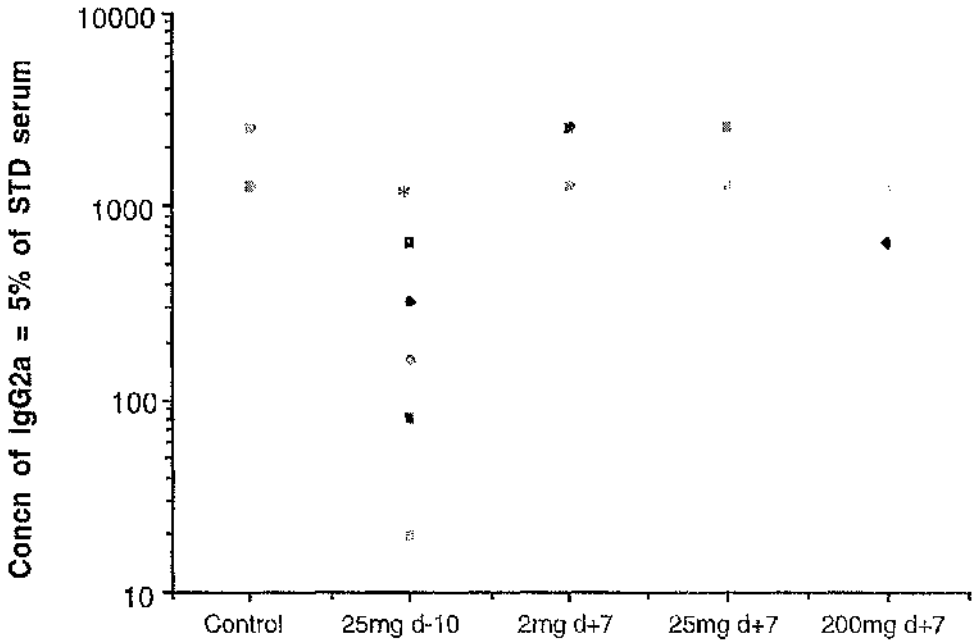


Figure 5.3 Induction of Oral Tolerance by Feeding Ovalbumin Before or After Immunisation in IL4$^{-/-}$ Mice.

Total serum IgG2a antibody levels in mice given a single feed of 25mg OVA 10 days prior to immunisation or of 2-200mg OVA 7 days after immunisation with OVA/CFA. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group and the reciprocal dilutions were done in duplicate. (*$p<0.05$ versus controls)

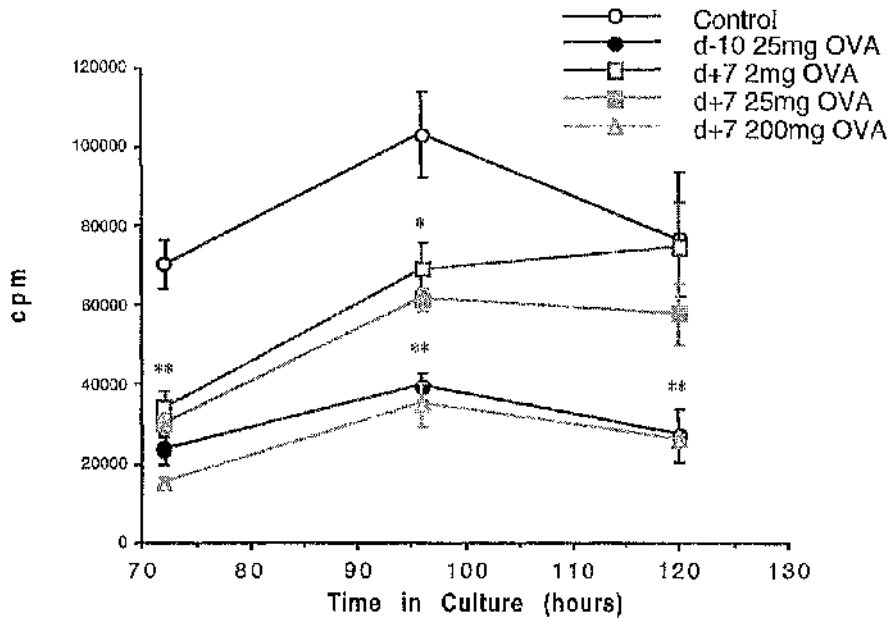


Figure 5.4 Induction of Oral Tolerance by Feeding Ovalbumin Before or After Immunisation in IL4$^{-/-}$ Mice.

OVA-specific proliferation response in mice given a single feed of 25mg OVA10 days prior to immunisation or of 2-200mg OVA 7 days after immunisation with OVA/CFA. The results shown are mean uptake of ^{3}H-TdR ± 1 SD for the last 24 hours of culture in quadruplicate cultures from lymph node cells pooled from 3 mice per group. (*p<0.002 versus controls,**p<0.001 versus controls)

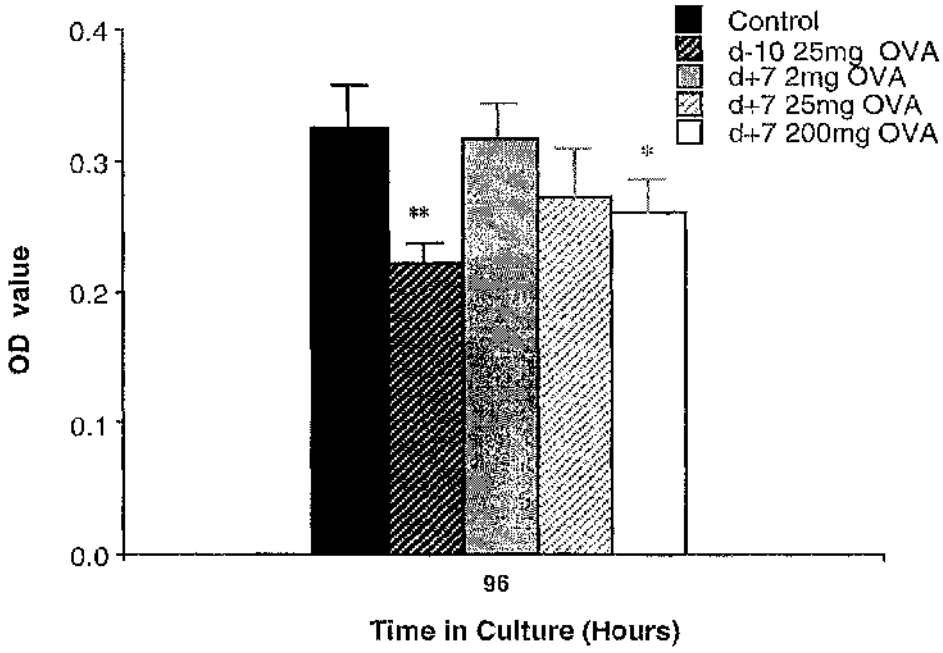


Figure 5.5 Induction of Oral Tolerance by Feeding Ovalbumin Before or After Immunisation in IL4$^{-/-}$ Mice.

OVA-specific IFNγ production in mice given a single feed of 25mg OVA 10 days prior to immunisation or of 2-200mg OVA 7 days after immunisation with OVA/CFA. The results shown are mean IFNγ level (OD value) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 4 days. (*$p<0.05$ versus controls,**$p<0.01$ versus controls)

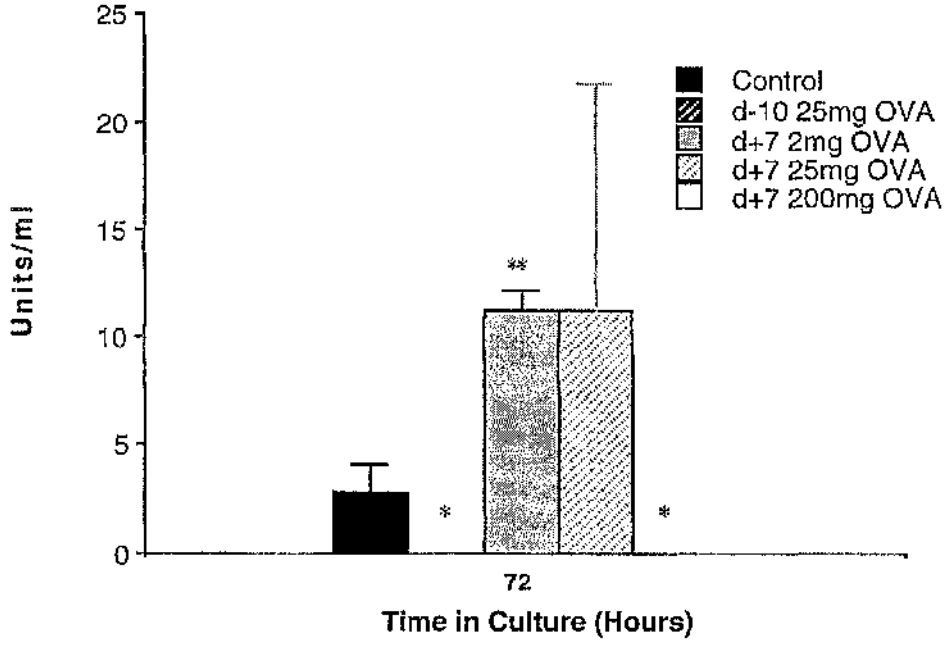


Figure 5.6 Induction of Oral Tolerance by Feeding Ovalbumin Before or After Immunisation in IL4$^{-/-}$ Mice.

OVA-specific IL5 production in mice given a single feed of 25mg OVA 10 days prior to immunisation or of 2-200mg OVA 7 days after immunisation with OVA/CFA. The results shown are mean IL5 level (U/ml) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 3 days. (*$p<0.05$ versus controls, **$p<0.002$ versus controls)

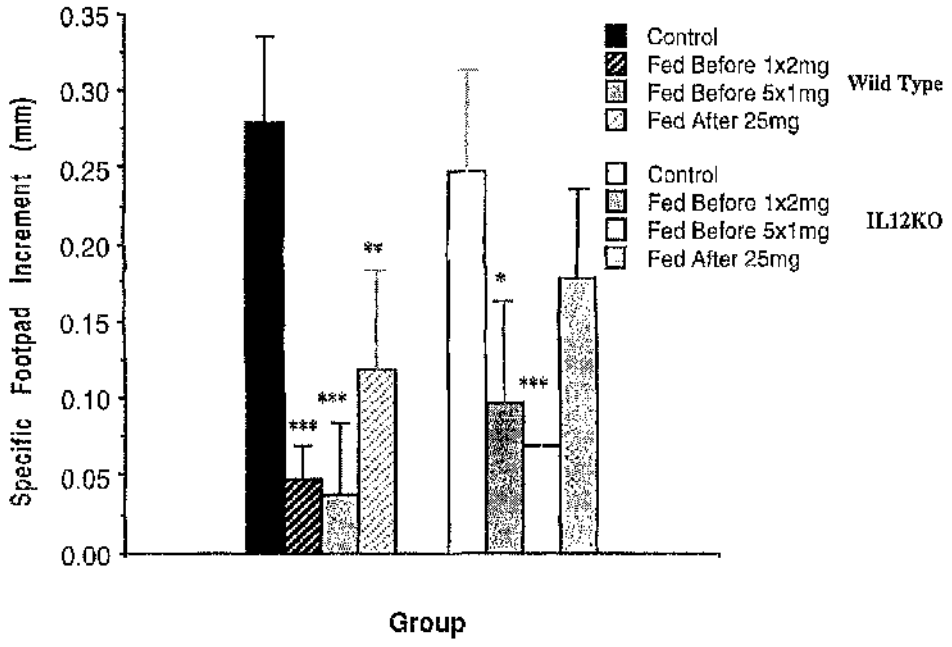


Figure 5.7 Induction of Oral Tolerance by Feeding Ovalbumin Before or After Immunisation in IL12$^{-/-}$ Mice.

OVA-specific DTH responses in wild type and IL12$^{-/-}$ BALB/c mice fed either a single dose of 2mg OVA 10 days prior to immunisation, or five feeds of 1mg OVA starting 10 days prior to immunisation or fed 25mg OVA 7 days after immunisation with OVA/CFA. Control mice were fed saline after immunisation. The results shown are mean specific increments in footpad thickness ± 1 SD for 5 mice per group. (*$p<0.01$ versus respective saline fed control, **$p<0.005$ versus respective saline fed control, ***$p<0.001$ versus respective saline fed control)

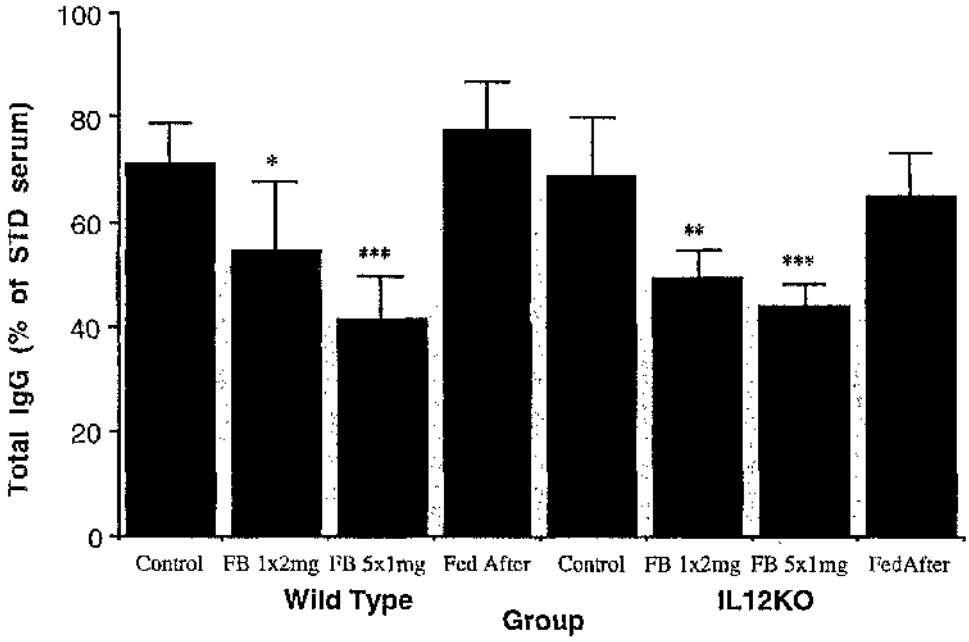


Figure 5.8 Induction of Oral Tolerance by Feeding Ovalbumin Before or After Immunisation in IL12$^{-/-}$ Mice.

Total serum IgG antibody levels in wild type and IL12$^{-/-}$ BALB/c mice fed either a single dose of 2mg OVA 10 days prior to immunisation, or five feeds of 1mg OVA starting 10 days prior to immunisation or fed 25mg OVA 7 days after immunisation with OVA/CFA. Control mice were fed saline after immunisation. The results shown are mean % of purified IgG standard ± 1 SD for individual sera from 5 mice per group. (*$p<0.05$ versus respective saline fed control, **$p<0.01$ versus respective saline fed control, ***$p<0.002$ versus respective saline fed control)

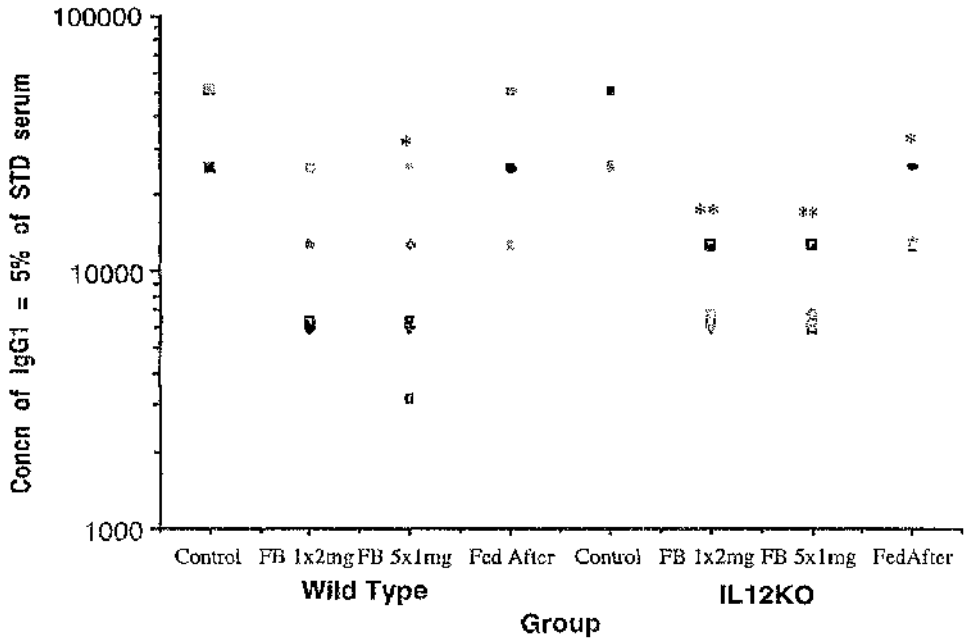


Figure 5.9 Induction of Oral Tolerance by Feeding Ovalbumin Before or After Immunisation in IL12$^{-/-}$ Mice.

Total serum IgG1 antibody levels in wild type and IL12$^{-/-}$ BALB/c mice fed either a single dose of 2mg OVA 10 days prior to immunisation, or five feeds of 1mg OVA starting 10 days prior to immunisation or fed 25mg OVA 7 days after immunisation with OVA/CFA. Control mice were fed saline after immunisation. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group and the reciprocal dilutions were done in duplicate. (*$p<0.05$ versus respective saline fed control, **$p<0.01$ versus respective saline fed control)

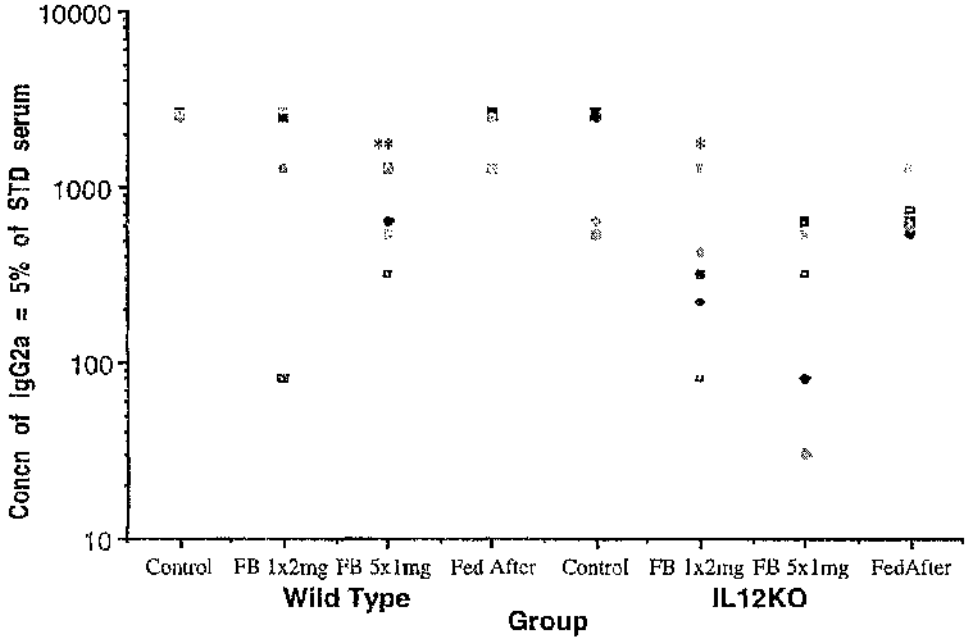


Figure 5.10 Induction of Oral Tolerance by Feeding Ovalbumin Before or After Immunisation in IL12$^{-/-}$ Mice.

Total serum IgG2a antibody levels in wild type and IL12$^{-/-}$ BALB/c mice fed either a single dose of 2mg OVA 10 days prior to immunisation, or five feeds of 1mg OVA starting 10 days prior to immunisation or fed 25mg OVA 7 days after immunisation with OVA/CFA. Control mice were fed saline after immunisation. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group and the reciprocal dilutions were done in duplicate. (*$p<0.05$ versus respective saline fed control, **$p<0.01$ versus respective saline fed control)

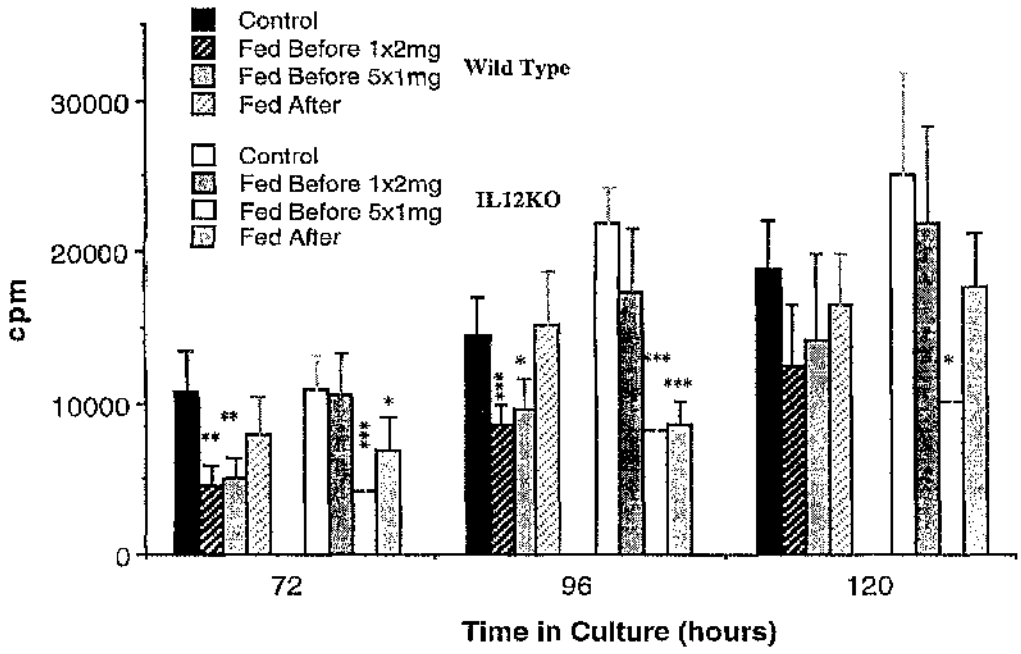


Figure 5.11 Induction of Oral Tolerance by Feeding Ovalbumin Before or After Immunisation in IL12$^{-/-}$ Mice.

OVA-specific proliferative responses in wild type and IL12$^{-/-}$ BALB/c mice fed either a single dose of 2mg OVA 10 days prior to immunisation, or five feeds of 1mg OVA starting 10 days prior to immunisation or fed 25mg OVA 7 days after immunisation with OVA/CFA. Control mice were fed saline after immunisation. The results shown are mean uptake of ^{3}H-TdR ± 1 SD for the last 24 hours of culture in quadruplicate cultures from lymph node cells pooled from 3 mice per group. (*$p<0.05$ versus respective saline fed control,**$p<0.01$ versus respective saline fed control, ***$p<0.005$ versus respective saline fed control)

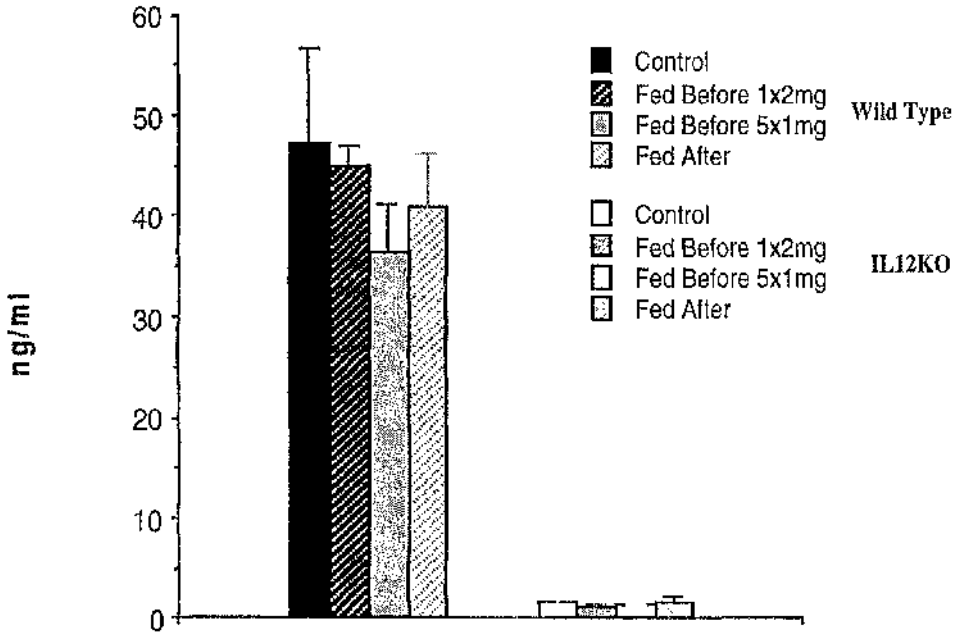


Figure 5.12 Induction of Oral Tolerance by Feeding Ovalbumin Before or After Immunisation in IL12$^{-/-}$ Mice.

OVA-specific IFNγ production in wild type and IL12$^{-/-}$ BALB/c mice fed either a single dose of 2mg OVA 10 days prior to immunisation, or five feeds of 1mg OVA starting 10 days prior to immunisation or fed 25mg OVA 7 days after immunisation with OVA/CFA. Control mice were fed saline after immunisation. The results shown are mean IFNγ level (OD value) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 4 days.

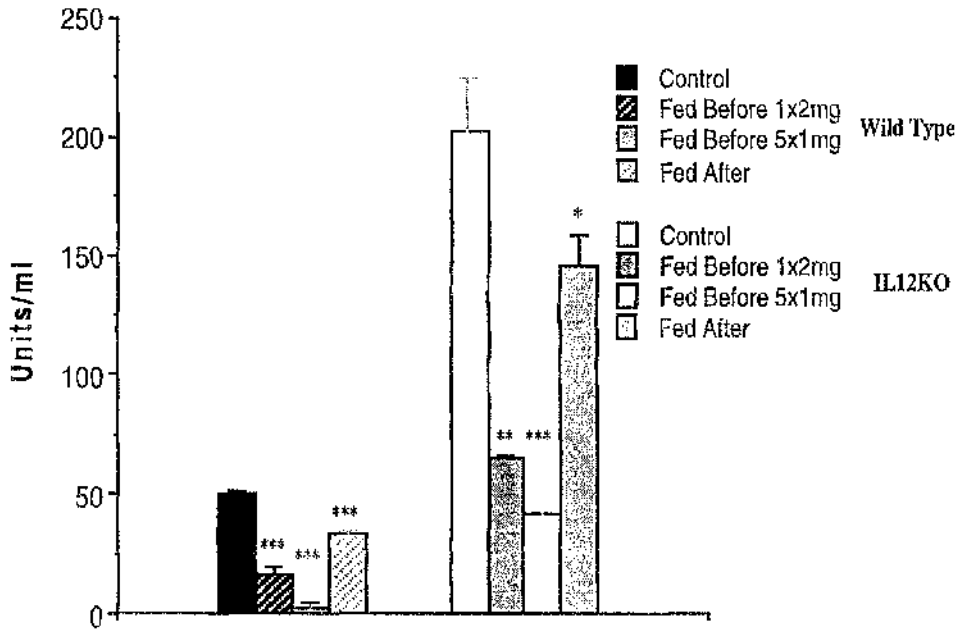


Figure 5.13 Induction of Oral Tolerance by Feeding Ovalbumin Before or After Immunisation in IL12$^{-/-}$ Mice.

OVA-specific IL5 production in wild type and IL12$^{-/-}$ BALB/c mice fed either a single dose of 2mg OVA 10 days prior to immunisation, or five feeds of 1mg OVA starting 10 days prior to immunisation or fed 25mg OVA 7 days after immunisation with OVA/CFA. Control mice were fed saline after immunisation. The results shown are mean IL5 level (U/ml) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 3 days. (*p<0.02 versus respective saline fed control, **p<0.002 versus respective saline fed control, ***p<0.001 versus respective saline fed control)

Chapter 6 Effect of Flt3L in Oral Tolerance in Primed Mice

6.1 Introduction

My experiments thus far have shown that feeding OVA to systemically primed mice results in *in vivo* and *in vitro* tolerance. However, the oral tolerance in primed mice is not as profound or wide ranging as that found when equivalent amounts of antigen are fed to naive mice. This is particularly manifested in the antibody response which I have found to be generally resistant to tolerance induction. In Chapter 5, I showed that this pattern of tolerance was not influenced by the absence of IL4 or upregulation of TGFβ in the absence of IL12 and therefore I was interested in finding an alternative strategy for enhancing tolerance in primed animals.

The haemopoetic growth factor Flt3ligand (FLT3L) increases the numbers of dendritic cells when it is given *in vivo* and recent work has shown that administration of Flt3L to mice enhances the induction of oral tolerance in naive mice (114). This affected all aspects of the immune responses and allowed normally non-tolerogenic doses of OVA to induce significant tolerance. The rationale for this effect appears to be that Flt3L expands the numbers of resting dendritic cells in the gut which then present fed antigen without costimulation to T cells, resulting in more profound anergy and/or clonal deletion, and thus function of tolerance. Thus, I decided to test the idea that expansion of DCs by Flt3L might also extend the scope of oral tolerance in primed mice.

6.2 Experimental Protocol

In the first experiment, BALB/c mice mice were challenged s.c. with OVA/CFA on d0 and oral tolerance was induced by feeding mice 25mg OVA 8 days after immunisation. Control mice were fed saline after immunisation. 10μg Flt3L saline was administered i.p. from d1 until d9 with saline being injected daily as a control. 14 days after immunisation PLN were taken and Ag specific proliferation and cytokine production was assessed. 20 days after immunisation mice were bled and

serum assessed for antibody levels. On the same day mice were given HAO in the other rear footpad and 24 hours later OVA-specific DTH responses were measured.

In separate experiments, BALB/c mice mice were challenged s.c. with OVA/CFA on d0 and oral tolerance was induced by feeding mice 25mg OVA 5 days after immunisation with 10μg Flt3L being administered from d-2 until d6. PLN were taken from d-1 to day 15, while DTH and serum antibodies were assessed as usual.

6.3 Results

6.3.1 Effects of Flt3L on Oral Tolerance in Primed mice

6.3.1.1 *In Vivo* Responses

OVA-specific DTH responses of mice given saline ip and fed OVA 8 days after priming were significantly lower than those of equivalent saline fed controls (Fig 6.1). DTH responses in Flt3L treated mice were difficult to interpret due to the fact that saline fed mice given Flt3L had very low DTH responses compared with equivalent saline treated controls. OVA fed, Flt3L treated mice had DTH responses that were not significantly different.

There was no tolerance of total IgG, IgG1 or IgG2a antibodies in saline treated mice fed OVA, consistent with my previous results. Administration of Flt3L revealed significant tolerance of total IgG production in OVA fed mice. However, there was still no tolerance of either IgG1 or IgG2a in Flt3L treated OVA fed mice. No effect of Flt3L on humoral immunity was seen in saline fed mice.

The total serum levels of OVA-specific IgG were only significantly reduced by feeding 25mg OVA after immunisation in the Flt3L treated mice (Fig 6.2). However, the OVA-specific IgG1 and IgG2a responses of mice fed OVA in the Flt3L treated groups were not reduced (Fig 6.3 and 6.4 respectively). In keeping with previous results in BALB/c mice, the antibody responses of mice fed after priming in the i.p. saline treated groups were not tolerised.

6.3.1.2 *In Vitro* Responses

As before, saline treated mice fed 25mg OVA after immunisation had significant tolerance of proliferative responses *in vitro* (Fig 6.5). There was also significant tolerance of proliferative responses in OVA fed, Flt3L treated mice compared with the appropriate controls, despite the fact that Flt3L markedly suppressed the response in saline fed controls.

Saline treated mice fed OVA after immunisation had no tolerance of IFNγ responses compared with controls, supporting my previous findings when primed mice were fed OVA 8 days after immunisation (Fig6.6). Flt3L had no significant effect on IFNγ responses in saline fed mice and did not reveal tolerance after feeding OVA. Significant tolerance of IL5 production in both saline treated mice fed OVA, while both groups of Flt3L treated mice had very low IL5 production irrespective of whether they were fed OVA or not(Fig 6.7).

The fact that Flt3L appeared to suppress systemic immune responses to OVA/CFA meant that it was difficult to determine the influence of this treatment on tolerance. Such effects of Flt3L have not been described previously and somewhat surprisingly given the fact that Flt3L expands dendritic cells, a procedure which one might anticipate would increase immunity. One possible explanation for my findings was that the systemic immune responses peaked more rapidly in Flt3L treated mice and to test this idea, I decided to reduce the length of the experiment by feeding mice sooner after immunisation. In addition, I assessed the time course of the developing immune response in OVA fed and control mice given Flt3L.

6.3.2 Effects of Flt3L on Early Induction of Oral Tolerance in Primed Mice.

In these experiments, mice were fed OVA 5 days after immunisation and given Flt3L or saline ip from d-2 to d+6.

6.3.2.1 *In Vivo* Responses

As I had found previously, OVA-specific DTH responses of mice given saline ip and fed OVA 5 days after priming were significantly lower than those of equivalent saline fed controls (Fig 6.8). Unlike the previous experiment, DTH responses in Flt3L treated mice were okay to interpret due to the fact that saline fed mice given Flt3L had DTH responses comparable with equivalent saline treated controls. OVA fed, Flt3L treated mice had DTH responses that were not significantly reduced compared to their saline fed controls.

As for the DTH responses, the total serum levels of OVA-specific IgG were significantly reduced by feeding 25mg OVA after immunisation in both the saline and the Flt3L treated mice (Fig 6.9). A significant reduction in serum IgG caused by feeding OVA to saline treated mice was surprising since I do not normally find that feeding 25mgOVA 5 days after priming significantly reduces serum IgG. Administration of Flt3L slightly enhanced the OVA-specific IgG tolerance at this time-point. Consistent with my previous findings, feeding OVA 5days after priming to saline treated mice did not significantly reduce IgG1 or IgG2a production compared with saline fed controls. However, OVA-specific IgG2a, but not IgG1, responses of mice fed OVA in the Flt3L treated groups were significantly reduced (Fig 6.10 and 6.11 respectively). These results do suggest that administration of Flt3L in this experiment did enhance oral tolerance induction compared with saline treated controls.

6.3.2.2 *In Vitro* Responses

Saline treated mice fed OVA after priming had significant tolerance of OVA-specific proliferation responses compared with controls when examined at both 8 (Fig 6.12a) and 14 days after immunisation, when the systemic response was at its peak (Fig 6.12b). Flt3L treated mice fed saline had normal proliferative responses 8 days after immunisation, but by 14 days, the responses of these animals were significantly reduced compared with saline treated controls. On day 8, OVA fed Flt3L treated mice

had much higher proliferative responses compared with saline fed mice. On day 14, the two groups had identical responses.

Saline treated mice fed OVA after priming had significant tolerance of OVA-specific IFNγ production compared with controls when examined 14 (Fig 6.13b) but not 8 days after immunisation (Fig 6.13a). Saline treated mice that were fed saline produced equivalent levels of IFNγ when compared with mice that were Flt3L treated and fed saline at both 8 and 14 days after immunistion. The production of IFNγ appeared to be slightly greater 8 days compared with 14 days after immunisation thus suggesting that the peak of the IFNγ production was 8 days after immunisation no matter whether mice were treated with saline or Flt3L. Flt3L treated mice fed OVA had significant tolerance of OVA-specific IFNγ production both 8 and 14 days after immunisation. This suggests that treatment with Flt3L enhanced IFNγ tolerance.

Saline treated mice fed OVA after priming did not have significant tolerance of OVA-specific IL5 production compared with controls when examined at both 8 (Fig 6.14a) and 14 days after immunisation (Fig 6.14b) which is in keeping with previous findings that IFNγ responses are easier to tolerise than IL5 responses. Saline treated mice that were fed saline produced much reduced levels of IL5 when compared with mice that were Flt3L treated and fed saline at both 8 and 14 days after immunistion. As was found for IFNγ production in the Flt3L treated mice, IL5 production was greater 8 days compared with 14 days after immunisation suggesting that the peak of the IL5 production was 8 days after immunisation. Flt3L treated mice fed OVA had significant tolerance of OVA-specific IL5 production both 8 and 14 days after immunisation. As was found with IFNγ production, this suggests that treatment with Flt3L enhanced IL5 tolerance.

These results show that administration of Flt3L to mice can enhance oral tolerance induced in mice fed OVA after immunisation compared with mice treated with saline, in terms of IFNγ and IL5 production. In view of this significant tolerance observed in Flt3L treated mice fed OVA it is somewhat surprising that these mice had very high proliferative responses 8 days after immunisation even when compared with

saline treated mice that were fed saline. This may, however, suggest that these cells from mice fed OVA and treated with Flt3L are undergoing some form of activation and expansion before being deleted in a manner similar to that described for AICD. This might imply that Flt3L may enhance oral tolerance induced when antigen is fed after immunisation.

6.3.3 Kinetics of Oral Tolerance Development in Flt3L Treated Primed Mice

As the results of the previous experiments suggested that the effects of Flt3L might depend on the time immune responsiveness was assessed, I carried out a more detailed time course of proliferative responses in primed mice fed OVA and treated with Flt3L or saline. In this experiment, mice were treated with saline or Flt3L ip from d-2 until d+6. Mice were fed saline or OVA on d+5 and lymph nodes were removed 4, 6, 8, 13 and 20 days after immunisation.

Four days after immunisation (1 day before feeding saline) both saline fed mice treated with saline or Flt3L had similar OVA-specific proliferation responses (Fig 6.15). Six days after immunisation, mice treated with saline and fed saline or OVA both had similar proliferative responses. However, eight days after immunisation which appeared to be the peak of the response, mice fed OVA and treated with saline had significantly reduced proliferation responses when compared with the equivalent mice fed saline. Thirteen days after immunisation mice fed OVA and treated with saline had more significantly reduced proliferation responses, compared to that found eight days after immunisation, than the appropriate mice fed saline . The level of suppression at this timepoint was the same as found in previous experiments. Twenty days after immunisation mice fed OVA and treated with saline only had slightly suppressed proliferation responses when compared to saline fed mice treated with saline. At all the time points looked at it did not appear that Flt3L increased or reduced proliferation responses when the responses of mice fed saline were compared.

Six days after immunisation there was a significant increase in the proliferation response of cells taken from mice fed OVA and treated with Flt3L when compared with cells from mice fed saline and treated with Flt3L. This appeared to be the peak of the proliferation response of cells from both groups of mice. As this is two days earlier than those cells taken from mice treated with saline these results further suggest that Flt3L increases the kinetics of the immune response to immunisation with OVA/CFA. By eight days after immunisation the proliferation response of cells taken from mice fed saline or OVA and treated with Flt3L were exactly the same. However, 13 days after immunisation, the proliferation response of cells taken from mice fed OVA were significantly reduced when compared with mice fed saline and also treated with Flt3L. This tolerance was shortlived however, as by 20 days after immunisation the proliferation response of mice fed OVA were not significantly different when compared with appropriate mice fed saline.

These results confirm the results of the previous experiment in this chapter in that treating mice with Flt3L slightly alters the kinetics of the response compared with saline treated controls. This is because it would appear that the peak of the proliferation response of Flt3L treated mice is achieved slightly more quickly than saline treated mice. Further, this experiment shows that feeding OVA to Flt3L treated mice results in a burst of proliferation early after feeding which then results in a significantly reduced proliferation response later after immunisation when compared with saline fed, Flt3L treated control mice. This was also found in the previous experiment.

6.4 Conclusions

Results show that expanding DCs with Flt3L does not interfere with the induction of oral tolerance in primed mice, confirming previous results in naive animals. Flt3L actually enhanced the induction of tolerance in some instances and occasionally allowed tolerance of some responses not normally susceptible to tolerance, such as antibody production. However, the effects of Flt3L were quite

variable, probably reflecting the influence that administration of Flt3L had on the level and the speed of the systemic immune responses after challenge with OVA/CFA. It proved difficult to determine precisely the best time course and protocol for examining the effects of Flt3L on oral tolerance in primed mice and I did not have time to extend these studies to obtain definitive results.

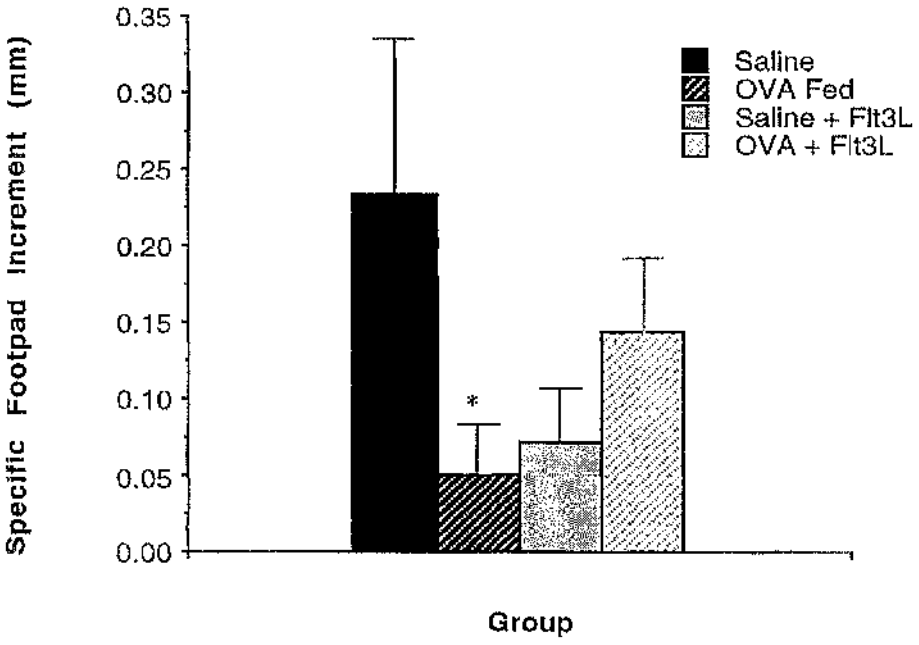


Figure 6.1 Effects of Flt3L on the Induction of Oral Tolerance in Primed Mice.

Systemic DTH responses in mice given a single feed of 25mg OVA 8 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. Mice were treated with saline or 10μg Flt3L daily from d1 until d9. The results shown are mean specific increments in footpad thickness ± 1 SD for 5 mice per group. (*$p<0.05$ versus appropriate saline fed control)

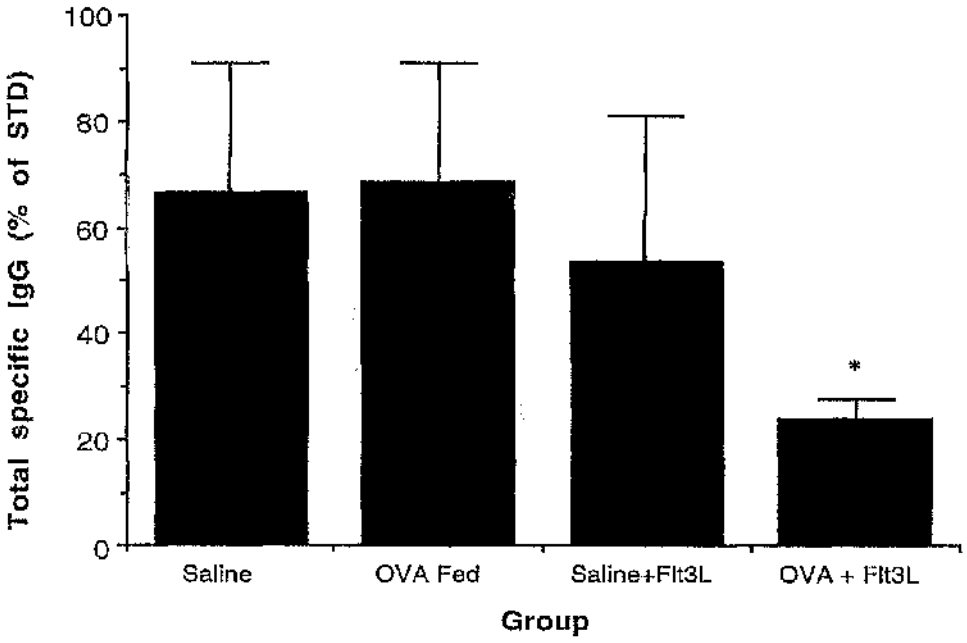


Figure 6.2 Effects of Flt3L on the Induction of Oral Tolerance in Primed Mice.

OVA-specific total serum IgG antibody responses in mice given a single feed of 25mg OVA 8 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. Mice were treated with saline or 10μg Flt3L daily from d1 until d9. The results shown are mean % of purified IgG standard ± 1 SD for individual sera from 5 mice per group. (*$p<0.05$ versus appropriate saline fed control)

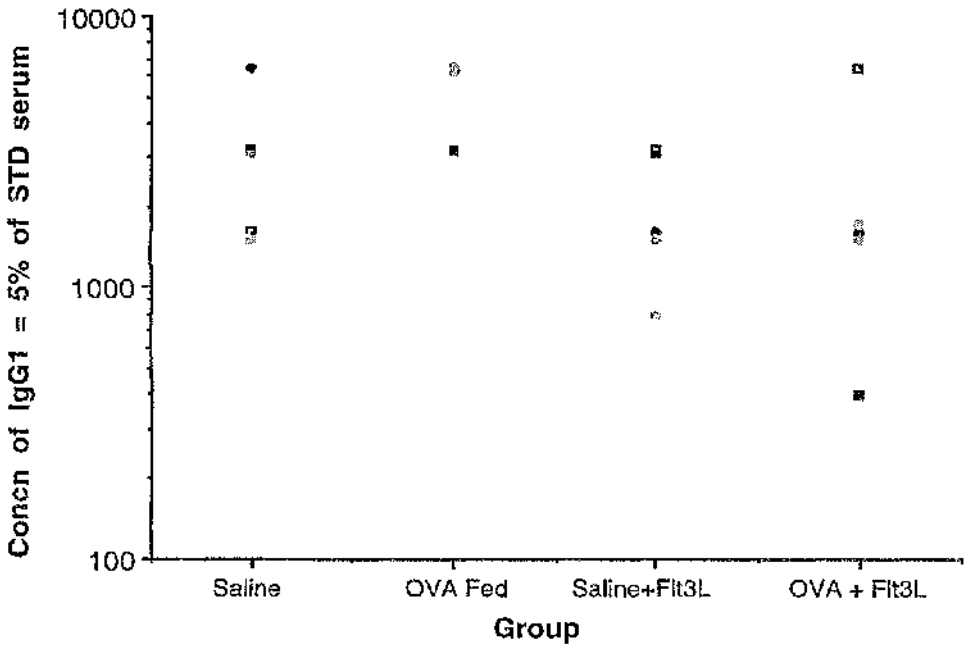


Figure 6.3 Effects of Flt3L on the Induction of Oral Tolerance in Primed Mice.

OVA-specific serum IgG1 antibody responses in mice given a single feed of 25mg OVA 8 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. Mice were treated with saline or 10μg Flt3L daily from d1 until d9. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group.

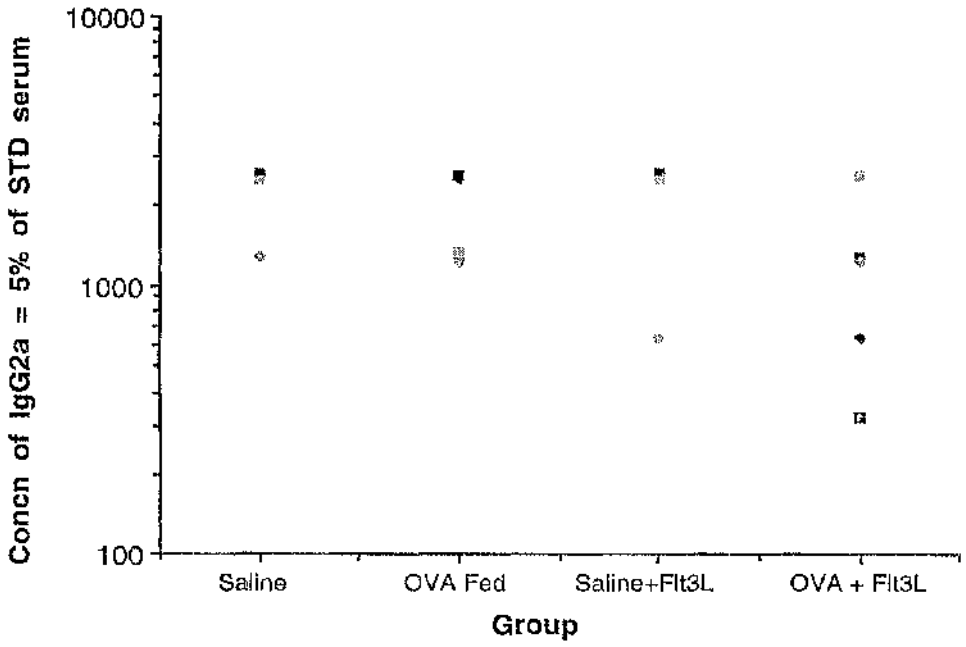


Figure 6.4 Effects of Flt3L on the Induction of Oral Tolerance in Primed Mice.

OVA-specific serum IgG2a antibody responses in mice given a single feed of 25mg OVA 8 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. Mice were treated with saline or 10µg Flt3L daily from d1 until d9. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group.

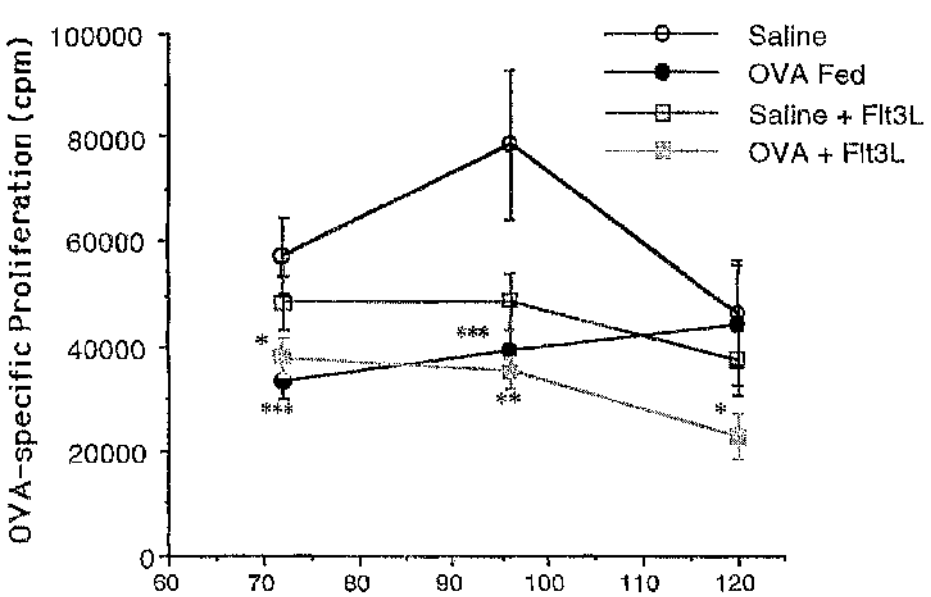


Figure 6.5 Effects of Flt3L on the Induction of Oral Tolerance in Primed Mice.

OVA-specific proliferative responses in draining lymph nodes of mice given a single feed of 25mg OVA 8 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 14 days after immunisation. Mice were treated with saline or 10μg Flt3L daily from d1 until d9. The results shown are mean uptake of ^{3}H-TdR ± 1 SD for the last 24 hours of culture in quadruplicate cultures from lymph node cells pooled from 3 mice per group. (*p<0.01 versus appropriate saline fed control **p<0.005 versus appropriate saline fed control, ***p<0.002 versus appropriate saline fed control)

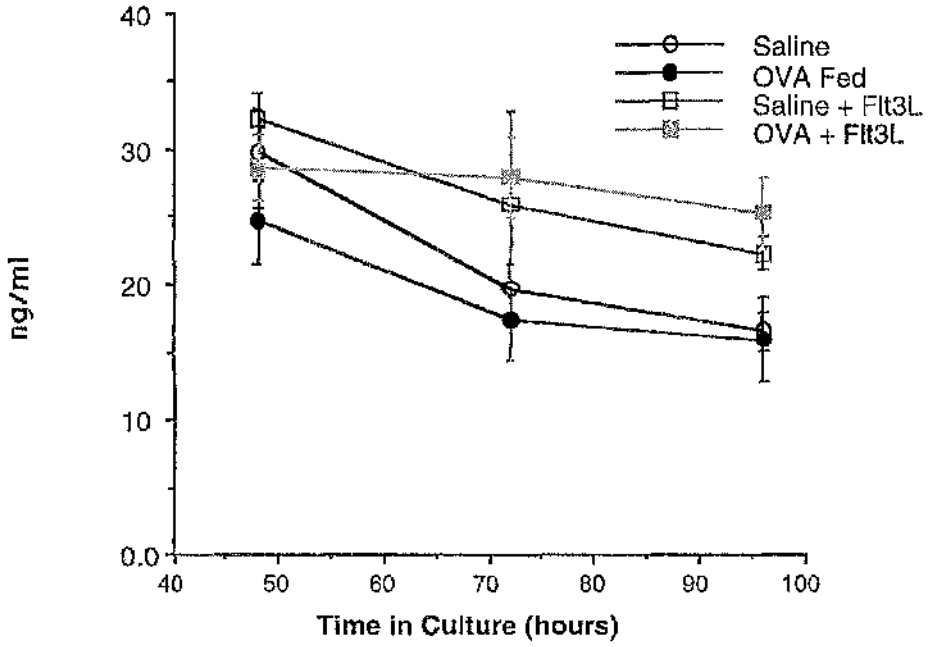


Figure 6.6 Effects of Flt3L on the Induction of Oral Tolerance in Primed Mice.

OVA-specific IFNγ production in draining lymph nodes of mice given a single feed of 25mg OVA 8 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 14 days after immunisation. Mice were treated with saline or 10μg Flt3L daily from d1 until d9. The results shown are mean IFNγ level (ng/ml) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 2-4 days. There was little or no production of IFNγ from cells cultured in the absence of antigen.

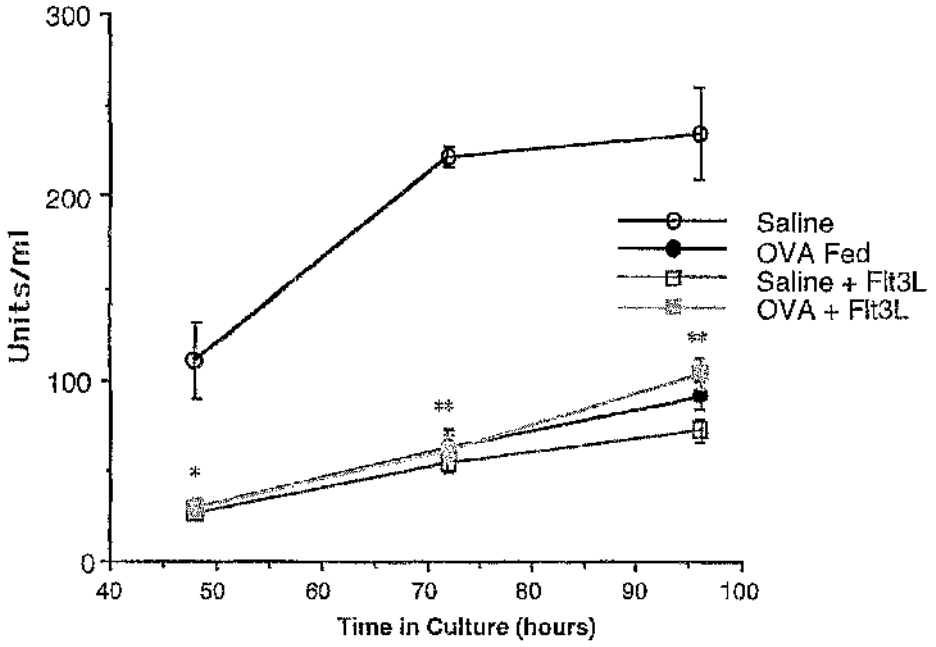


Figure 6.7 Effects of Flt3L on the Induction of Oral Tolerance in Primed Mice.

OVA-specific IL5 production in draining lymph nodes of mice given a single feed of 25mg OVA 8 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 14 days after immunisation. Mice were treated with saline or 10μg Flt3L daily from d1 until d9. The results shown are mean IL5 level (U/ml) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 2-4 days. There was little or no production of IL5 from cells cultured in the absence of antigen. (*$p<0.005$ versus appropriate saline fed control, *$p<0.001$ versus appropriate saline fed control)

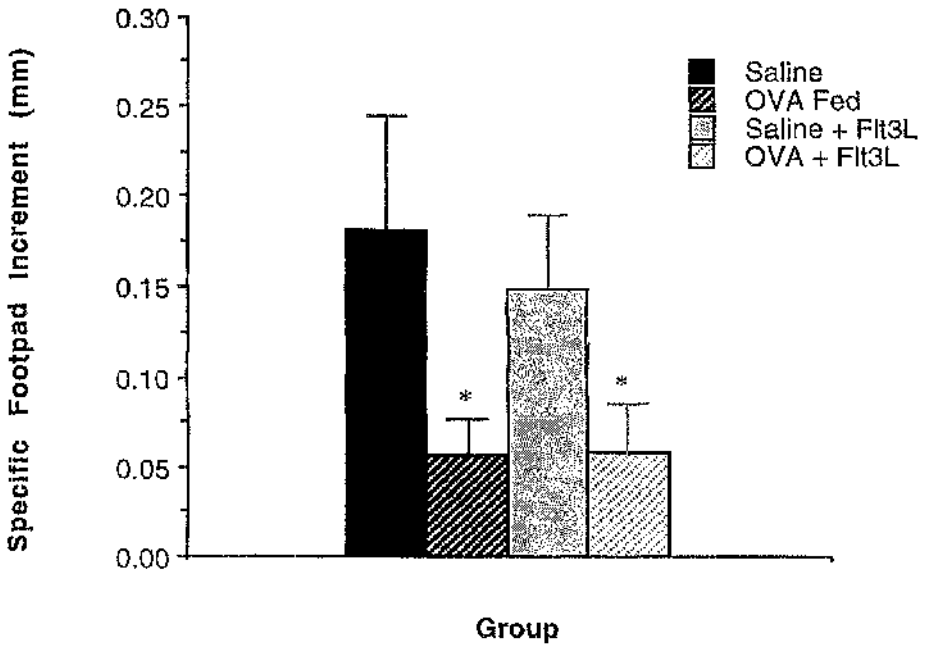


Figure 6.8 Effects of Flt3L on the Induction of Oral Tolerance in Primed Mice.

Systemic DTH responses in mice given a single feed of 25mg OVA 5 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. Mice were treated with saline or 10μg Flt3L daily from d-2 until d+6. The results shown are mean specific increments in footpad thickness ± 1 SD for 5 mice per group. (*$p<0.005$ versus appropriate saline fed control)

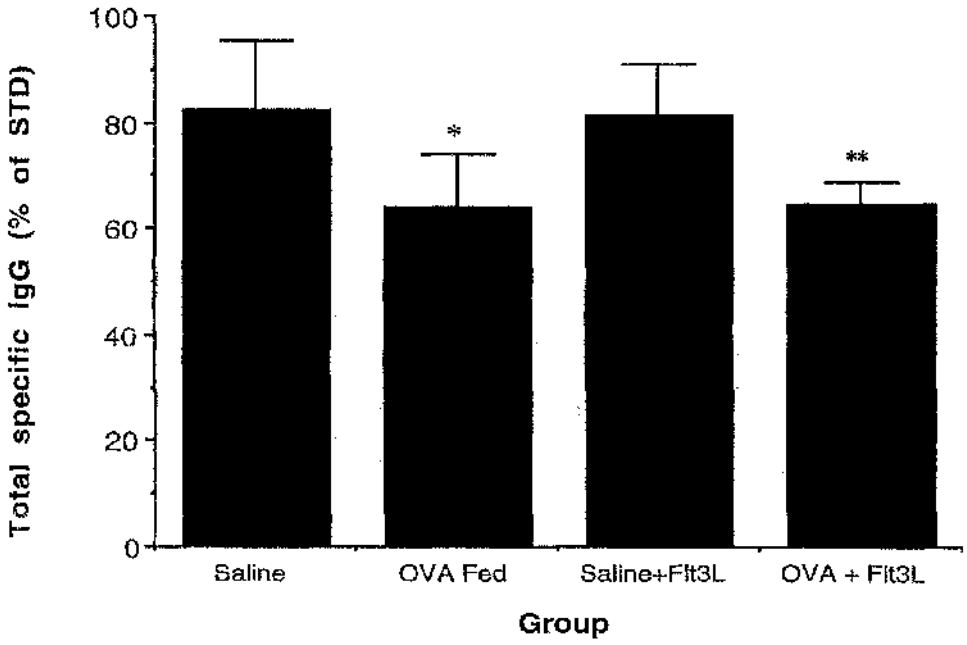


Figure 6.9 Effects of Flt3L on the Induction of Oral Tolerance in Primed Mice.

OVA-specific total serum IgG antibody responses in mice given a single feed of 25mg OVA 5 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. Mice were treated with saline or 10μg Flt3L daily from d-2 until d+6. The results shown are mean % of purified IgG standard ± 1 SD for individual sera from 5 mice per group. (*$p<0.05$ versus appropriate saline fed control, **$p<0.01$ versus appropriate saline fed control)

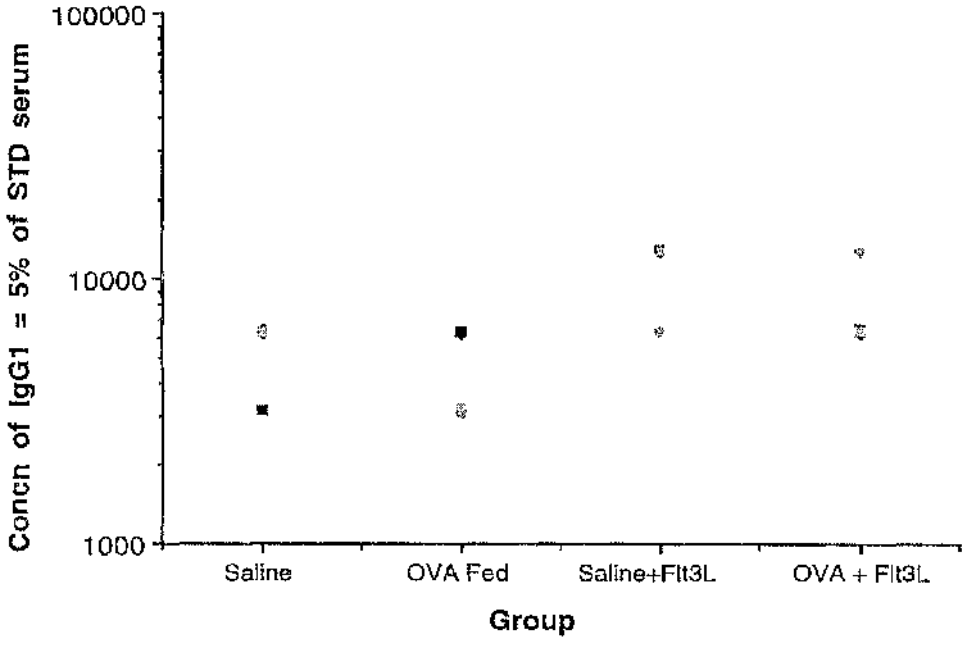


Figure 6.10 Effects of Flt3L on the Induction of Oral Tolerance in Primed Mice.

OVA-specific serum IgG1 antibody responses in mice given a single feed of 25mg OVA 5 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. Mice were treated with saline or 10μg Flt3L daily from d-2 until d+6. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group.

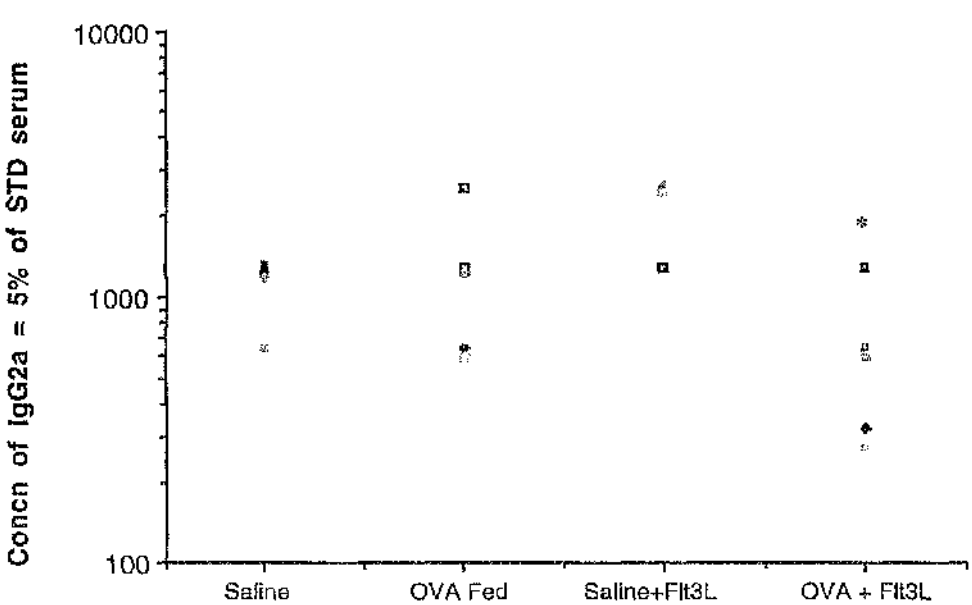


Figure 6.11 Effects of Flt3L on the Induction of Oral Tolerance in Primed Mice.

OVA-specific serum IgG2a antibody responses in mice given a single feed of 25mg OVA 5 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 21 days after immunisation. Mice were treated with saline or 10μg Flt3L daily from d-2 until d+6. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group. (*$p<0.05$ versus appropriate saline fed control)

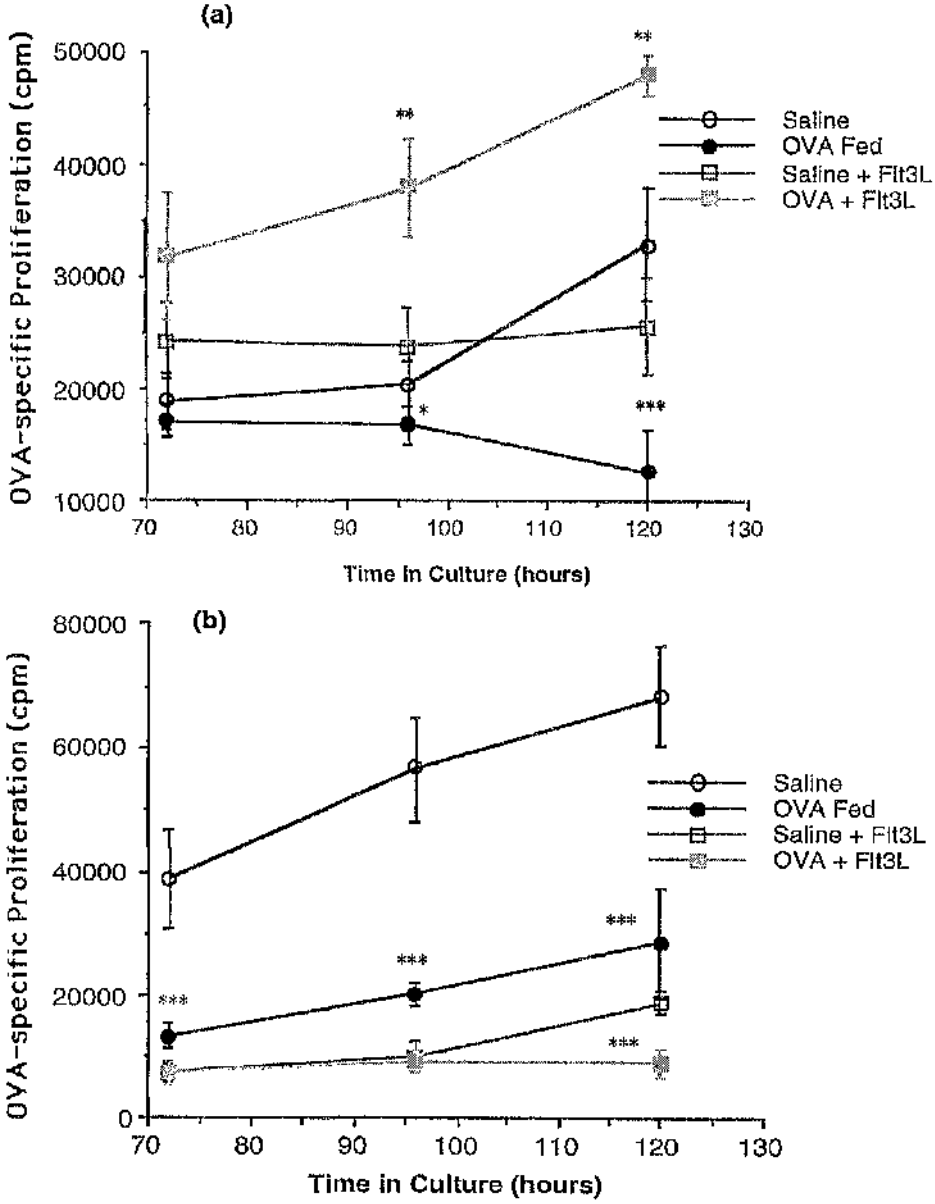


Figure 6.12 Effects of Flt3L on the Induction of Oral Tolerance in Primed Mice.

OVA-specific proliferative responses in draining lymph nodes of mice given a single feed of 25mg OVA 5 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 8 (**a**) and 14 (**b**) days after immunisation. Mice were treated with saline or 10μg Flt3L daily from d-2 until d+6. The results shown are mean uptake of ^{3}H-TdR ± 1 SD for the last 24 hours of culture in quadruplicate cultures from lymph node cells pooled from 3 mice per group. (*$p<0.05$ versus appropriate saline fed control, **$p<0.01$ versus appropriate saline fed control, ***$p<0.005$ versus appropriate saline fed control)

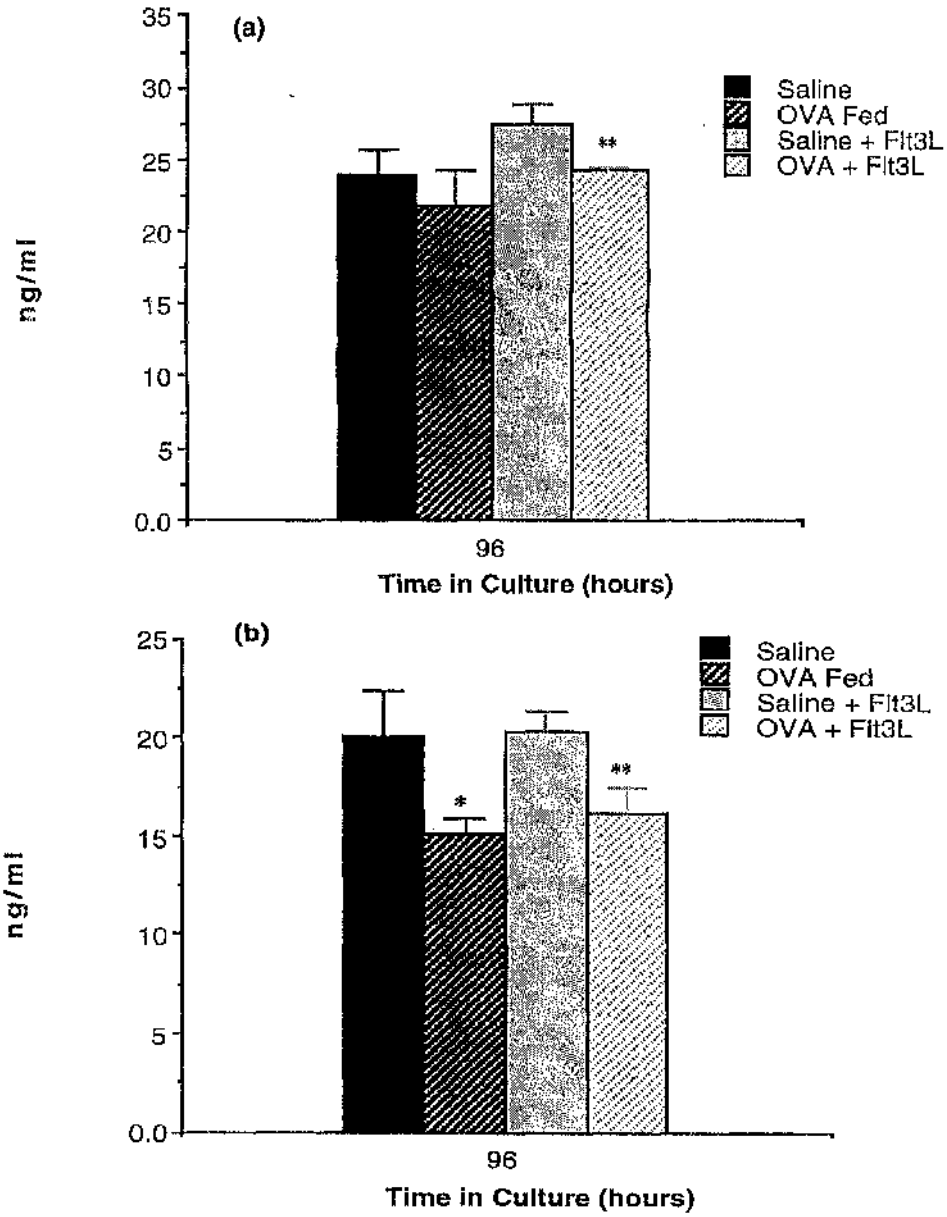


Figure 6.13 Effects of Flt3L on the Induction of Oral Tolerance in Primed Mice.

OVA-specific IFNγ production in draining lymph nodes of mice given a single feed of 25mg OVA 5 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 8 **(a)** and 14 **(b)** days after immunisation. Mice were treated with saline or 10μg Flt3L daily from d-2 until d+6. The results shown are mean IFNγ level (OD value) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 2-4 days. There was little or no production of IFNγ from cells cultured in the absence of antigen. (*$p<0.05$ versus appropriate saline fed control, **$p<0.01$ versus appropriate saline fed control)

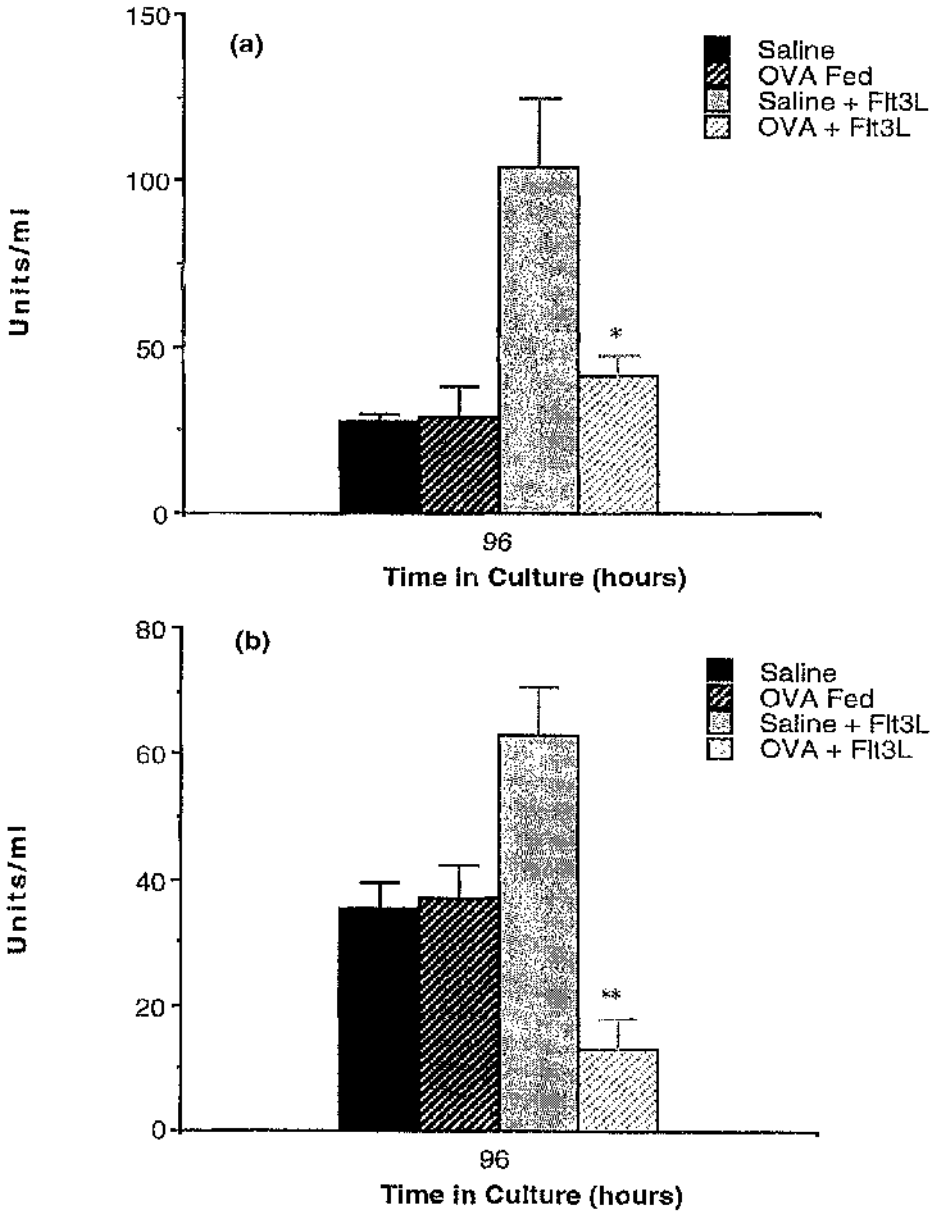


Figure 6.14 Effects of Flt3L on the Induction of Oral Tolerance in Primed Mice.

OVA-specific IL5 production in draining lymph nodes of mice given a single feed of 25mg OVA 5 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 8 **(a)** and 14 **(b)** days after immunisation. Mice were treated with saline or 10μg Flt3L daily from d-2 until d+6. The results shown are mean IL5 level (U/ml) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 2-4 days. There was little or no production of IL5 from cells cultured in the absence of antigen. (*$p<0.01$ versus appropriate saline fed control, **$p<0.001$ versus appropriate saline fed control)

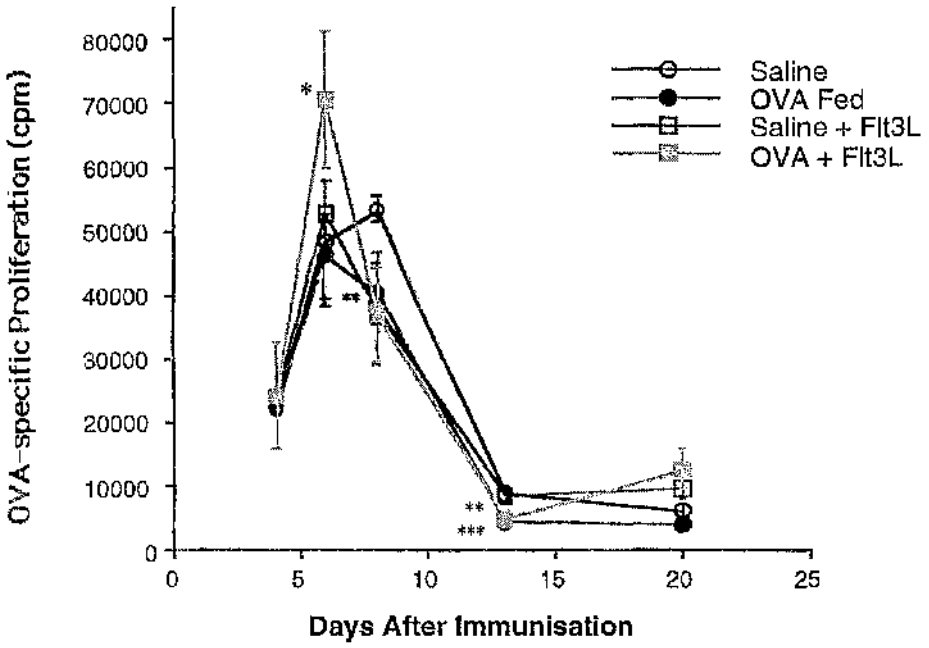


Figure 6.15 Effects of Flt3L on the Induction of Oral Tolerance in Primed Mice.

OVA-specific proliferative responses in draining lymph nodes of mice given a single feed of 25mg OVA 5 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 4, 6, 8, 13 and 20 days after immunisation. Mice were treated with saline or 10μg Flt3L daily from d-2 until d+6. The results shown are mean uptake of ^{3}H-TdR ± 1 SD for the last 24 hours of culture in quadruplicate cultures from lymph node cells pooled from 3 mice per group. (*$p<0.02$ versus appropriate saline fed control, **$p<0.005$ versus appropriate saline fed control, ***$p<0.001$ versus appropriate saline fed control)

Chapter7 Use of the Adoptive Transfer System in Oral Tolerance in Primed Mice

7.1 Introduction

Recently there have been many advances in techniques and experimental systems allowing antigen specific T cell responses to be followed *in vivo*. This is useful for my studies because I wanted to look directly at what happened to an established antigen-specific immune response when I fed antigen. One of the most useful approaches has been to transfer TCR transgenic T cells into normal mice and follow them using a clonotypic antibody. The advantage of this system for my studies was that I was able to look directly at the expansion and/or deletion of antigen-specific T cells when I fed my model antigen, ovalbumin, to mice to try and induce oral tolerance. As previous chapters in my thesis have shown that the time the antigen was fed after priming effects the oral tolerance induced, another advantage of this system was that it enabled me to investigate more thoroughly the effects that feeding antigen had on antigen-specific T cells at various stages of a developing immune response. The first of these TCR transgenic T cell transfer systems to be described was that using DO11.10 transgenic T cells specific for the OVA323-339 immunodominant peptide and I-A^d which are recognised by KJ1-26 antibody (143). This has now been widely used to study peripheral immune responses and also more recently oral tolerance in naive mice (114,179,180,225).

I thought this might be an appropriate means of studying fate of antigen-specific T cells in primed mice fed OVA and also of assessing directly if primed T cells are inherently resistant to the induction of oral tolerance. Therefore, I decided to try and establish the adoptive transfer model as a means of explaining my model of oral tolerance.

7.2 Experimental Protocol

Normal sex-matched BALB/c mice were adoptively transferred with systemic DO11.10 TCR transgenic T cells as described by Kearney *et al* (143) The percentage of KJ1-26$^+$CD4$^+$ cells present in suspensions of spleen and lymph node DO11.10 cells was calculated by flow cytometry and mice were injected with 1-3 x 10^6 KJ1-26$^+$CD4$^+$ cells intravenously. 2 days after the adoptive cell transfer, BALB/c mice were immunised s.c. with OVA/CFA (d0) and oral tolerance was induced by feeding mice OVA at various times thereafter, Control mice were fed saline, and to assess the effects of an immunogenic stimulus, additional mice were fed OVA and 10μg cholera toxin (CT). The expansion of Ag-specific T cells was then assessed in the draining PLN by determining the proportions and absolute numbers of KJ1-26$^+$CD4$^+$ T cells. Functional responses were assessed after restimulation of these cell suspensions with OVA *in vitro* by measuring antigen specific proliferation and production of IFNγ and IL5.

7.3 Results

7.3.1 Effects of Oral Tolerance on Primed Antigen-Specific T cells

Mice were transferred with 3 x 10^6 KJ1-26$^+$CD4$^+$ cells and two days later were immunised with OVA/CFA sc. 1, 2, 3, 4, 7 or 10 days after immunisation mice were fed 0.2ml saline, 50mg OVA / 0.2ml saline or 50mg OVA / 10μg CT / 0.2ml saline. It is known that the peak of expansion of antigen-specific T cells occurs around 5 days after immunisation with OVA/CFA. Thus, antigen was fed at various times before, during and after this peak of antigen-specific T cell expansion so that I could investigate the effects that feeding antigen had on these antigen-specific T cells as they respond to immunisation with OVA/CFA . Mice adoptively transferred with transgenic T cells would have a larger number of antigen-specific T cells than an untransferred animal and so I chose to feed a higher dose of OVA, 50mg, compared with my normal feeding dose of 25mg OVA so that there would be enough antigen

presented to all of the transgenic T cells. 1 and 5 days after feeding the percentage and function of the KJ1-26$^+$CD4$^+$ cells in the draining PLN of the mice were assessed.

7.3.1.1 Expansion of Primed T cells *in vivo* After Feeding OVA

The percentage of KJ1-26$^+$CD4$^+$ cells present in the draining lymph nodes of mice was assessed at frequent intervals over a period of 13 days after feeding/immunisation. In mice fed saline, OVA-specific T cell expansion was apparent at the first time point, day 2 after immunisation. This peaked at 3-6 days, before falling. This profile was then used as a basis for examining the effects of feeding antigen on the kinetics of the evolving response.

When mice were fed soluble OVA 1, 2, 3, 4, and 7 days after immunisation, there was no obvious effect on the kinetics of the developing systemic immune response in terms of percentage (Fig7.1a, b, c, d and e) and numbers (Fig7.2a, b, c, d and e) of KJ1-26$^+$CD4$^+$. However, feeding OVA 10 days after immunisation led to a small but significant reduction in the proportion (Fig7.1f) and absolute numbers (Fig7.2f) of KJ1-26$^+$CD4$^+$ cells in the draining lymph at later time points compared with saline fed controls. Feeding OVA + CT had no effect on T cell expansion at any time.

7.3.1.2 *In Vitro* Responses

The function of the KJ1-26$^+$CD4$^+$ cells present in the draining lymph nodes of the mice *in vitro* was measured by OVA-specific proliferation at 24 and 72 hours of culture to examine for alterations in the kinetics of responsiveness *in vitro*.

The OVA specific proliferative responses of PLN cells taken from mice fed OVA or OVA + CT 1, 2, 3, 4, and 7 days after immunisation and harvested after 24 (Fig7.3a, b, c, d and e) and 72hrs (Fig7.4a, b, c, d and e) in culture were not significantly different compared with control saline fed mice. However, feeding OVA or OVA/CT 10 days after immunisation significantly reduced the OVA specific proliferative responses of PLN cells harvested after 24 (Fig7.3f) and 72hr (Fig7.4f) in

culture compared with saline fed controls, whose response had declined more markedly by this time.

Thus it would appear that feeding OVA early after immunisation does not alter the kinetics of the response of KJ1-26$^+$CD4$^+$ cells to OVA/CFA, feeding OVA to mice 10 days after immunisation did reduce the expansion and proliferative capacity of primed OVA-specific T cells.

7.3.2 Effects of Feeding High Doses on Adoptively Transferred Primed T Cells

The apparent ability to tolerise primed transgenic T cells late, but not early after immunisation, contrasted with my previous results in normal mice. As I thought this might reflect the greater number of OVA specific T cells present in mice adoptively transferred mice, I decided to examine the effects of feeding higher doses of antigen.

In these experiments, mice were transferred with 3 x 10^6 KJ1-26$^+$CD4$^+$ cells and two days later were immunised with OVA/CFA sc. 5 or 12 days after immunisation, the mice were fed saline, 200mg OVA or 200mg OVA + 10μg CT and the numbers and proliferation of the KJ1-26$^+$CD4$^+$ cells in the draining PLN assessed 1-7 days later.

7.3.2.1.1 Expansion of Antigen Specific T cells *in vivo* After Feeding High Doses of OVA Early After Immunisation

In the first experiment, T cell expansion in saline fed controls was at its peak at the first time of sacrifice, 6 days after immunisation. T cell expansion was significantly higher in mice fed soluble OVA 5 days after immunisation immediately after feeding, suggesting that this procedure had restimulated primed T cells in the periphery. However, there were no differences between the proportions of OVA-specific T cells at later times in OVA fed and control mice (Fig 7.5). In addition, the absolute numbers of KJ1-26$^+$CD4$^+$ T cells were not significantly altered at any time in OVA fed mice (Fig7.6). However, the proportion and number of OVA-specific T cells was significantly higher in mice fed soluble OVA + CT 5 days after immunisation

immediately after feeding, suggesting that this procedure had restimulated primed T cells in the periphery. As for OVA fed mice, there were no differences between the proportions and numbers of OVA-specific T cells at later times in OVA + CT fed and control mice.

7.3.2.1.2 *In Vitro* Responses of OVA-specific T cells in Mice Fed High Doses of OVA Early After Immunisation

Lymph node cells from mice fed OVA 5 days after immunisation showed some increased proliferative activity when assessed 1 day after feeding, consistent with the increased expansion of KJ1-26$^+$CD4$^+$ T cells (Figure 7.7). Therefore, proliferative responses were normal until 7 days, when significantly suppressed responses were observed. Conversely, mice fed OVA + CT had a significant increase in proliferation at this time, although these responses were normal at other times.

Because of the apparent tolerance of proliferating T cells in OVA fed primed mice, I also measured the OVA-specific IFNγ and IL5 production by cells from these mice. However, feeding OVA or OVA + CT did not significantly alter IFNγ (Figure7.8a) or IL5 (Figure7.8b) production compared with saline fed controls.

7.3.2.2.1 Expansion of Antigen Specific T cells *in vivo* After Feeding High Doses of OVA Late After Immunisation

In the second experiment, where I fed 12 days after priming and analysed 1 and 5 days after feeding, T cell expansion in all the groups would have past its peak in response to immunisation. T cell expansion was reduced, but not significantly, in mice fed soluble OVA 12 days after immunisation immediately after feeding, suggesting that this procedure had reduced the amount of primed T cells in the periphery (Fig 7.9 and 7.10). However, there were no differences between the proportions or numbers of OVA-specific T cells 5 days after feeding in OVA fed and control mice. Further, there

were no differences between the proportions or numbers of OVA-specific T cells early or late after feeding in OVA + CT fed and control mice.

7.3.2.2.2 *In Vitro* Responses of OVA-specific T cells in Mice Fed High Doses of OVA Late After Immunisation

Lymph node cells from mice fed OVA 12 days after immunisation showed significantly decreased proliferative activity when assessed 1 day after feeding, consistent with the apparent decreased expansion of KJ1-26^{+}CD4^{+} T cells at this time (Figure 7.11a). However, as for proportion and numbers of OVA-specific T cells, proliferative responses were unaltered 5 days after feeding, when compared with saline fed controls. Mice fed OVA + CT appeared to have slightly decreased proliferation responses compared with saline fed controls early after feeding. However, mice fed OVA + CT had no significant differences in proliferation 1 and 5 days after feeding compared with saline fed controls which was also found for the proportion and numbers of OVA-specific T cells.

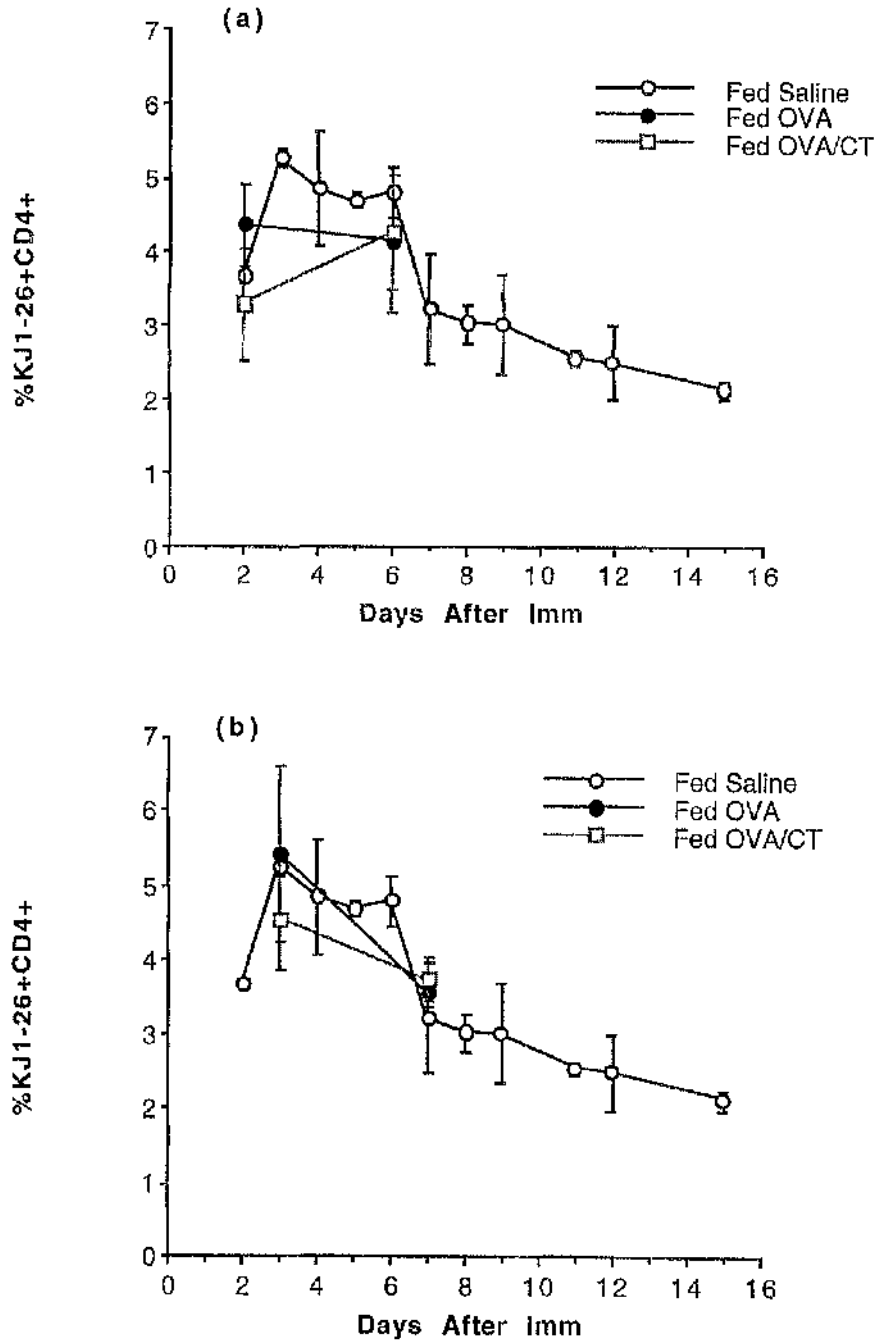
Because of the apparent tolerance of proliferating T cells in OVA fed primed mice, I also measured the OVA-specific IFNγ and IL5 production by cells from these mice 1 and 5 days after feeding. However, feeding OVA or OVA + CT did not significantly alter IFNγ (Figure7.12) or IL5 (Figure7.13) production compared with saline fed controls.

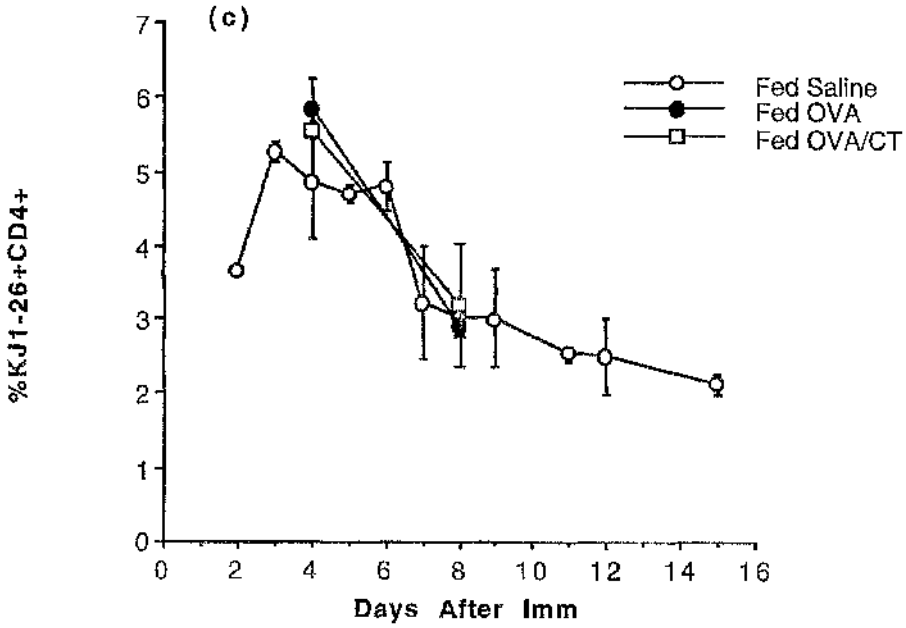
Thus, feeding OVA later after immunisation may induce some degree of tolerance in primed T cells, although this may not be a general effect and was only partly reproduced by an immunogenic feeding protocol.

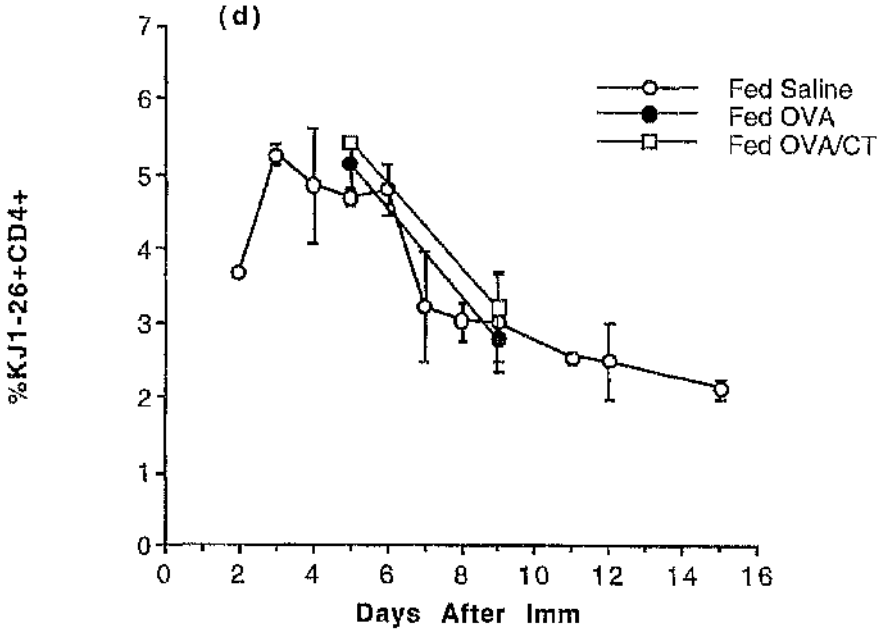
7.4 Conclusions

In this chapter I attempted to examine the induction of oral tolerance in primed T cells using the adoptive transfer of Ag-specific transgenic T cells. In the first experiments, I found that feeding soluble OVA after priming could alter the kinetics of T cell expansions, but that this only occurred if feeding was delayed until 10 days. This

did not simply appear to be due to re-exposure to antigen, as there was no equivalent effect of feeding OVA in an immunogenic manner with CT. In addition, the inhibited expansion caused by feeding soluble antigen after priming was accompanied by suppressed proliferation responses to OVA. However, these results contrast to my earlier findings in normal mice, where tolerance could be induced early, but not late after priming. To try and investigate why this might be the case, I fed a higher dose of OVA, as I thought the increase in numbers of Ag-specific T cells in transfer recipients might complicate the system. These experiments showed again that tolerance might occur late after priming but there was still no effect earlier and also functional tolerance was variable in that proliferative responses were easier to tolerise than cytokines responses. Also, the effects of multiple exposure to antigen on antigen-specific T cells, as revealed by feeding OVA + CT, show that this did not radically alter the kinetics and level of the primary response to antigen.


(a)
%KJ1-26+CD4+
Fed Saline
Fed OVA
Fed OVA/CT
Days After Imm
(b)
%KJ1-26+CD4+
Fed Saline
Fed OVA
Fed OVA/CT
Days After Imm


(c)
%KJ1-26+CD4+
Fed Saline
Fed OVA
Fed OVA/CT
0
1
2
3
4
5
6
7
0
2
4
6
8
10
12
14
16
Days After Imm


(d)
%KJ1-26+CD4+
Fed Saline
Fed OVA
Fed OVA/CT
0
1
2
3
4
5
6
7
0
2
4
6
8
10
12
14
16
Days After Imm

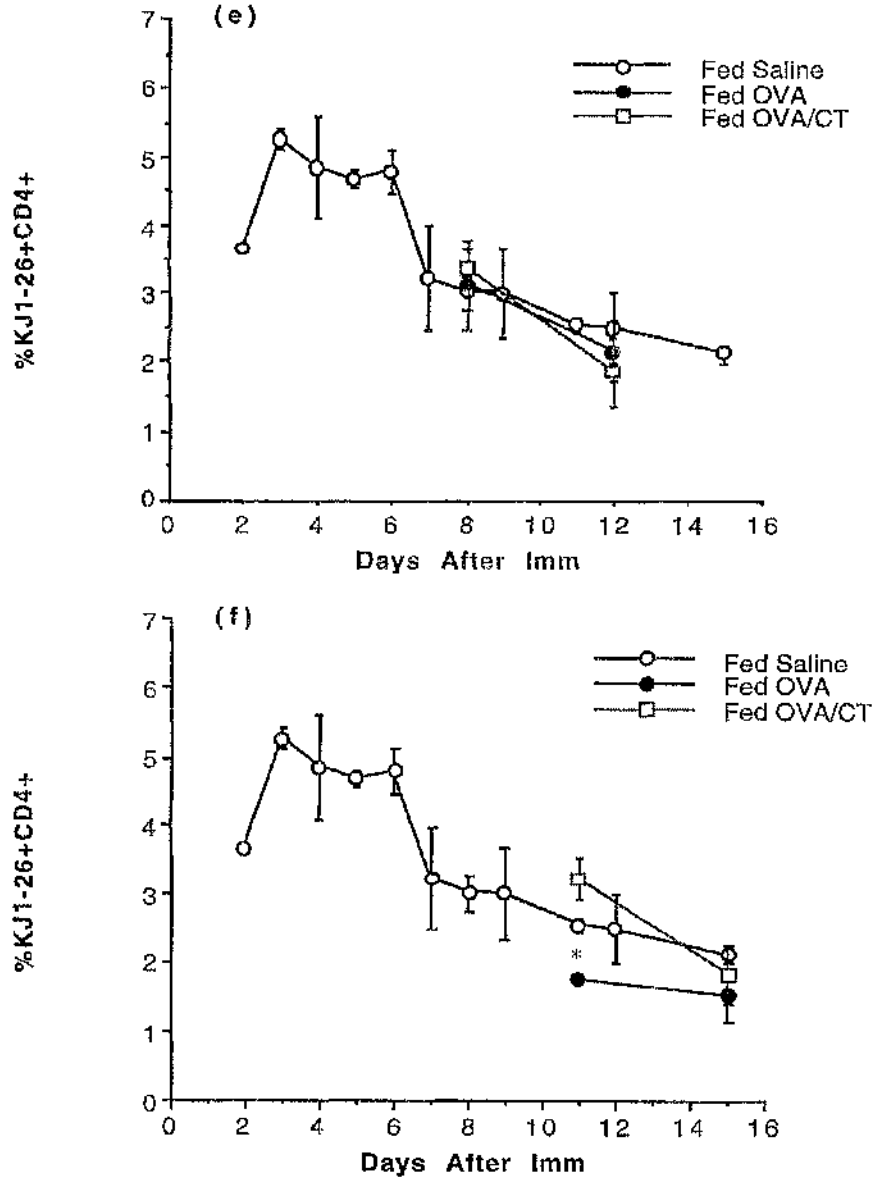
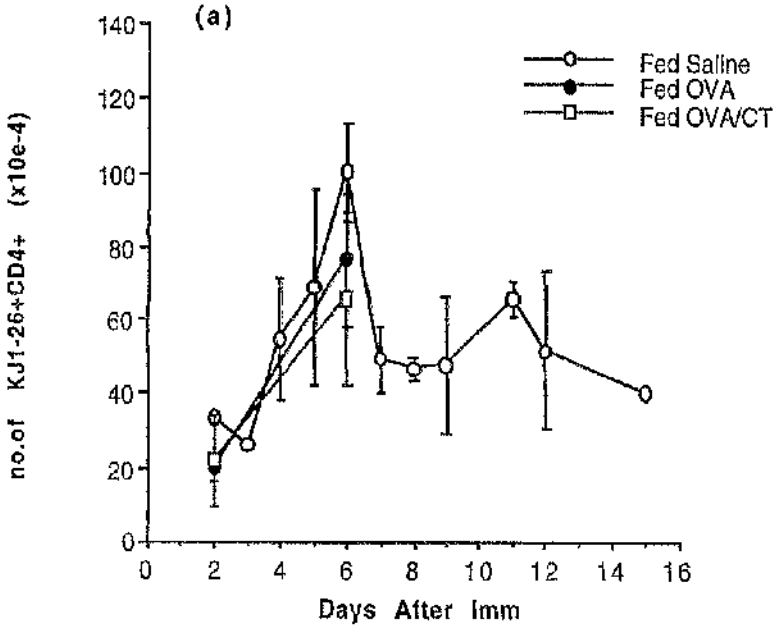
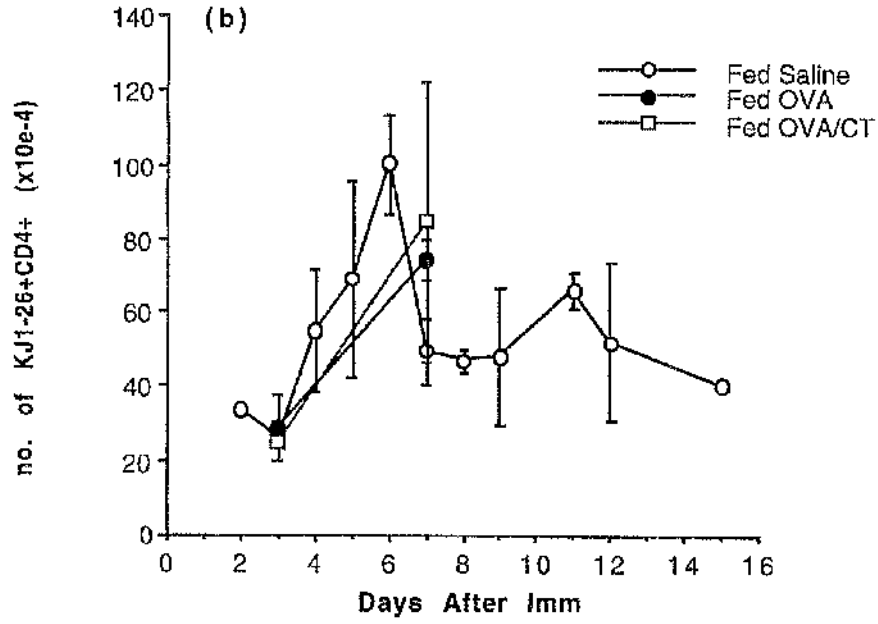
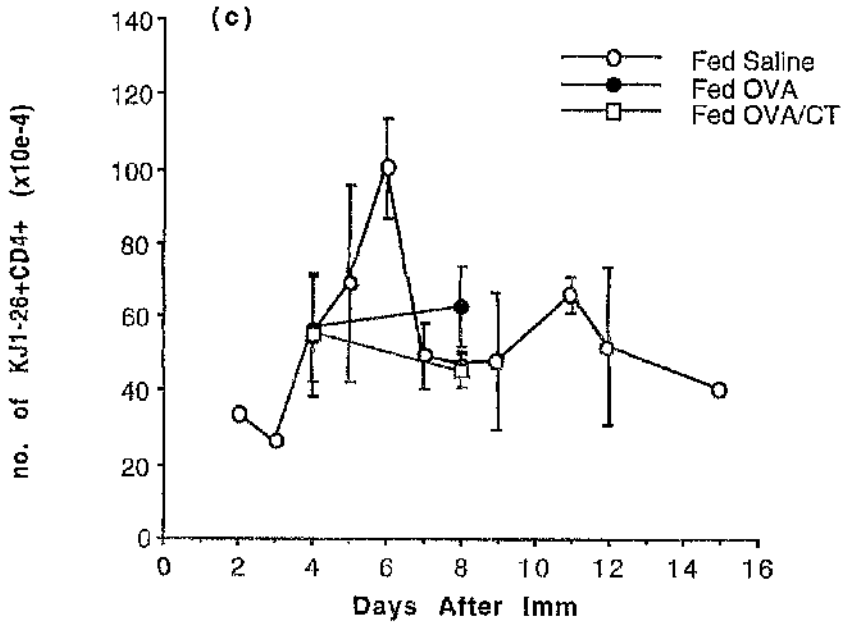


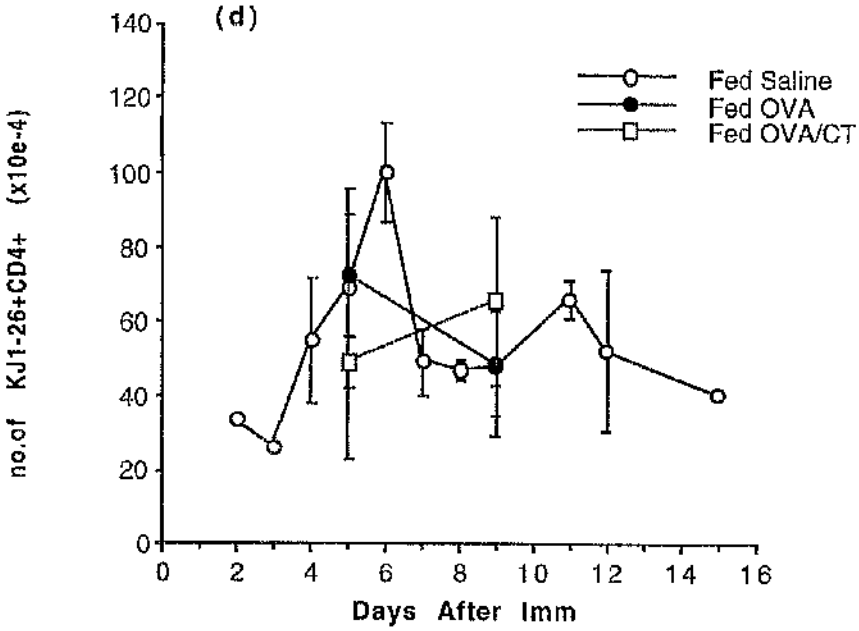
Figure 7.1 Kinetics of Antigen Specific T Cell Expansion After Feeding Antigen to Primed Mice

Percentage of KJ1-26$^+$CD4$^+$ cells in the draining lymph nodes of mice adoptively transferred with 3 x 10^6 KJ1-26$^+$CD4$^+$ cells and given a single feed of 50mg OVA or 50mg OVA + CT 1(**a**), 2(**b**), 3(**c**), 4(**d**), 7(**e**) or 10(**f**) days after subcutaneous immunisation with OVA/CFA, and in saline fed controls. The results shown are the mean percentage of KJ1-26$^+$CD4$^+$ cells ± 1 SD in lymph nodes from 2 mice per group. (*$p<0.01$ versus controls)


(a)
Fed Saline
Fed OVA
Fed OVA/CT
no.of KJ1-26+CD4+ (x10e-4)
140
120
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16
Days After Imm


(b)
Fed Saline
Fed OVA
Fed OVA/CT
no. of KJ1-26+CD4+ (x10e-4)
140
120
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16
Days After Imm


(c)
Fed Saline
Fed OVA
Fed OVA/CT
no. of KJ1-26+CD4+ (x10e-4)
140
120
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16
Days After Imm


(d)
Fed Saline
Fed OVA
Fed OVA/CT
no.of KJ1-26+CD4+ (x10e-4)
140
120
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16
Days After Imm

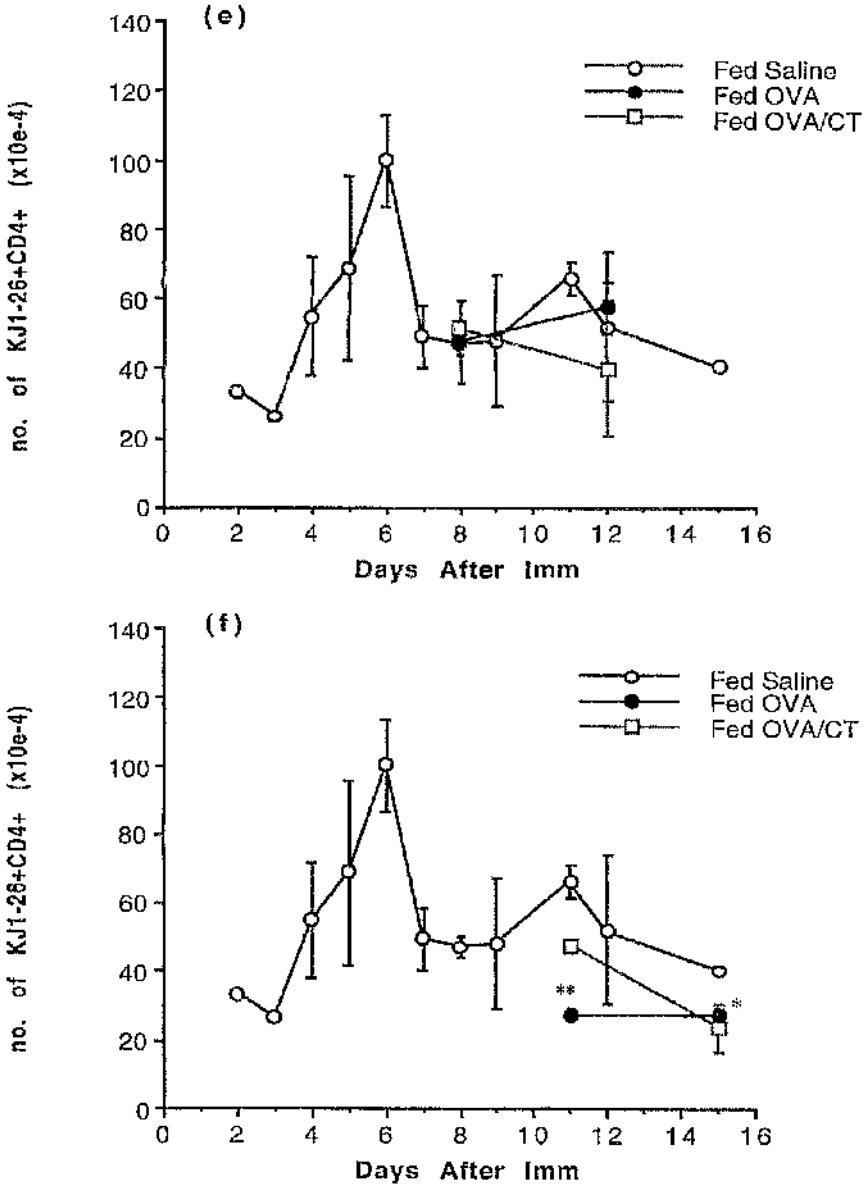
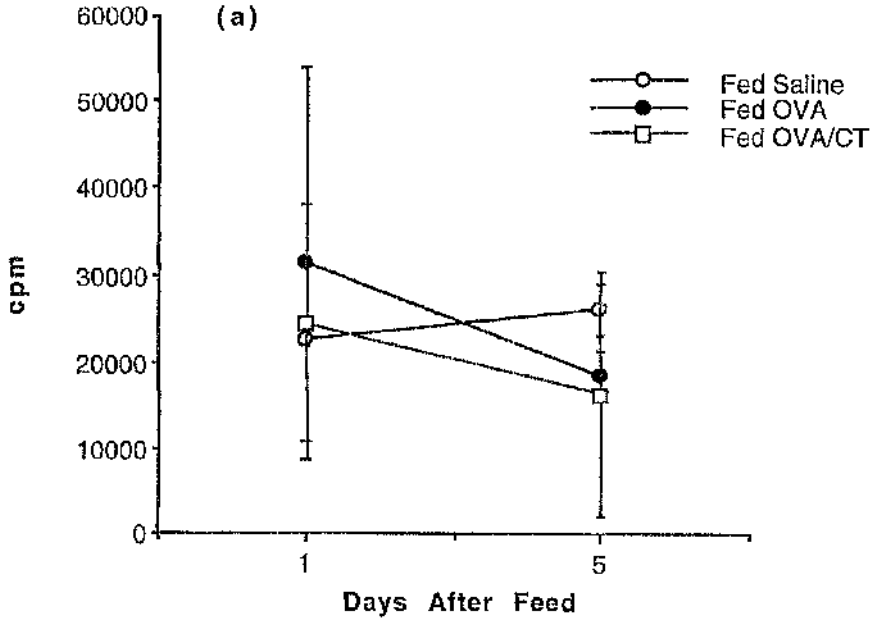
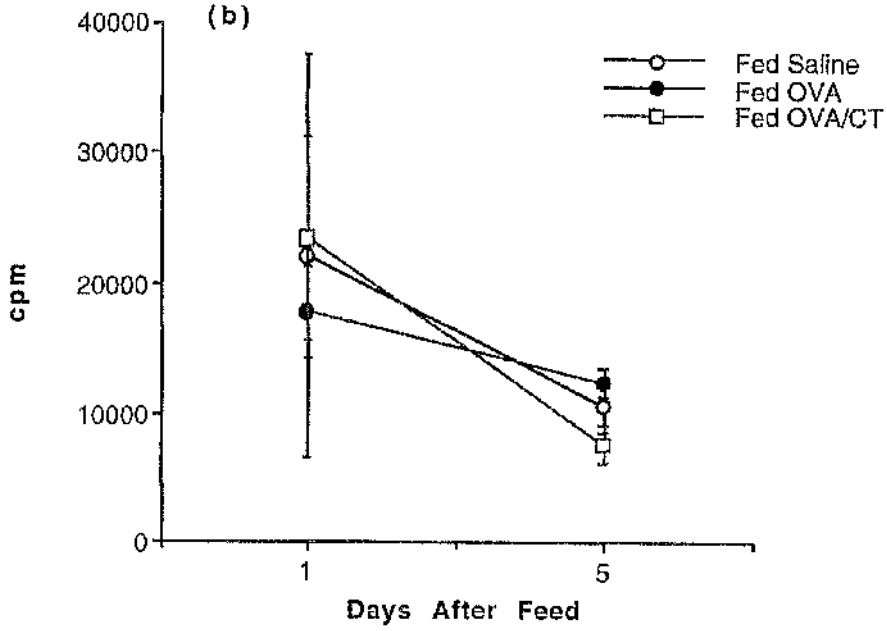
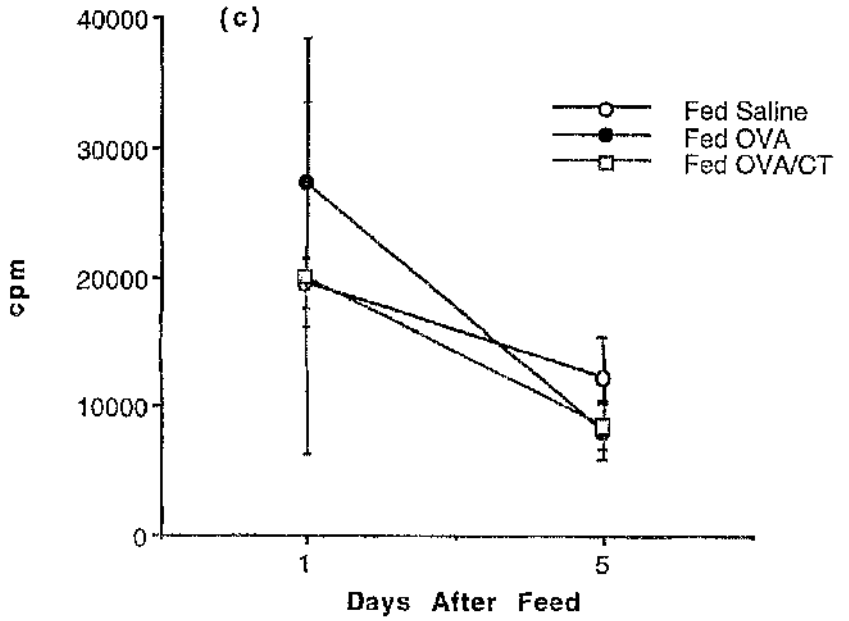


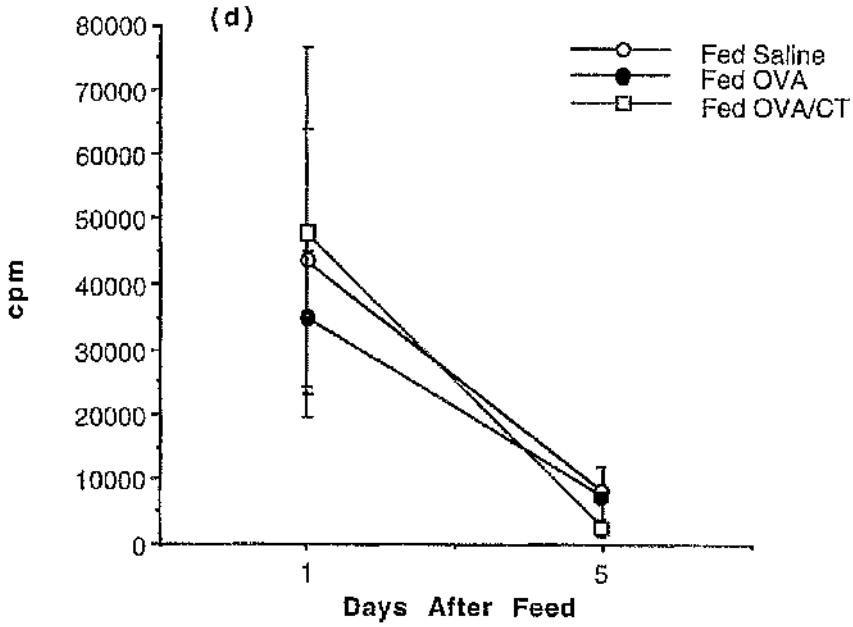
Figure 7.2 Kinetics of Antigen Specific T Cell Expansion After Feeding Antigen to Primed Mice

Numbers of KJ1-26$^+$CD4$^+$ cells in the draining lymph nodes of mice adoptively transferred with 3 x 10^6 KJ1-26$^+$CD4$^+$ cells and given a single feed of 50mg OVA or 50mg OVA + CT 1(**a**), 2(**b**), 3(**c**), 4(**d**), 7(**e**) or 10(**f**) days after subcutaneous immunisation with OVA/CFA, and in saline fed controls. The results shown are the mean absolute numbers of KJ1-26$^+$CD4$^+$ cells ± 1 SD in lymph nodes from 2 mice per group. (*$p<0.05$ versus controls, **$p<0.01$ versus controls)


(a)
60000
50000
40000
30000
20000
10000
0
cpm
Fed Saline
Fed OVA
Fed OVA/CT
1
5
Days After Feed


(b)
40000
30000
20000
10000
0
cpm
Fed Saline
Fed OVA
Fed OVA/CT
1
5
Days After Feed


(c)
40000
30000
20000
10000
0
cpm
1
5
Days After Feed
Fed Saline
Fed OVA
Fed OVA/CT


(d)
80000
70000
60000
50000
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20000
10000
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1
5
Days After Feed
Fed Saline
Fed OVA
Fed OVA/CT

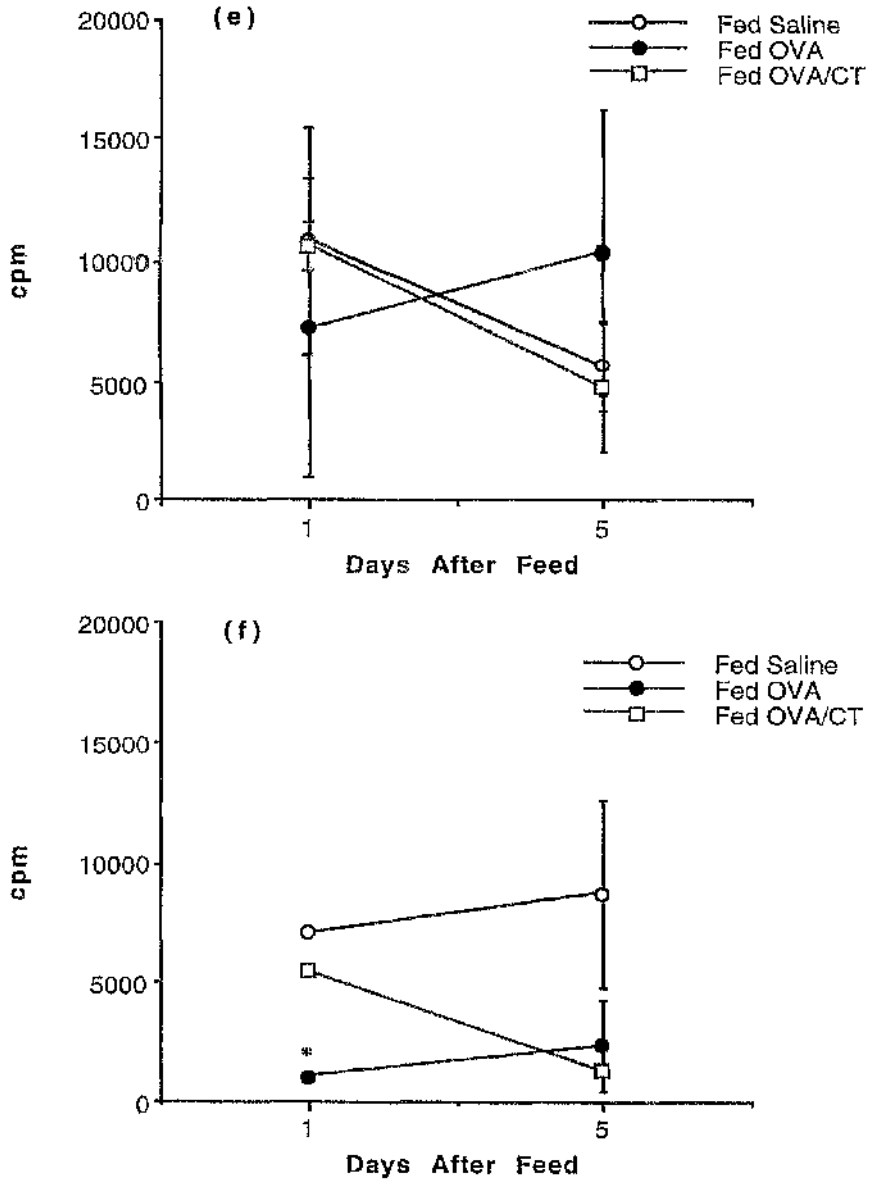
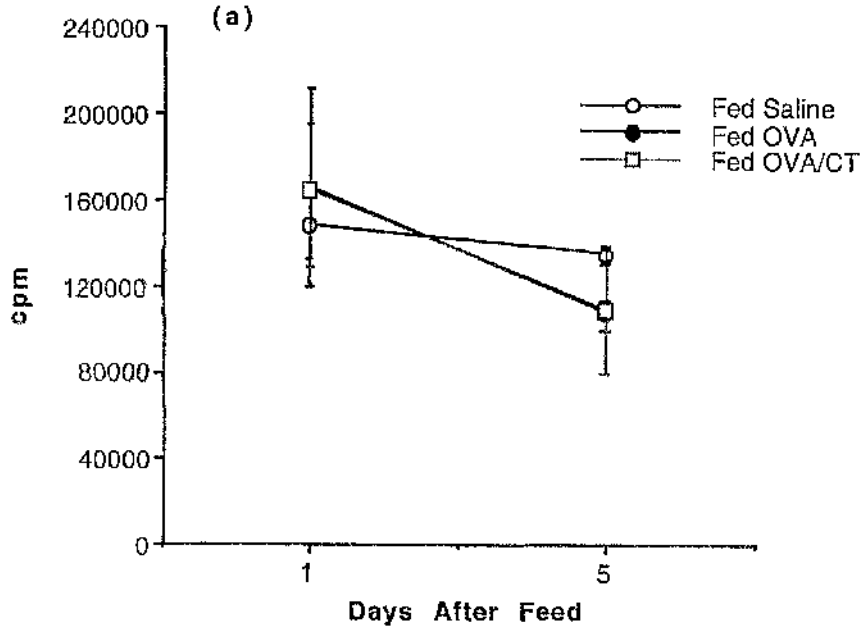
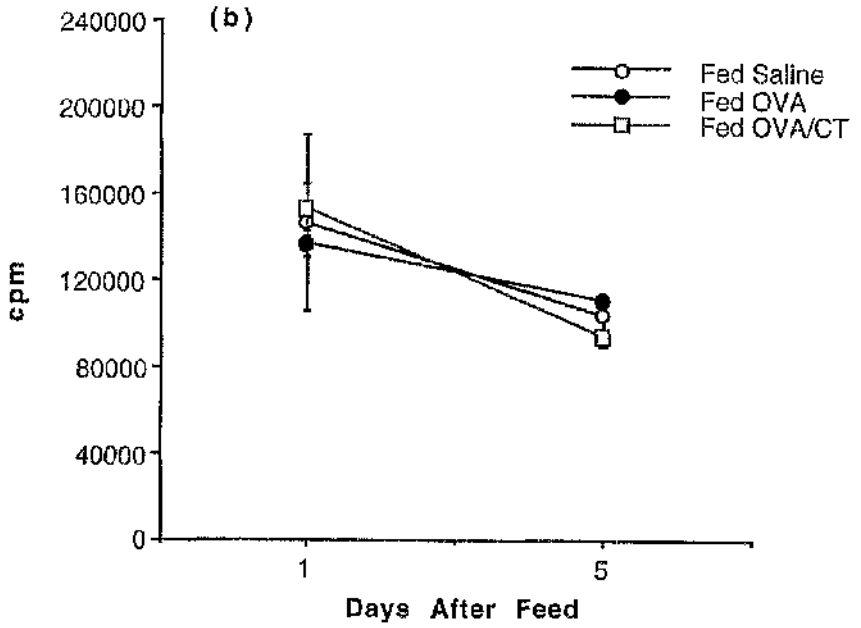
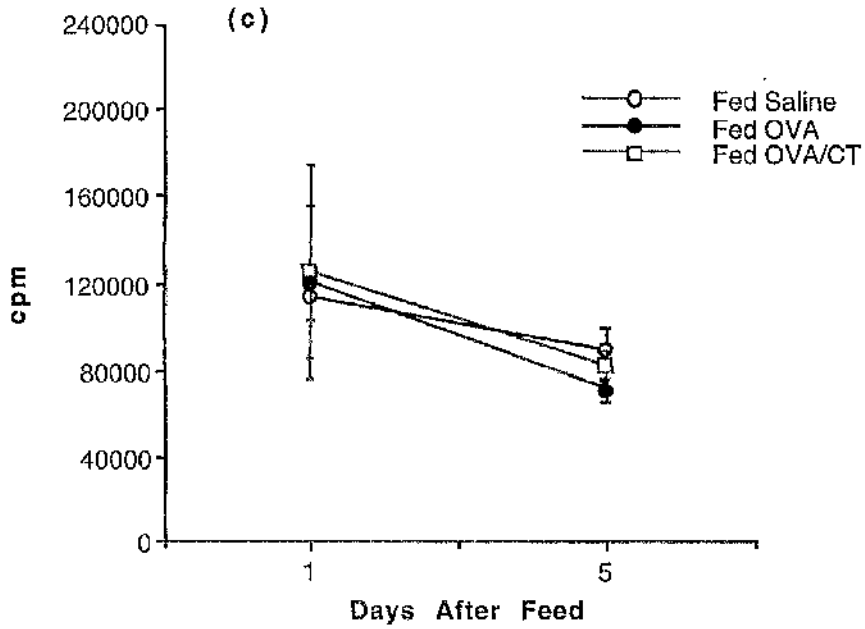


Figure 7.3 Kinetics of Antigen Specific T Cell Expansion After Feeding Antigen to Primed Mice

OVA-specific proliferative responses in the draining lymph nodes of mice adoptively transferred with 3 x 10^6 KJ1-26$^+$CD4$^+$ cells given a single feed of 50mg OVA or 50mg OVA + CT 1(**a**), 2(**b**), 3(**c**), 4(**d**), 7(**e**) or 10(**f**) days after subcutaneous immunisation with OVA/CFA, and in saline fed controls. The results shown are the mean uptake of ^{3}H-TdR ± 1 SD for quadruplicate cultures of lymph node cells from 2 mice per group after 24 hours in culture. (*$p<0.005$ versus controls)


(a)
240000
200000
160000
120000
80000
40000
0
cpm
1
5
Days After Feed
Fed Saline
Fed OVA
Fed OVA/CT


(b)
240000
200000
160000
120000
80000
40000
0
cpm
1
5
Days After Feed
Fed Saline
Fed OVA
Fed OVA/CT


(c)
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Days After Feed
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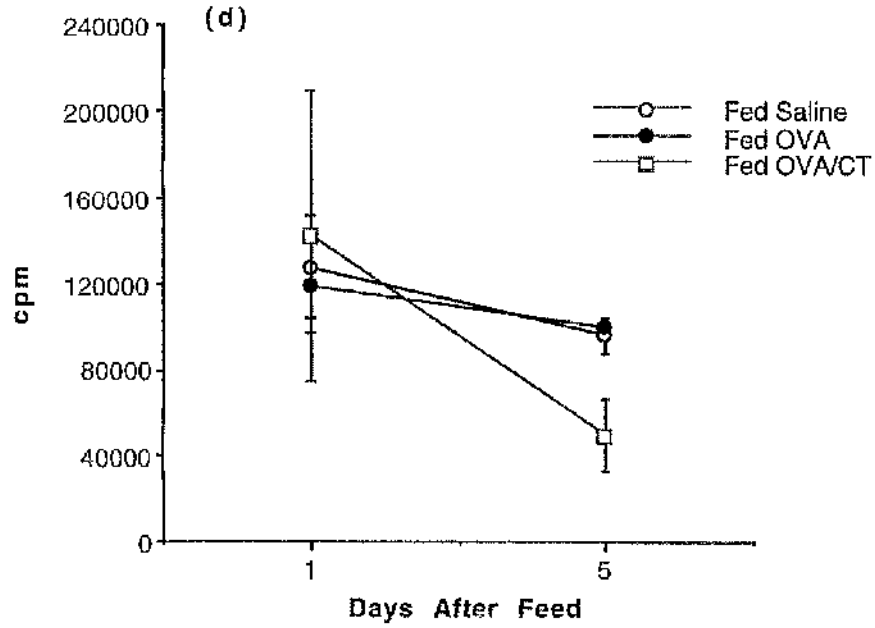
Fed OVA/CT


(d)
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Days After Feed
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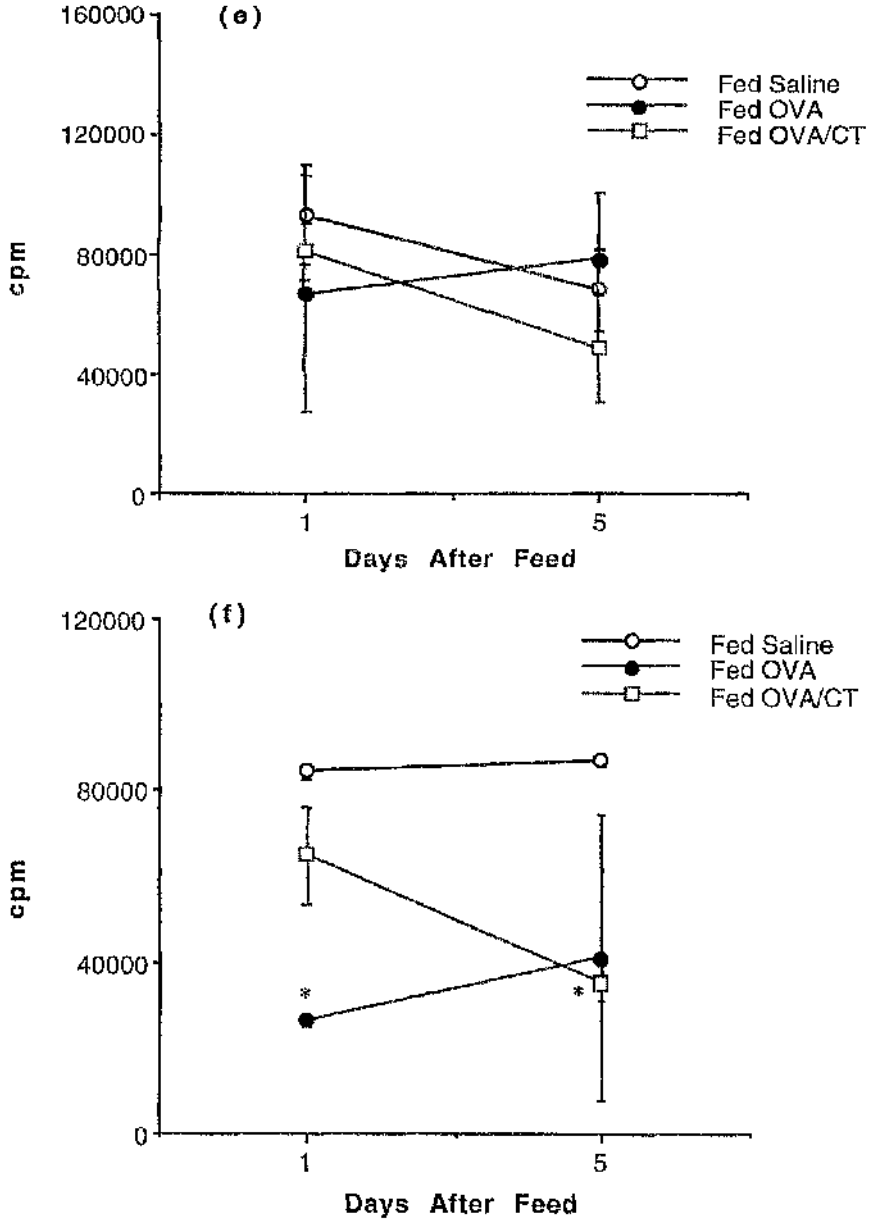


Figure 7.4 Kinetics of Antigen Specific T Cell Expansion After Feeding Antigen to Primed Mice

OVA-specific proliferative responses in the draining lymph nodes of mice adoptively transferred with 3 x 10^6 KJ1-26$^+$CD4$^+$ cells given a single feed of 50mg OVA or 50mg OVA + CT 1(**a**), 2(**b**), 3(**c**), 4(**d**), 7(**e**) or 10(**f**) days after subcutaneous immunisation with OVA/CFA, and in saline fed controls. The results shown are the mean uptake of ^{3}H-TdR ± 1 SD for quadruplicate cultures of lymph node cells from 2 mice per group after 72 hours in culture. (*$p<0.005$ versus controls)

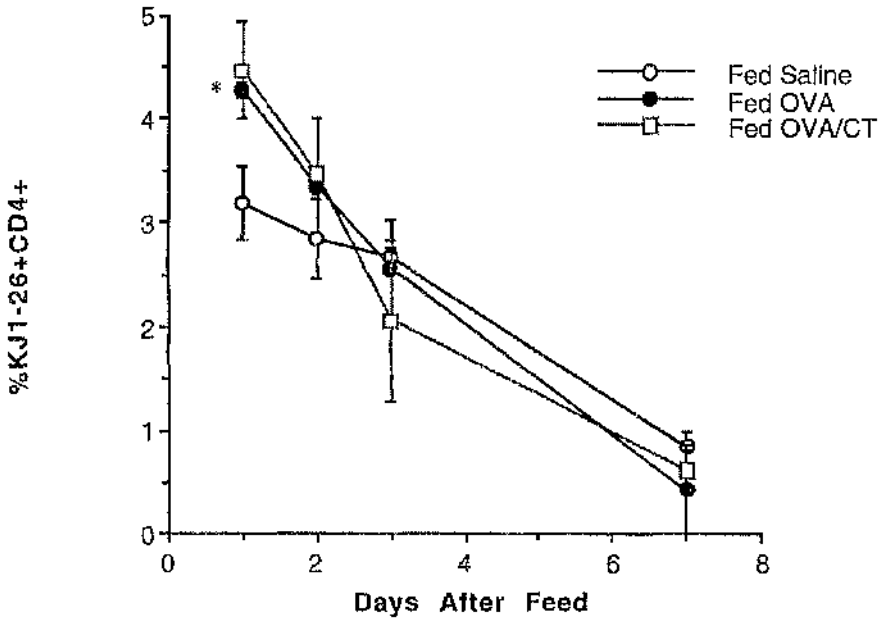


Figure 7.5 Expansion of Antigen Specific T Cells in Mice Fed a High Dose of Antigen 5 Days After Priming

Percentage of KJ1-26^{+}CD4^{+} cells in the draining lymph nodes of mice adoptively transferred with 3 x 10^{6} KJ1-26^{+}CD4^{+} cells given a single feed of 200mg OVA or 200mg OVA + CT 5 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls, 1, 2, 3 and 7 days after feeding. The results shown are the mean percentage of KJ1-26^{+}CD4^{+} cells ± 1 SD in lymph node cells from 2 mice per group. (*$p<0.05$ versus controls)

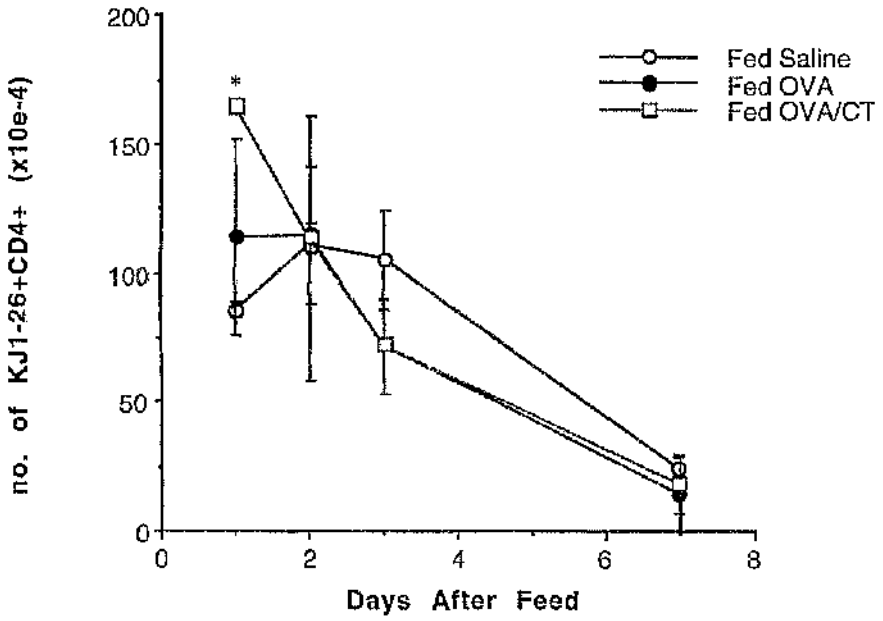
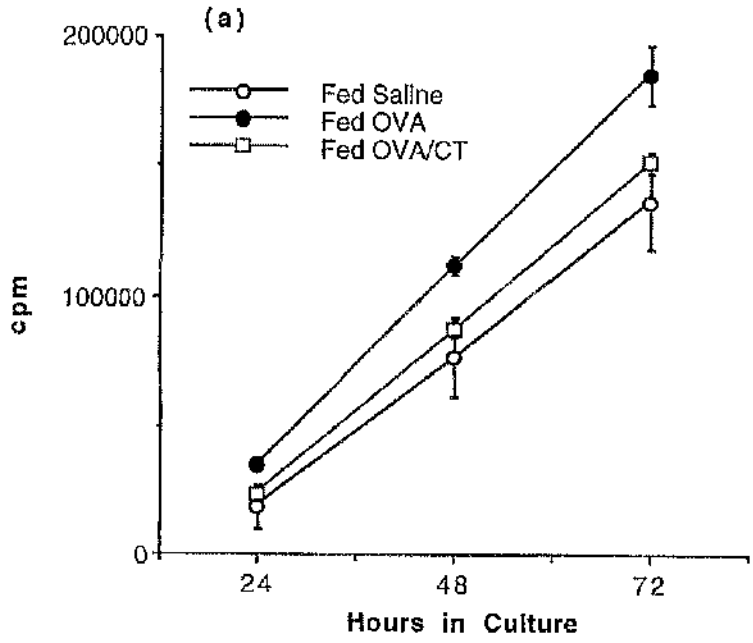
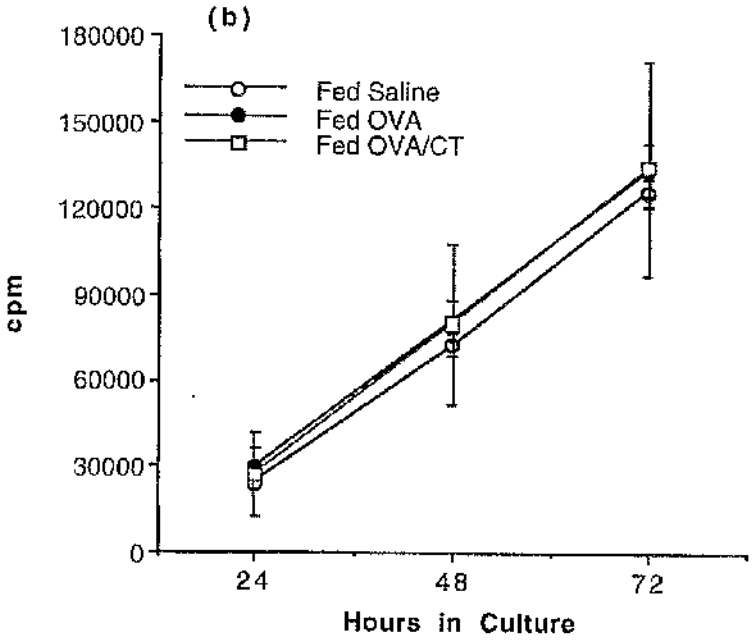


Figure 7.6 Expansion of Antigen Specific T cells in Mice Fed a High Dose of Antigen 5 Days After Priming

Numbers of KJ1-26$^+$CD4$^+$ cells in the draining lymph nodes of mice adoptively transferred with 3 x 10^6 KJ1-26$^+$CD4$^+$ cells given a single feed of 200mg OVA or 200mg OVA + CT 5 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls, 1, 2, 3 and 7 days after feeding. The results shown are mean absolute numbers of KJ1-26$^+$CD4$^+$ cells ± 1 SD in lymph node cells from 2 mice per group. (*$p<0.005$ versus controls)


(a)
200000
100000
0
cpm
Fed Saline
Fed OVA
Fed OVA/CT
24
48
72
Hours in Culture


(b)
180000
150000
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Fed Saline
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Hours in Culture

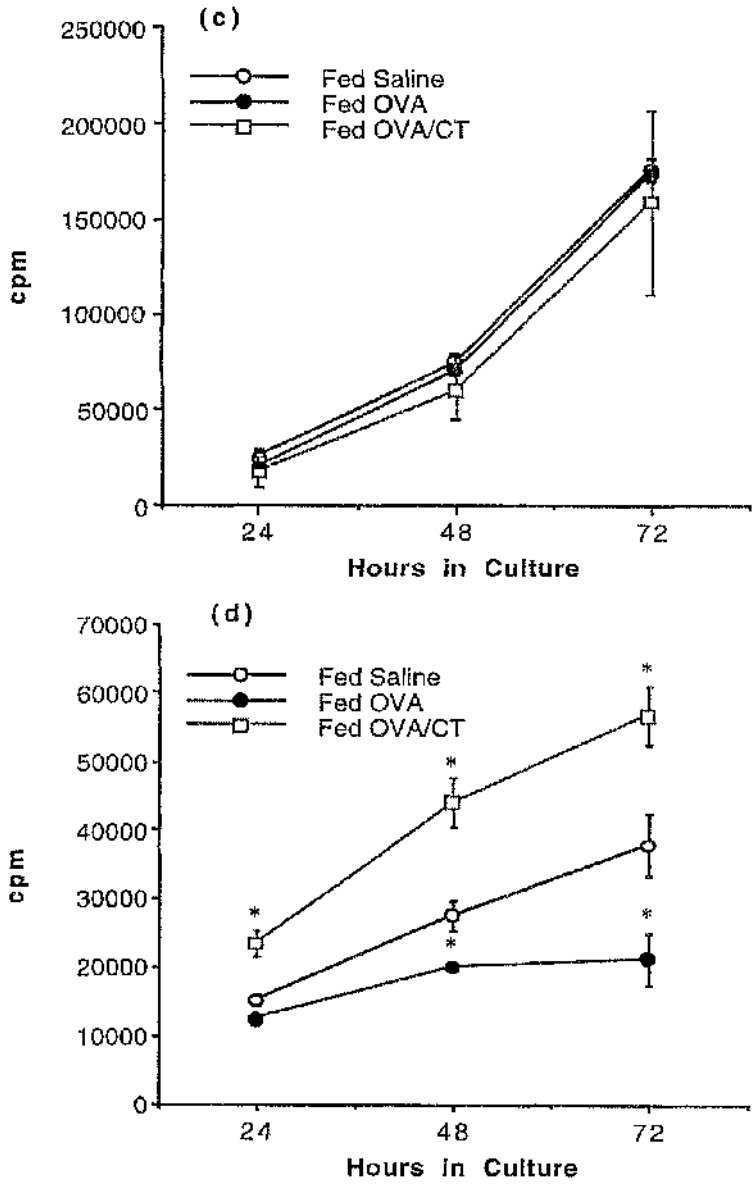


Figure 7.7 Expansion of Antigen Specific T cells in Mice Fed a High Dose of Antigen 5 Days After Priming

OVA-specific proliferative responses in the draining lymph nodes of mice adoptively transferred with 3 x 10^6 KJ1-26$^+$CD4$^+$ cells given a single feed of 200mg OVA or 200mg OVA + CT 5 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls, 1(**a**), 2(**b**), 3(**c**) and 7(**d**) days after feeding. The results shown are the mean uptake of ^{3}H-TdR ± 1 SD for quadruplicate cultures of lymph node cells from 2 mice per group after 72 hours in culture. (*$p<0.05$ versus controls)

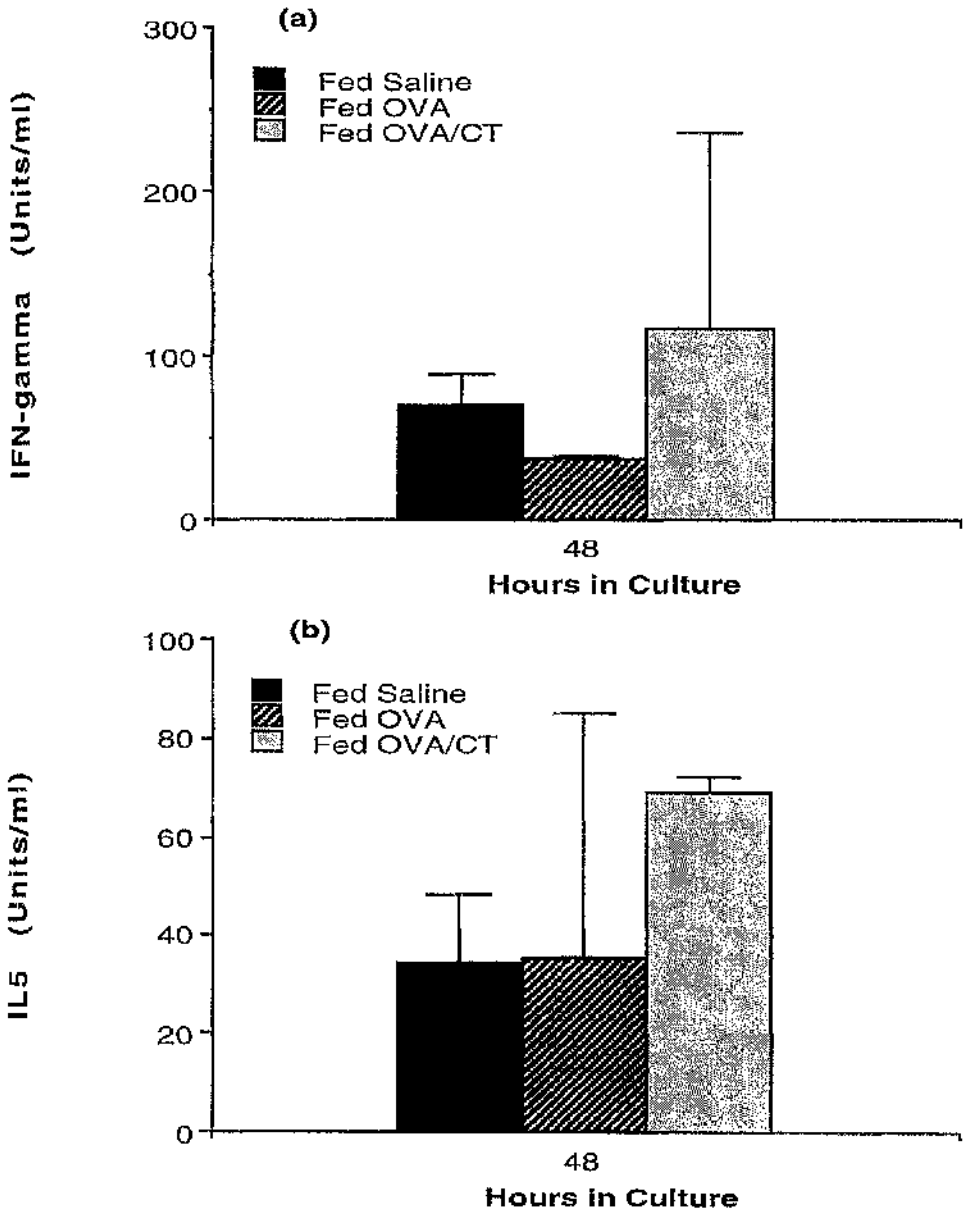


Figure 7.8 Expansion of Antigen Specific T cells in Mice Fed a High Dose of Antigen 5 Days After Priming

OVA-specific IFNγ (**a**) and IL5 (**b**) production in the draining lymph nodes of mice adoptively transferred with 3 x 10^6 KJ1-26$^+$CD4$^+$ cells given a single feed of 200mg OVA or 200mg OVA + CT 5 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls, 7 days after feeding. The results shown are mean cytokine levels (Units/ml) ± 1 SD of triplicate samples from supernatants of cells from 2 mice per group cultured *in vitro* for 2-4 days. There was little or no production of IFNγ or IL5 from cells cultured in the absence of antigen.

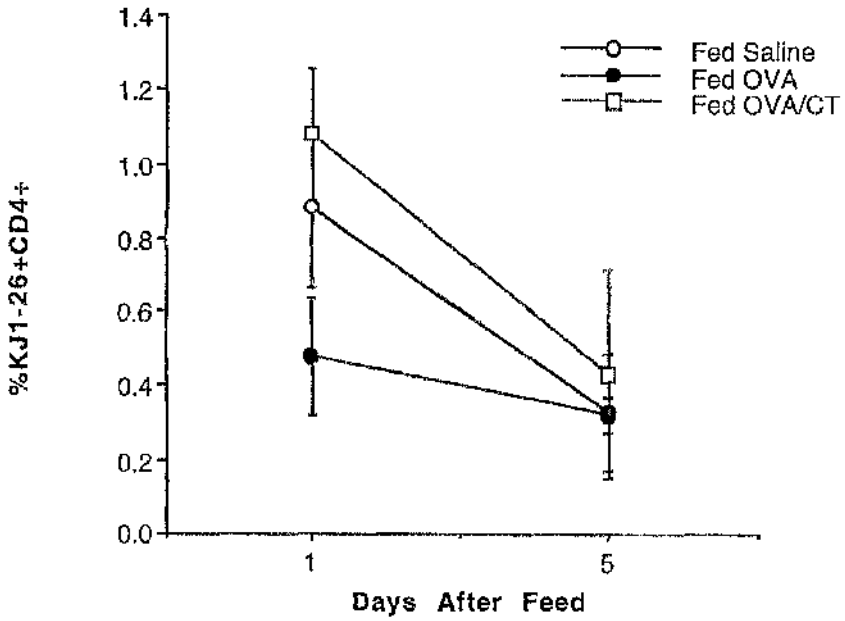


Figure 7.9 Reduction of Antigen Specific T cells in Mice Fed a High Dose of Antigen 12 Days After Priming

Percentage of KJ1-26$^+$CD4$^+$ cells in the draining lymph nodes of mice adoptively transferred with 3 x 10^6 KJ1-26$^+$CD4$^+$ cells and given a single feed of 200mg OVA or 200mg OVA + CT 12 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls. The results shown are the mean percentage of KJ1-26$^+$CD4$^+$ cells $\pm$ 1 SD in lymph node cells from 2 mice per group.

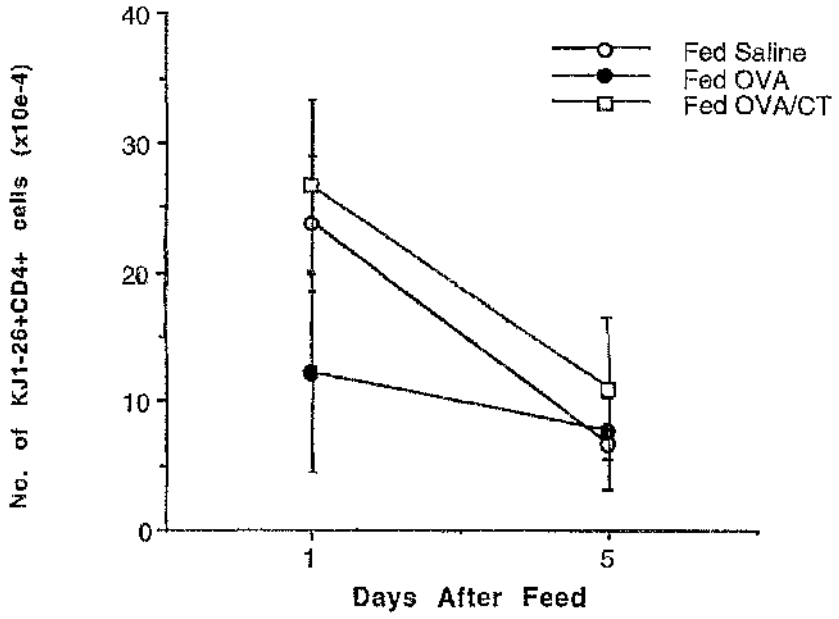


Figure 7.10 Reduction of Antigen Specific T cells in Mice Fed a High Dose of Antigen 12 Days After Priming

Numbers of KJ1-26^{+}CD4^{+} cells in the draining lymph nodes of mice adoptively transferred with 3 x 10^{6} KJ1-26^{+}CD4^{+} cells given a single feed of 200mg OVA or 200mg OVA+ CT 12 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls. The results shown are the mean absolute numbers of KJ1-26^{+}CD4^{+} cells ± 1 SD in lymph node cells from 2 mice per group.

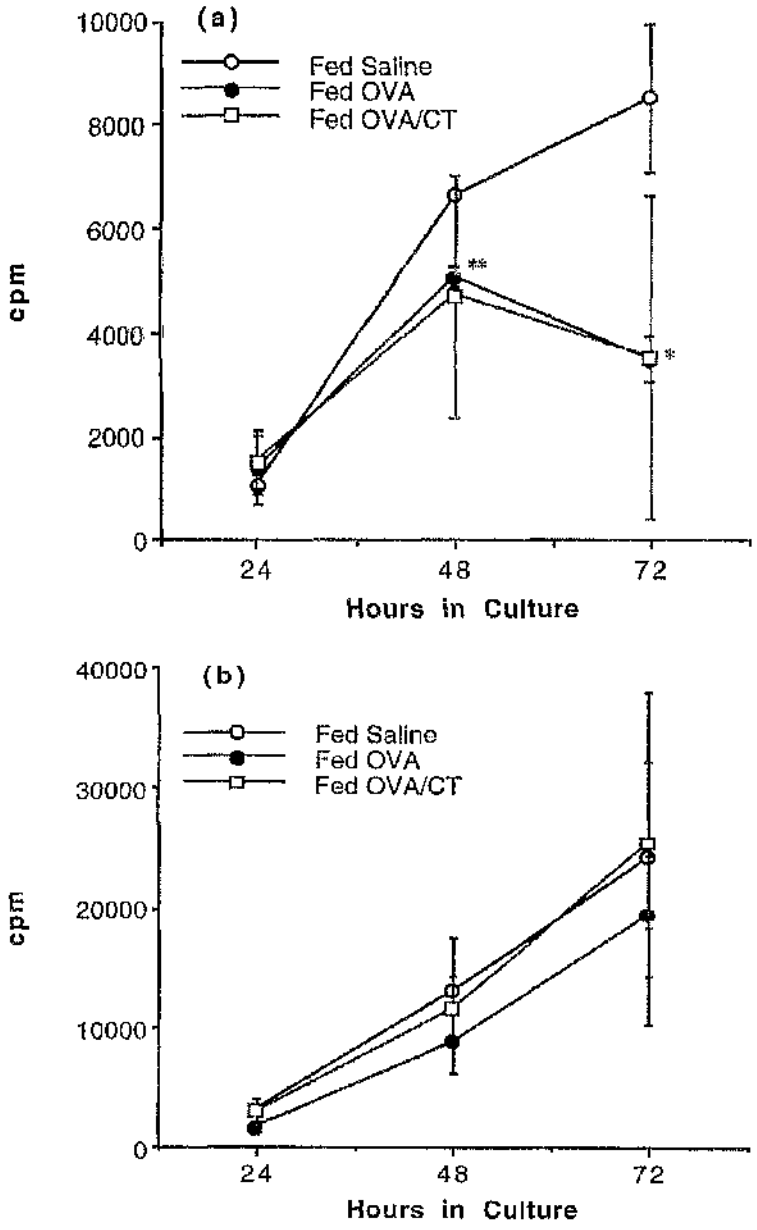


Figure 7.11 Reduction of Antigen Specific T cells in Mice Fed a High Dose of Antigen 12 Days After Priming

OVA-specific proliferative responses in the draining lymph nodes of mice adoptively transferred with 3 x 10^6 KJ1-26$^+$CD4$^+$ cells and given a single feed of 200mg OVA or 200mg OVA+ CT 12 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls, 1(**a**), and 5(**b**) days after feeding. The results shown are the mean uptake of ^{3}H-TdR ± 1 SD for quadruplicate cultures of lymph node cells from 2 mice per group after 72 hours in culture. (*$p<0.05$ versus controls, **$p<0.01$ versus controls)

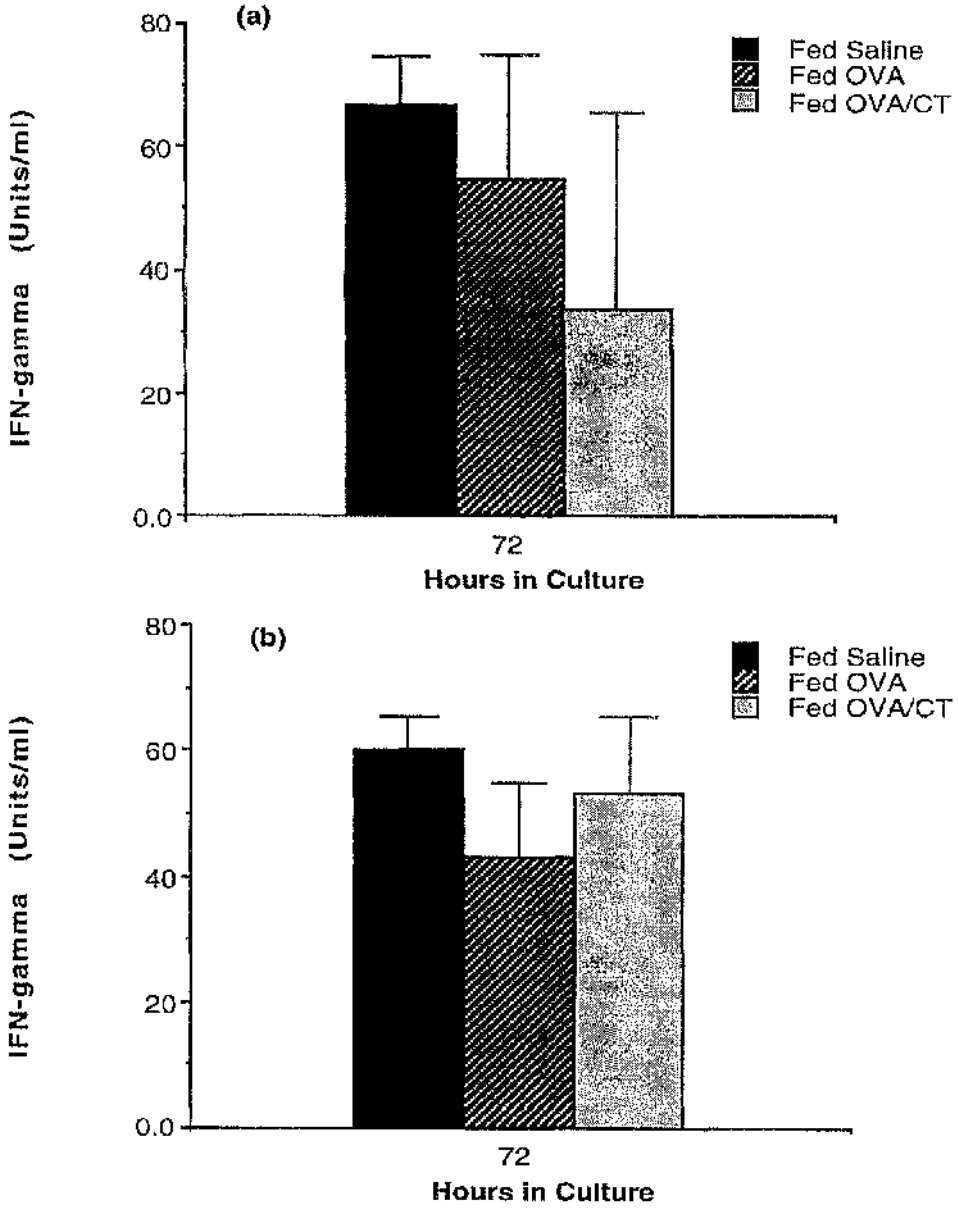


Figure 7.12 Reduction of Antigen Specific T cells in Mice Fed a High Dose of Antigen 12 Days After Priming

OVA-specific IFNγ production in the draining lymph nodes of mice adoptively transferred with 3 x 10^6 KJ1-26$^+$CD4$^+$ cells and given a single feed of 200mg OVA or 200mg OVA + CT 12 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls, 1(**a**), and 5(**b**) days after feeding. The results shown are the mean cytokine levels (Units/ml) ± 1 SD of triplicate samples from supernatants of cells from 2 mice per group cultured *in vitro* for 2-4 days. There was little or no production of IFNγ from cells cultured in the absence of antigen. (*$p<0.05$ versus controls, **$p<0.005$ versus controls)

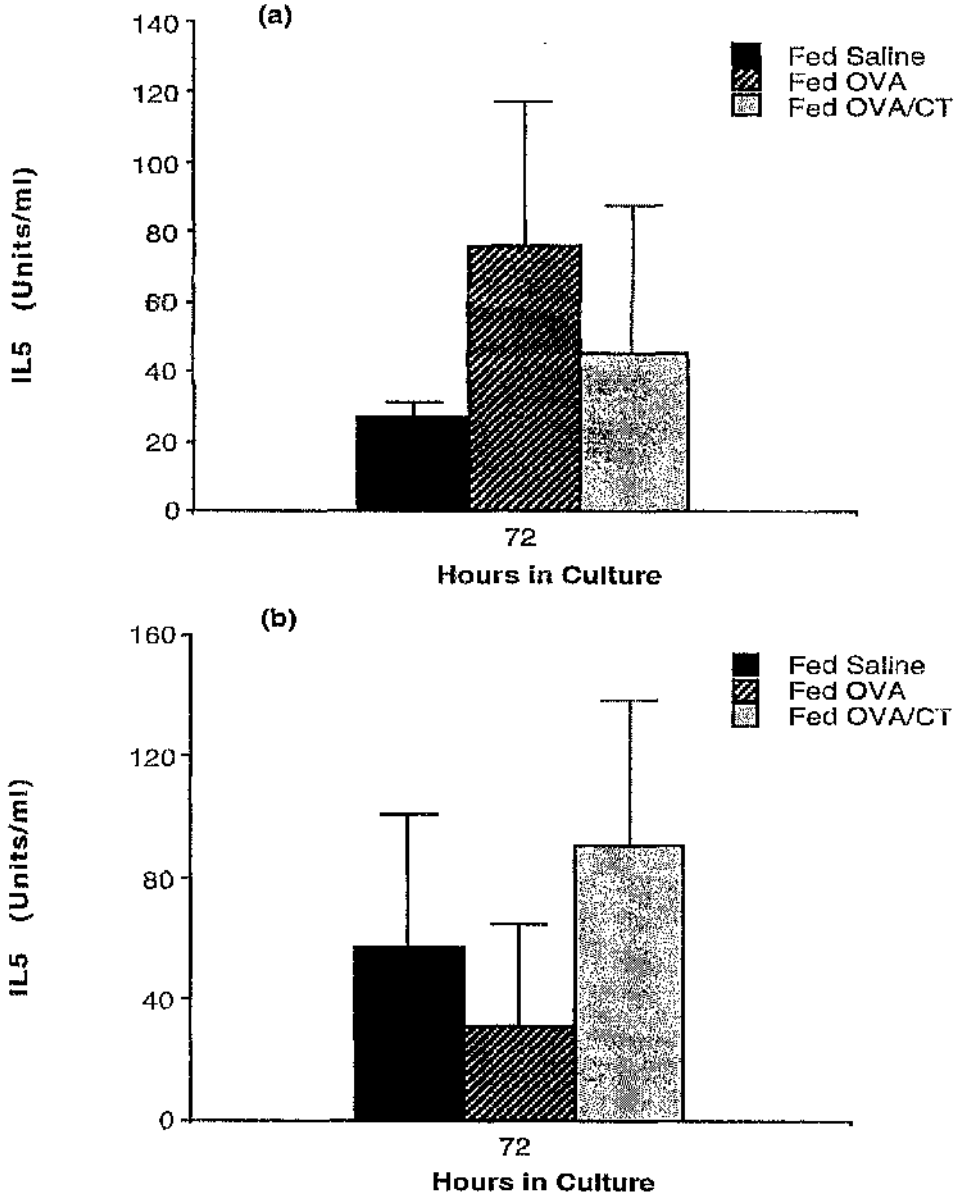


Figure 7.13 Reduction of Antigen Specific T cells in Mice Fed a High Dose of Antigen 12 Days After Priming

OVA-specific IL5 production in the draining lymph nodes of mice adoptively transferred with 3 x 10^6 KJ1-26$^+$CD4$^+$ cells and given a single feed of 200mg OVA or 200mg OVA + CT 12 days after subcutaneous immunisation with OVA/CFA, and in saline fed controls, 1(**a**), and 5(**b**) days after feeding. The results shown are the mean cytokine levels (Units/ml) ± 1 SD of triplicate samples from supernatants of cells from 2 mice per group cultured *in vitro* for 2-4 days. There was little or no production of IL5 from cells cultured in the absence of antigen. (*$p<0.05$ versus controls, **$p<0.005$ versus controls)

Chapter 8 Influence of Antigen Persistence and Memory Response on Oral Tolerance in Primed Mice

8.1 Introduction

The results I have obtained thus far have shown that it is *harder* to induce oral tolerance of CMI in primed mice than in naive mice and together these findings raise doubts about the general applicability of oral tolerance for therapeutic use and I decided to investigate some of the possible reasons underlying the resistance of the primed immune system to oral tolerance. The factors I considered were the effects of persisting antigen and adjuvant activity after initial priming and the possibilities that actively expanding or Ag-specific memory T cells were inherently resistant to tolerance induction.

To do this, I first fed antigen six weeks after immunisation with OVA/CFA, by which time I considered the immune response would be established and T cell expansion would have ceased. Under these conditions, there would still be a depot of antigen in an immunogenic form and I therefore repeated this experiment by feeding antigen six weeks after immunisation with OVA/LPS, where there would not be the same residual depot of antigen and adjuvant. Finally, I also assessed the susceptibility of antigen-specific memory T cells to tolerance induction using an adoptive transfer model in which primed transgenic T cells were given to normal mice.

8.2 Experimental Protocol

BALB/c mice mice were primed systemically in the footpad with 100µg OVA in 50µl CFA or with 100µg OVA + 50µg LPS in 0.2ml saline subcutaneously at the base of the tail on d0 and fed 25mg OVA at intervals thereafter. Control mice were fed saline after immunisation. 7 days after feeding, PLN were taken and Ag specific proliferation and cytokine production was assessed. 14 days after feeding mice were bled and serum assessed for antibody levels. On the same day mice were given HAO

in the other rear footpad and 24 hours later OVA-specific DTH responses were measured.

To obtain primed OVA-specific transgenic T cells, BALB/c mice were adoptively transferred with DO11.10 TCR transgenic T cells as described by Kearney *et al* (143) Mice were injected with 7.5 x 10^6 KJ1-26$^+$CD4$^+$ cells intravenously and two days later mice were immunised sc with OVA/CFA. Two weeks later, lymph node cells were prepared from these mice and the percentage of primed KJ1-26$^+$ cells present calculated by flow cytometry and 0.5 x 10^6 KJ1-26$^+$CD4$^+$ cells were then transferred into naive mice. These secondary recipient mice were fed either saline or 200mg OVA 2 days after receiving the primed Tg T cells and assessed for tolerance 1, 3 and 5 days after feeding by *in vitro* proliferation and cytokine production. Control groups of naive recipient mice received 0.5 x 10^6 naive KJ1-26$^+$CD4$^+$ cells and tolerised.

8.3 Results

8.3.1 Effects of Feeding Antigen on Fully Established Immune Responses.

In the first experiment, I set out to eliminate the possibility that the resistance of primed mice reflected the fact that in my experiments I was feeding mice relatively soon after priming when it might be anticipated that T cell expansion was proceeding. To examine this, mice were fed 25mg OVA 6 weeks after immunisation with OVA/CFA by which time the immune response would be fully established.

8.3.1.1 *In vivo* Responses

Saline fed control mice still had significant DTH and serum IgG responses when assessed 8 weeks later. However, mice fed 25mg OVA six weeks after immunisation had no significant tolerance of DTH (Figure 8.1), total OVA-specific

IgG antibody (Figure 8.2a), IgG1 (Figure 8.2b) or IgG2a (Figure 8.2c) antibodies compared with controls.

8.3.1.2 *In vitro* Responses

Proliferation responses were low in saline fed mice by this time point (Figure 8.3), although these mice still had significant IFNγ and IL5 production (Figure 8.4). OVA-specific IFNγ production was significantly decreased in mice fed OVA 6 weeks after immunisation, but proliferative activity was markedly and significantly enhanced in OVA fed animals. OVA-specific IL5 production was also significantly enhanced, but to a lower extent.

Thus feeding OVA to mice with a fully established immune response does not improve tolerance. Indeed, feeding OVA at this time appeared to stimulate a secondary response for some aspects of the systemic immune response.

8.3.2 Effects of Feeding OVA After Priming with OVA/LPS on Subsequent Effector Functions.

In all the experiments performed thus far, I used CFA as the adjuvant to induce systemic immunity. I considered it was possible that the long term resistance of these mice to oral tolerance might reflect the depot effect of this adjuvant, with persistently high levels of antigen being generated in the context of chronic inflammation and active costimulatory activity. To examine this possibility, I primed mice with OVA and LPS as a means of administering antigen in an adjuvant without long term depot effects.

8.3.2.1 *In vivo* Responses

I first examined the induction of tolerance using the protocols I had carried out with CFA, by feeding feeding mice 2 or 7 days after priming. Compared with CFA, immunisation with OVA and LPS induced lower levels of antigen-specific primary immune responses, especially those measured *in vivo*. DTH responses were

reasonable, but antibody production was low. Nevertheless, as with mice primed with CFA mice fed 25mg OVA 2 or 7 days after immunisation with OVA + LPS had significant tolerance of DTH (Figure 8.5) compared with unfed controls. No significant tolerance of total OVA-specific IgG antibody (Figure 7.8a) and OVA-specific IgG1 (Figure 8.6b) and IgG2a (Figure 8.6c) antibodies was found in any OVA fed mice.

8.3.2.2 *In vitro* Responses

OVA + LPS immunised mice had reasonable proliferative responses and IFNγ / IL5 production *in vitro*. Mice fed 25mg OVA 2 days after immunisation had significant tolerance of OVA-specific proliferation (Figure7.7), IFNγ and IL5 production (Figure7.8). However, OVA-specific proliferation was normal in mice fed 7 days after immunisation, while IFNγ production was significantly increased in these animals and IL5 production was decreased.

Thus feeding OVA early after immunisation with OVA/LPS results in a pattern of tolerance similar to that found when mice are primed using CFA as an adjuvant.

8.3.3 Effects of Feeding OVA Six Weeks After Priming with OVA/LPS on Subsequent Effector Functions

As oral tolerance was normal early after priming with OVA/LPS, I went on to feed 25mg OVA 6 weeks after immunisation with OVA/LPS by which time the immune response would be fully established and I reasoned that there would be no lingering effects of the adjuvant.

Under these conditions, OVA-specific DTH responses (Figure 8.9) were reduced compared with controls, but there was no significant tolerance of total IgG antibody (Figure 8.10a), IgG1 (Figure 8.10b) and IgG2a (Figure 8.10c) antibodies.

There was also no significant tolerance of OVA-specific proliferation (Figure 8.11), IFNγ and IL5 production (Figure 8.12).

Thus, as I found earlier when mice were primed with OVA/CFA, feeding OVA to mice with a fully established immune response does not improve tolerance. I therefore concluded that the difficulty in inducing oral tolerance six weeks after priming is not due to a depot of antigen or adjuvant, but rather the presence of an established immune response.

8.3.4 Effects of Feeding Antigen on Adoptively Transferred Antigen Experienced or Naive Transgenic T Cells

As the results above suggested that the resistance of primed mice to oral tolerance was not simply due to persisting antigen, I decided to test the idea that activated/memory CD4 T cells were inherently resistant to tolerance induction after initial exposure to a priming dose of antigen. To examine this possibility directly, I made use of an adoptive transfer system, in which naive or previously activated OVA-specific transgenic T cells were transferred into naive BALB/c mice which were then tolerised by feeding 200mg OVA.

8.3.4.1 *In vivo* and *In vitro* Responses

Antigen experienced 'memory' cells were obtained from recipients of naive DO11.10 Tg T cells which had been immunised with OVA/CFA 14 days earlier. This protocol for obtaining antigen experienced 'memory' cells is the same as that used by Pape, *et al* who showed that these Tg cells have a reduced expression of CD45RB (180).

As controls in this experiment, I adoptively transferred mice with naive DO11.10 Tg T cells and fed them a tolerogenic dose of OVA. Under these conditions, the expansion of Tg T cells was identical to that found in recipients of naive Tg T cells fed saline (Figure 8.13), with no evidence of the early expansion and subsequent reduction compared with saline fed controls, as has been reported in a previous study (179). However, cells isolated 10 days after feeding OVA had reduced proliferative

responses to OVA *in vitro* when compared with unfed mice transferred with naive cells, confirming the induction of functional tolerance (Figure 8.14d). It should be noted here that the OVA-specific proliferation results in Figure 8.14c+d are calculated per individual Tg T cell present in the cultured population as previously calculated by Pape *et al* for IL2 production (180). The proliferation results of the whole cultured populations follow a similar pattern to those proliferation results calculated per individual Tg T cell present in the cultured populations, however, in some cases apparent increases or decreases were only significant when calculated one way or the other. Thus, I used both methods for calculating OVA-specific proliferation. As expected, T cells from mice immunised with an immunogenic protocol of OVA/LPS sc after receiving naive cells expanded in peripheral lymphoid tissues and proliferated well when restimulated with OVA *in vitro*.

When antigen experienced 'memory' cells were transferred into secondary recipients, only small numbers of these cells could be detected in the peripheral lymphoid tissues, especially in the group of secondary recipients examined 10 days after feeding. In addition, these cells showed reduced expansion in response to an immunogenic challenge with OVA/LPS sc compared with naive Tg cells (Figure 8.13) and also had reduced proliferative responses to further restimulation with OVA *in vitro* after *in vivo* priming with OVA/LPS (Figure 8.14). This poor clonal expansion was not surprising given that this was their third exposure to antigen and so confirms the 'memory' nature of the transferred cells. When memory cells were isolated from saline fed secondary recipients at 4 and 10 days, these cells responded to restimulation with OVA *in vitro* as did cells from recipients of naive Tg cells (Figure 8.14). Although feeding OVA to the secondary recipients had no effect on the numbers or proportions of Tg cells in peripheral lymphoid tissues, there were reduced antigen-specific proliferative responses in OVA fed mice 4 days after feeding (Figure 8.14c) suggesting that activated/memory CD4 T cells are not inherently resistant to tolerance induction. By 10 days after feeding these responses were normal (Figure 8.14d).

8.4 Conclusions

In this chapter I attempted to examine some of the possible reasons underlying the resistance of the primed immune system to oral tolerance. In the first experiment, I found that feeding OVA to mice with a fully established immune response did not improve tolerance. Indeed, feeding OVA at this time appeared to stimulate a secondary response for some aspects of the systemic immune response. As I thought this long term resistance to oral tolerance might reflect the depot effect of this adjuvant, I decided to prime mice with OVA and LPS as a means of administering antigen in an adjuvant without long term depot effects. I found that feeding OVA early after immunisation with OVA/LPS results in a pattern of tolerance similar to that found when mice are primed using CFA as the adjuvant. Next, I went on to find that feeding OVA to mice with a fully established immune response to immunisation with OVA/LPS did not improve tolerance. Thus, I concluded that the difficulty in inducing oral tolerance six weeks after priming was not due to a depot of antigen or adjuvant, but rather the presence of an established immune response. Therefore, I decided to test the idea that activated/memory CD4 T cells were inherently resistant to tolerance induction after initial exposure to a priming dose of antigen. When OVA-specific memory Tg T cells were transferred into secondary recipients, they expanded and proliferated less well than naive Tg cells in response to immunogenic challenge *in vivo* and *in vitro*. However, my experiments showed that the proliferation of these cells could be further reduced by feeding a tolerogenic dose of OVA. Thus, I conclude from the experiments presented in this chapter that activated/memory CD4 T cells are not inherently resistant to orally induced tolerance and that their resistance to tolerance is due to factors present in the microenvironment of the primed mouse such as the persistence of antigen or presence of costimulation.

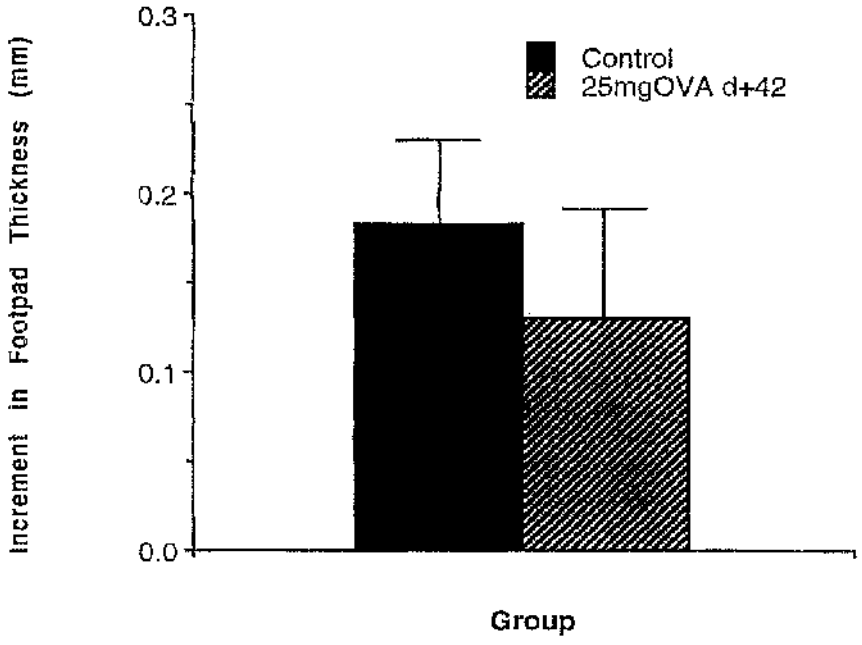
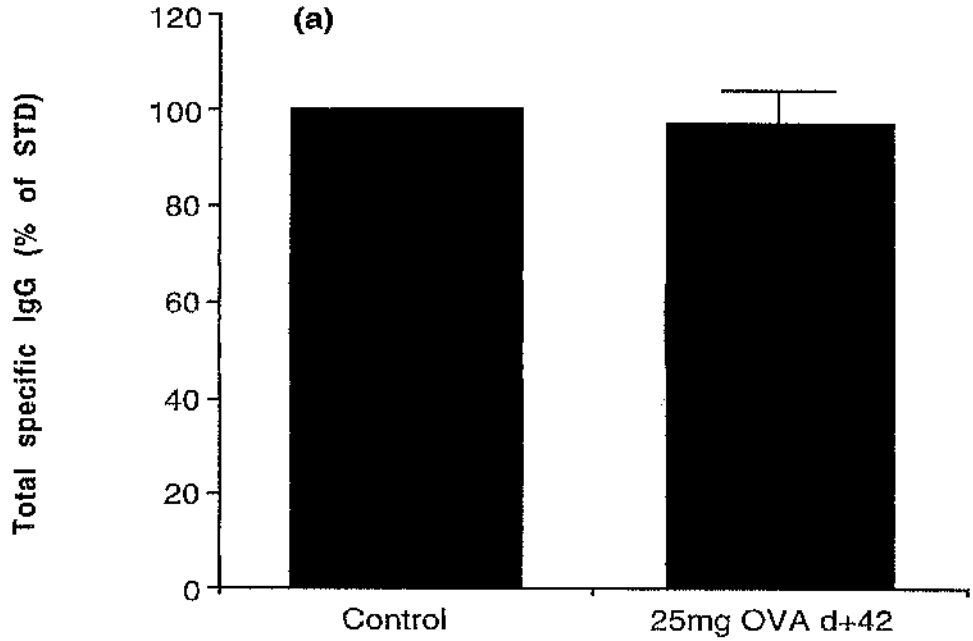
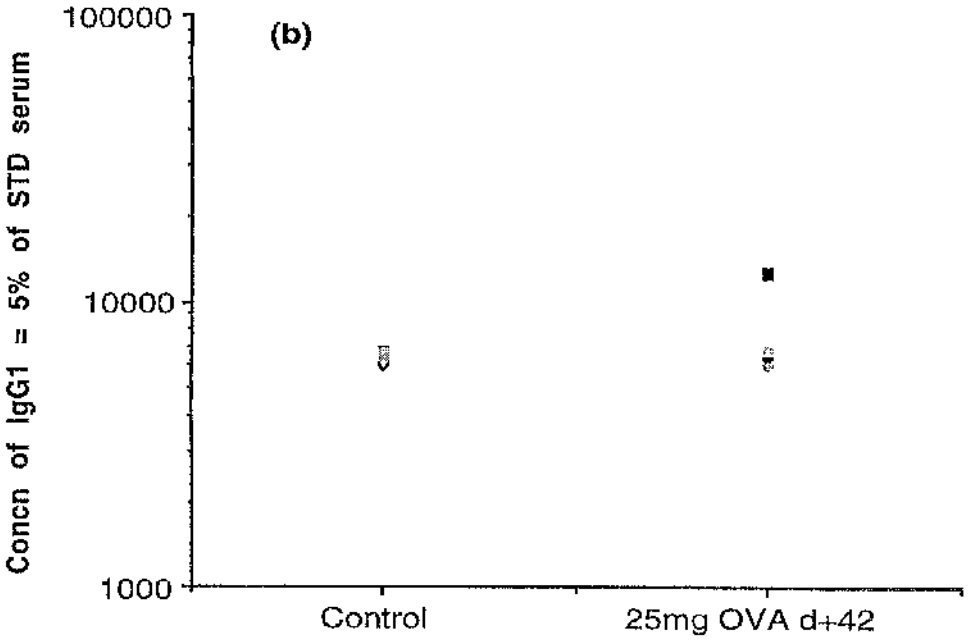


Figure 8.1 Effects of Feeding Antigen Six Weeks After Immunisation on Systemic Immunity.

Systemic DTH responses in mice given a single feed of 25mg OVA six weeks after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 14 days after feeding. The results shown are mean specific increments in footpad thickness ± 1 SD for 5 mice per group.


(a)
Total specific IgG (% of STD)
120
100
80
60
40
20
0
Control
25mg OVA d+42


(b)
Concn of IgG1 = 5% of STD serum
100000
10000
1000
Control
25mg OVA d+42

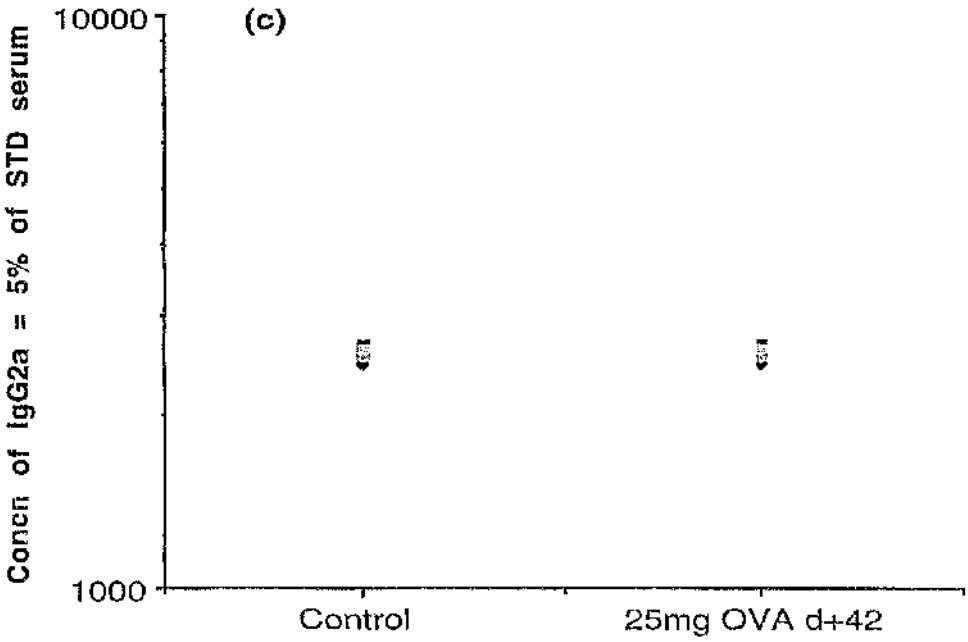


Figure 8.2 Effects of Feeding Antigen Six Weeks After Immunisation on Systemic Immunity.

(a) OVA-specific total serum IgG antibody responses in mice given a single feed of 25mg OVA six weeks after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 14 days after feeding. The results shown are mean % of purified IgG standard ± 1 SD for individual sera from 5 mice per group. (b) OVA-specific IgG1 and (c) IgG2a antibody responses in control and OVA fed mice. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group.

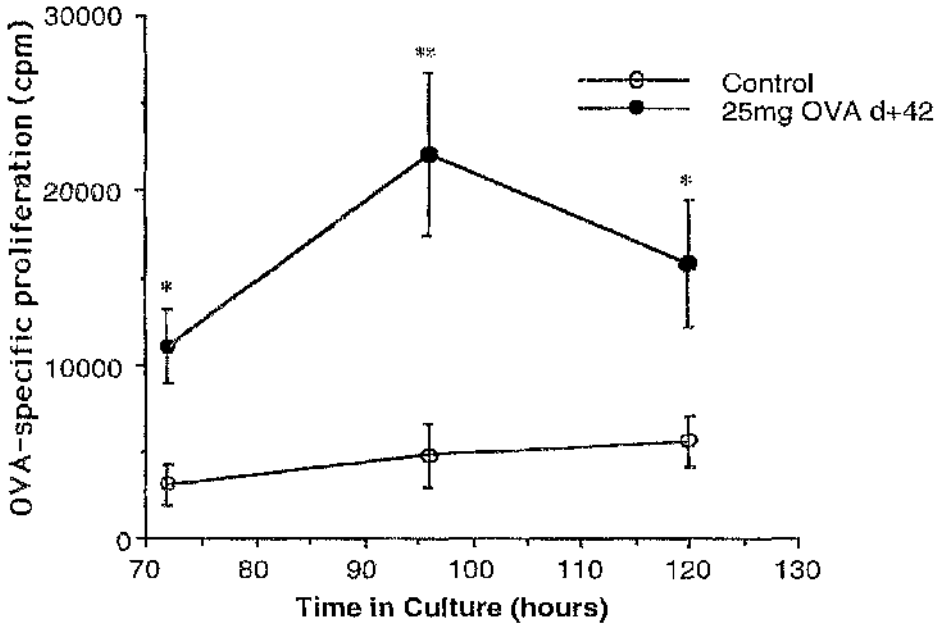


Figure 8.3 Effects of Feeding Antigen Six Weeks After Immunisation on Systemic Immunity.

OVA-specific proliferative responses in draining lymph nodes of mice given a single feed of 25mg OVA six weeks after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 7 days after feeding. The results shown are mean uptake of ^{3}H-TdR ± 1 SD for quadruplicate cultures of lymph node cells pooled from 3 mice per group. (*$p<0.002$ versus Control, **$p<0.001$ versus Control)

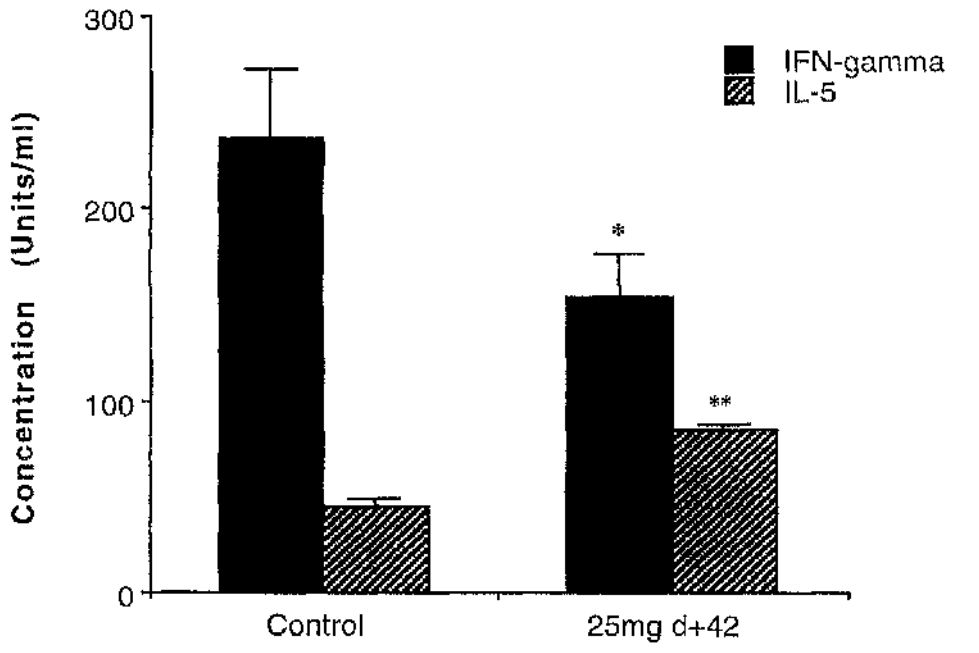


Figure 8.4 Effects of Feeding Antigen Six Weeks After Immunisation on Systemic Immunity.

OVA-specific IFNγ and IL5 production in draining lymph nodes of mice given a single feed of 25mg OVA six weeks after subcutaneous immunisation with OVA/CFA, and in saline fed controls measured 7 days after feeding. The results shown are mean cytokine levels (U/ml) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 2-4 days. (*$p<0.05$ versus Control, **$p<0.001$ versus Control)

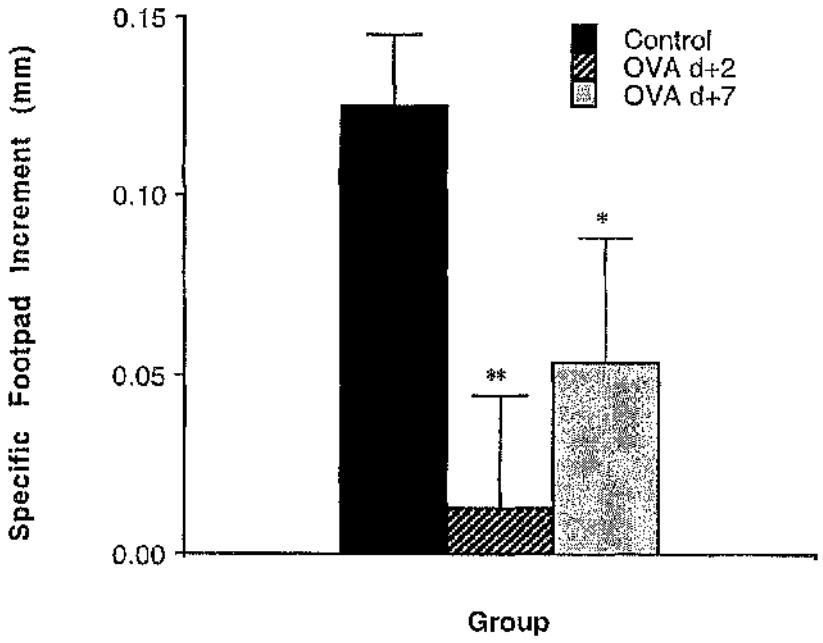
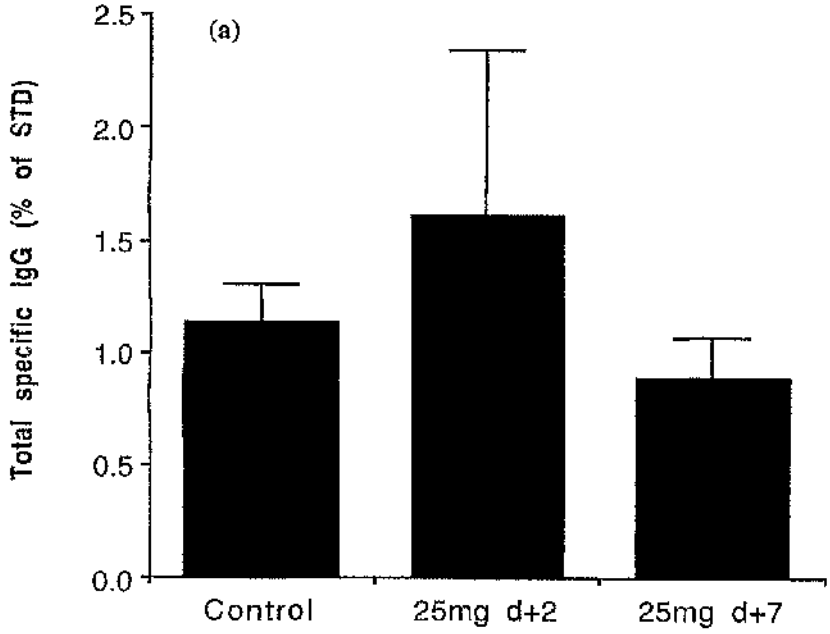
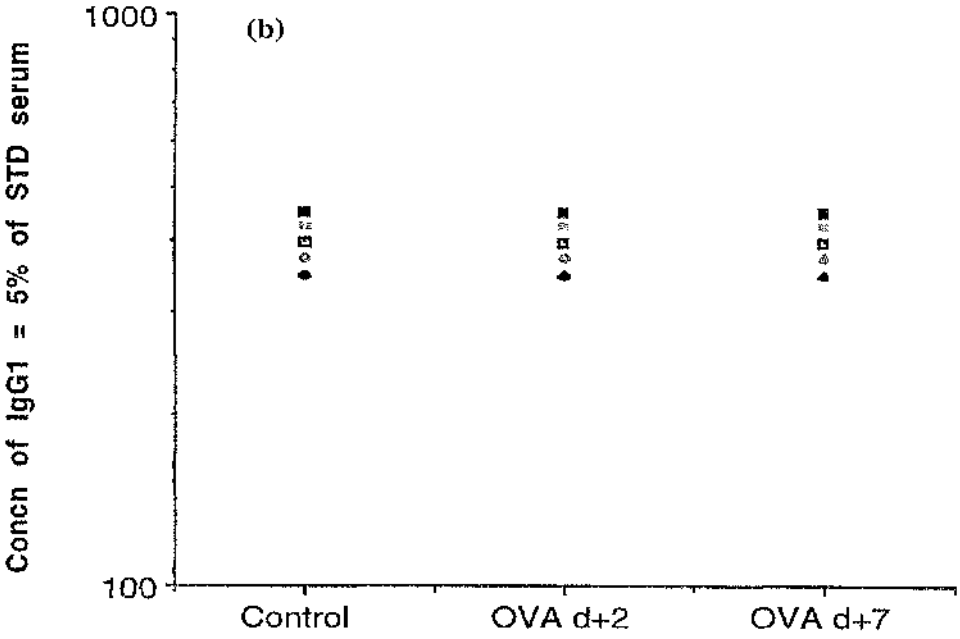


Figure 8.5 Effects of Feeding Antigen After Immunisation with OVA/LPS on Systemic Immunity.

Systemic DTH responses in mice given a single feed of 25mg OVA 2 or 7 days after subcutaneous immunisation with OVA/LPS, and in saline fed controls measured 21 days after immunisation. The results shown are mean specific increments in footpad thickness ± 1 SD for 5 mice per group. (*$p<0.005$ versus Control, **$p<0.001$ versus Control)


(a)
Total specific IgG (% of STD)
2.5
2.0
1.5
1.0
0.5
0.0
Control
25mg d+2
25mg d+7


(b)
Concn of IgG1 = 5% of STD serum
1000
100
Control
OVA d+2
OVA d+7

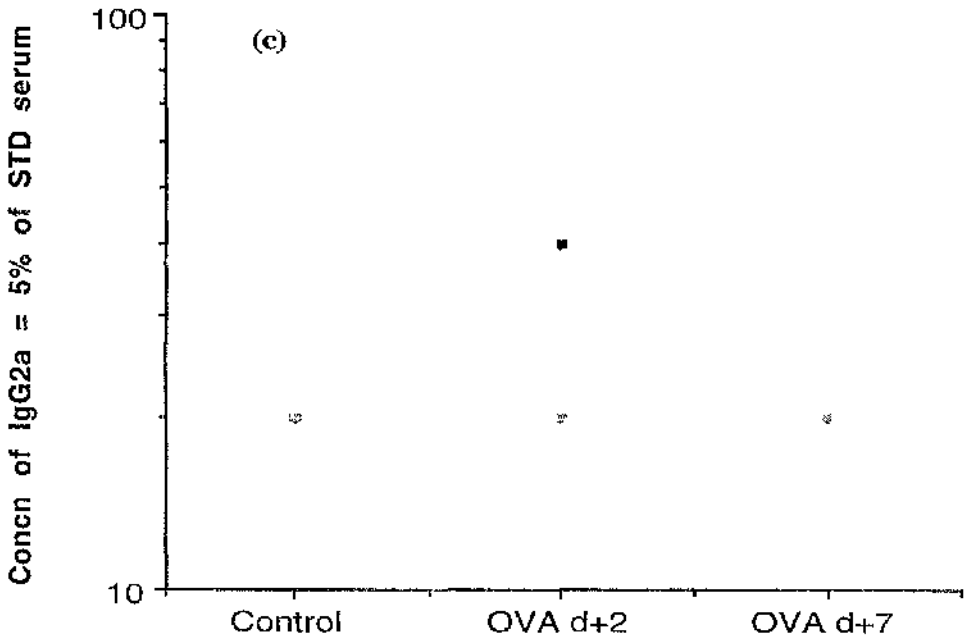


Figure 8.6 Effects of Feeding Antigen After Immunisation with OVA/LPS on Systemic Immunity.

(a) OVA-specific total serum IgG antibody responses in mice given a single feed of 25mg OVA 2 or 7 days after subcutaneous immunisation with OVA/LPS, and in saline fed controls measured 21 days after immunisation. The results shown are mean % of purified IgG standard ± 1 SD for individual sera from 5 mice per group. **(b)** OVA-specific IgG1 and **(c)** IgG2a antibody responses in control and OVA fed mice. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group.

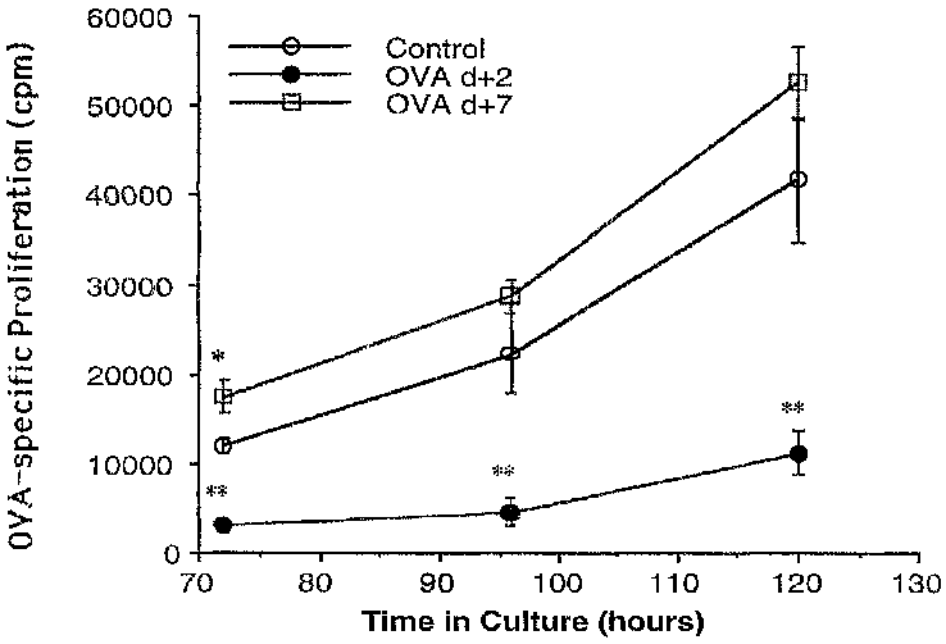


Figure 8.7 Effects of Feeding Antigen After Immunisation with OVA/LPS on Systemic Immunity.

OVA-specific proliferative responses in draining lymph nodes of mice given a single feed of 25mg OVA 2 or 7 days after subcutaneous immunisation with OVA/LPS, and in saline fed controls measured 14 days after immunisation. The results shown are mean uptake of ^{3}H-TdR ± 1 SD for quadruplicate cultures of lymph node cells pooled from 3 mice per group. (*$p<0.002$ versus Control, **$p<0.001$ versus Control)

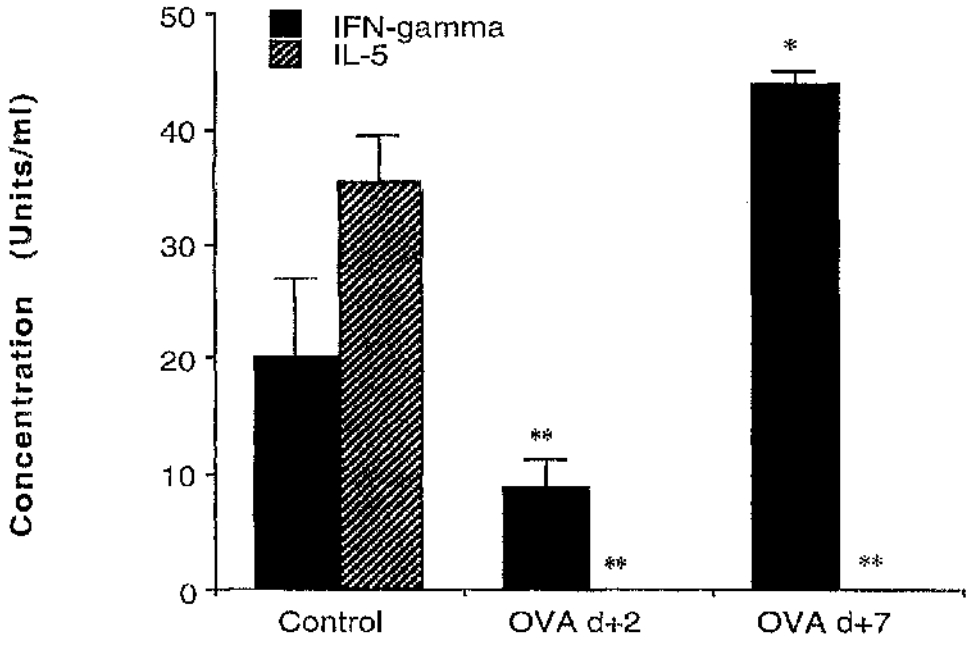


Figure 8.8 Effects of Feeding Antigen After Immunisation with OVA/LPS on Systemic Immunity.

OVA-specific IFNγ and IL5 production in draining lymph nodes of mice given a single feed of 25mg OVA 2 or 7 days after subcutaneous immunisation with OVA/LPS, and in saline fed controls measured 14 days after immunisation. The results shown are mean cytokine levels (U/ml) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 2-4 days. (*$p<0.01$ versus Control, **$p<0.005$ versus Control)

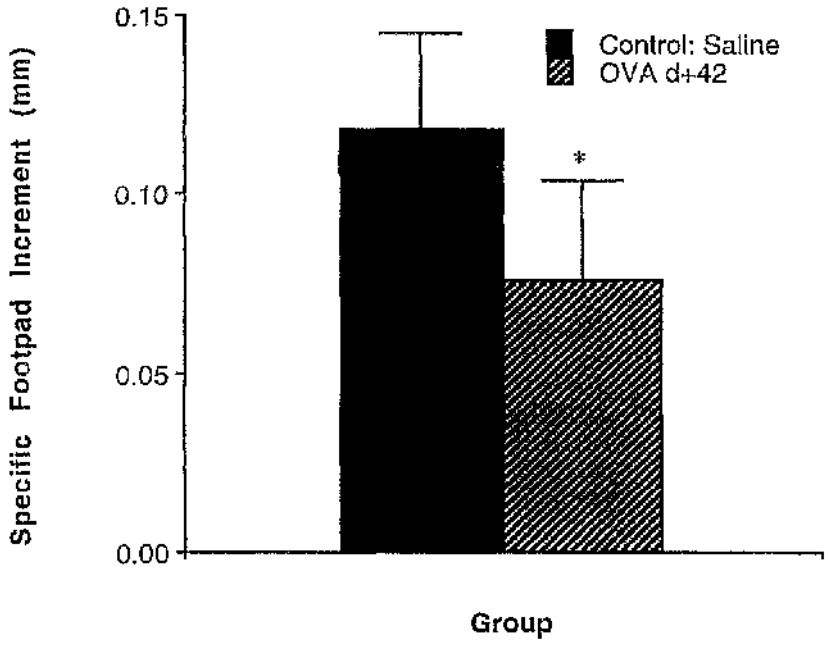
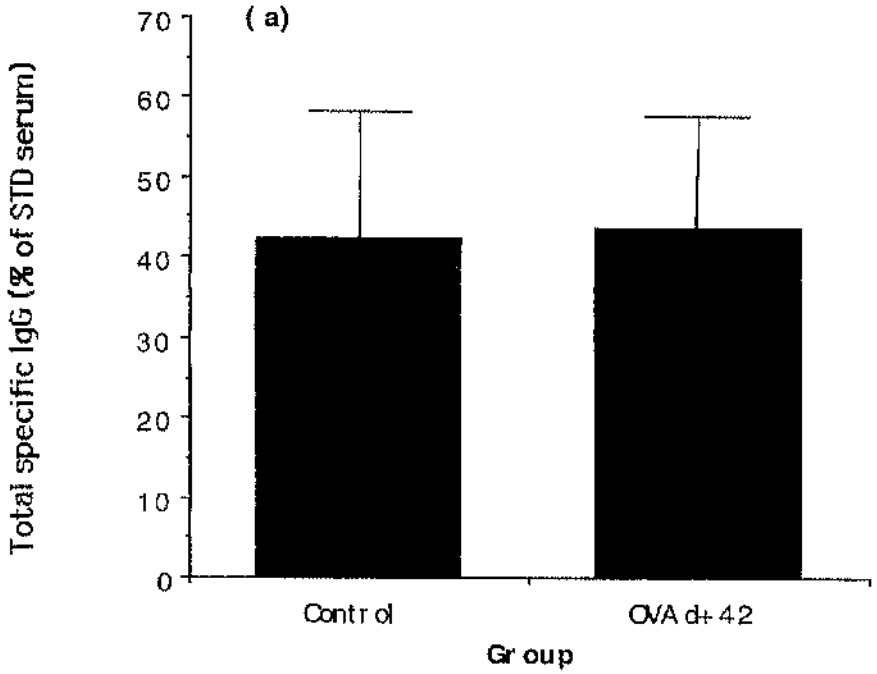
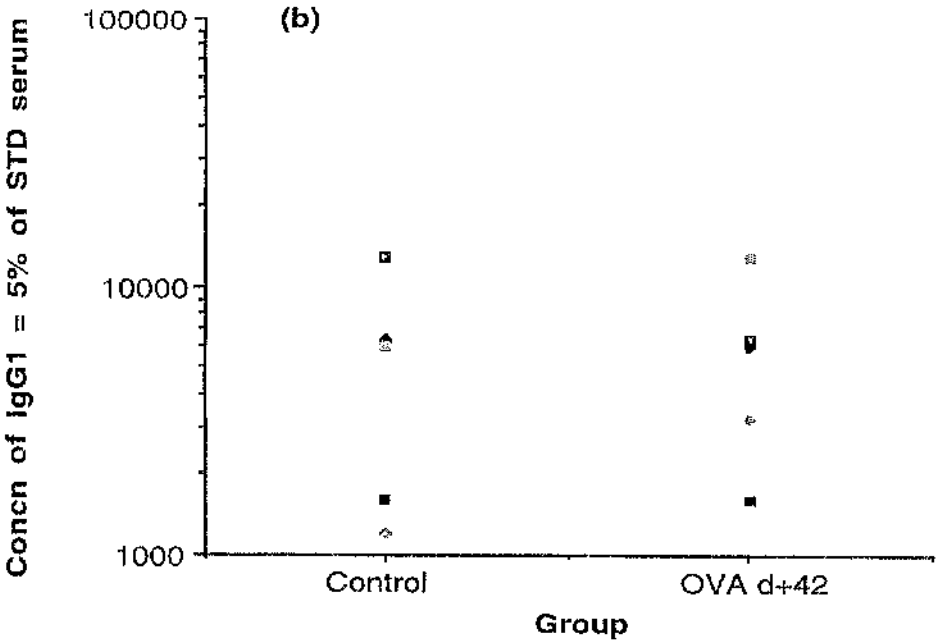


Figure 8.9 Effects of Feeding Antigen Six Weeks After Immunisation with OVA/LPS on Systemic Immune Responses.

Systemic DTH responses in mice given a single feed of 25mg OVA six weeks after subcutaneous immunisation with OVA/LPS, and in saline fed controls measured 14 days after feeding. The results shown are mean specific increments in footpad thickness ± 1 SD for 5 mice per group. (*$p<0.05$ versus Control)


(a)
70
60
50
40
30
20
10
0
Total specific IgG (% of STD serum)
Contr ol
OVA d+ 42
Gr oup


(b)
100000
10000
1000
Concn of IgG1 = 5% of STD serum
Control
OVA d+42
Group

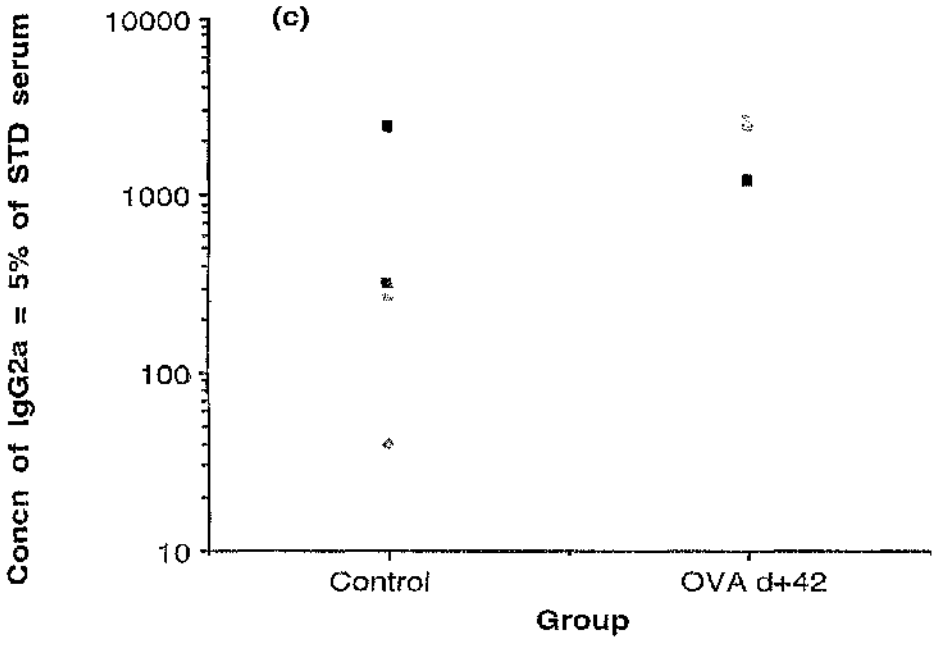


Figure 8.10 Effects of Feeding Antigen Six Weeks After Immunisation with OVA/LPS on Systemic Immune Responses.

(a) OVA-specific total serum IgG antibody responses in mice given a single feed of 25mg OVA six weeks after subcutaneous immunisation with OVA/LPS, and in saline fed controls measured 14 days after feeding. The results shown are mean % of purified IgG standard ± 1 SD for individual sera from 5 mice per group. (b) OVA-specific IgG1 and (c) IgG2a antibody responses in control and OVA fed mice. The results shown are the reciprocal dilutions which give an OD equal to 5% of that given by a standard serum from hyperimmunised control mice for 5 mice per group.

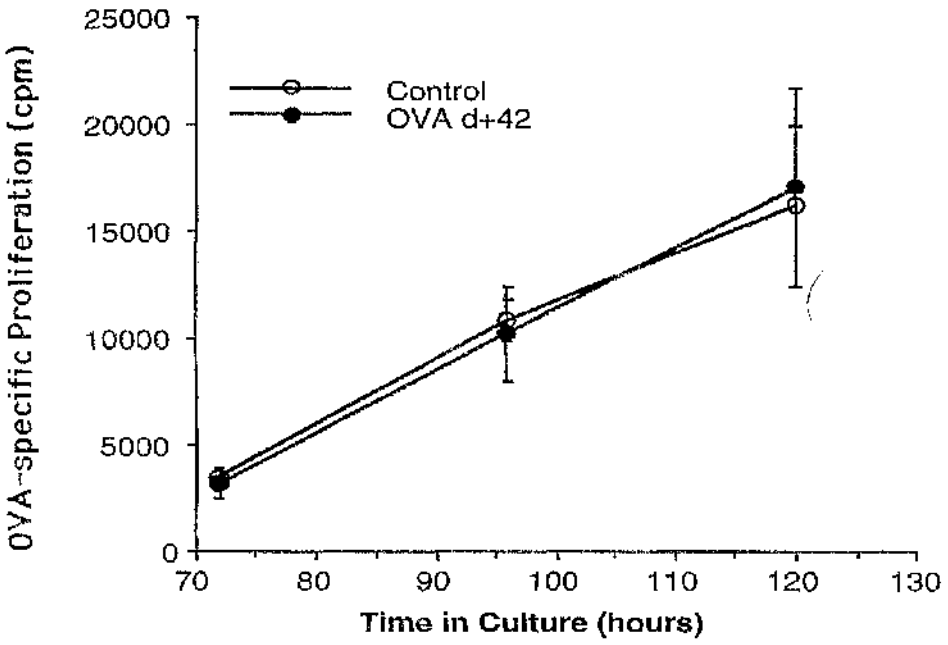


Figure 8.11 Effects of Feeding Antigen Six Weeks After Immunisation with OVA/LPS on Systemic Immune Responses.

OVA-specific proliferative responses in draining lymph nodes of mice given a single feed of 25mg OVA six weeks after subcutaneous immunisation with OVA/LPS, and in saline fed controls measured 7 days after feeding. The results shown are mean uptake of ^{3}H-TdR ± 1 SD for quadruplicate cultures of lymph node cells pooled from 3 mice per group.

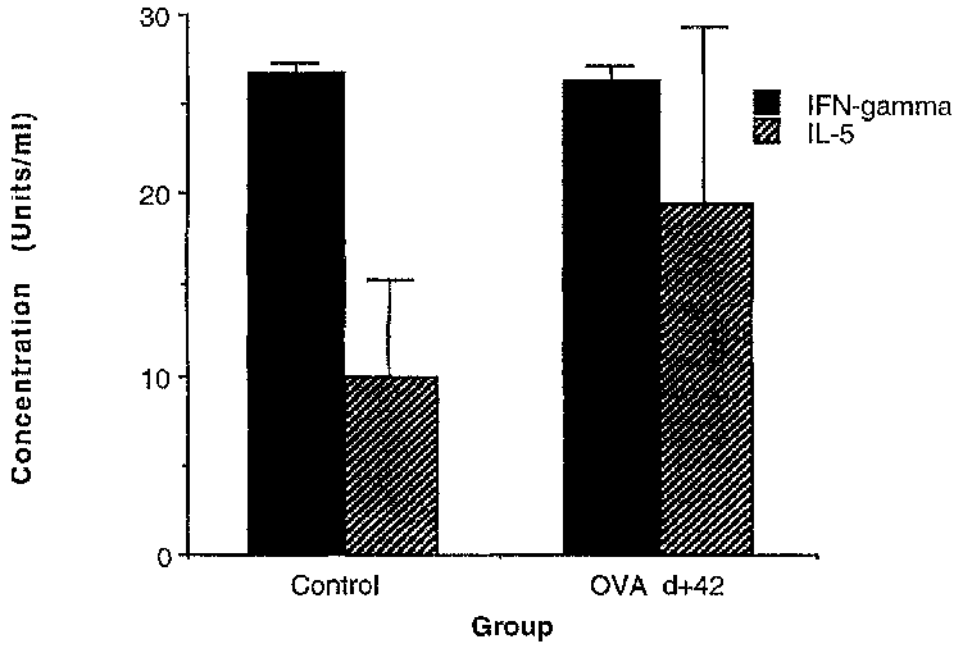


Figure 8.12 Effects of Feeding Antigen Six Weeks After Immunisation with OVA/LPS on Systemic Immune Responses.

OVA-specific IFNγ and IL5 production in draining lymph nodes of mice given a single feed of 25mg OVA six weeks after subcutaneous immunisation with OVA/LPS, and in saline fed controls measured 7 days after feeding. The results shown are mean cytokine levels (U/ml) ± 1 SD of triplicate samples from supernatants of cells pooled from 3 mice per group cultured *in vitro* for 2-4 days.

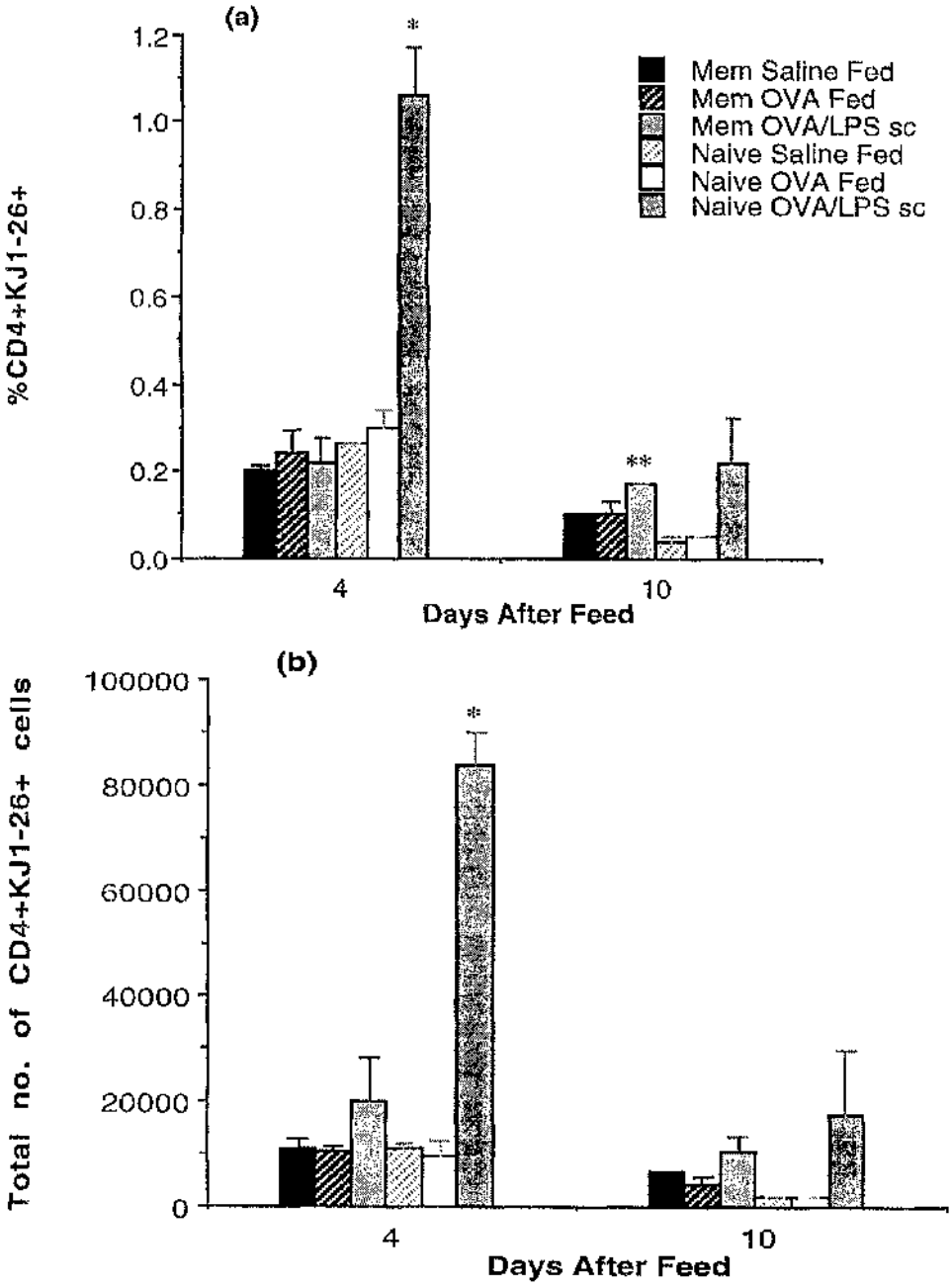
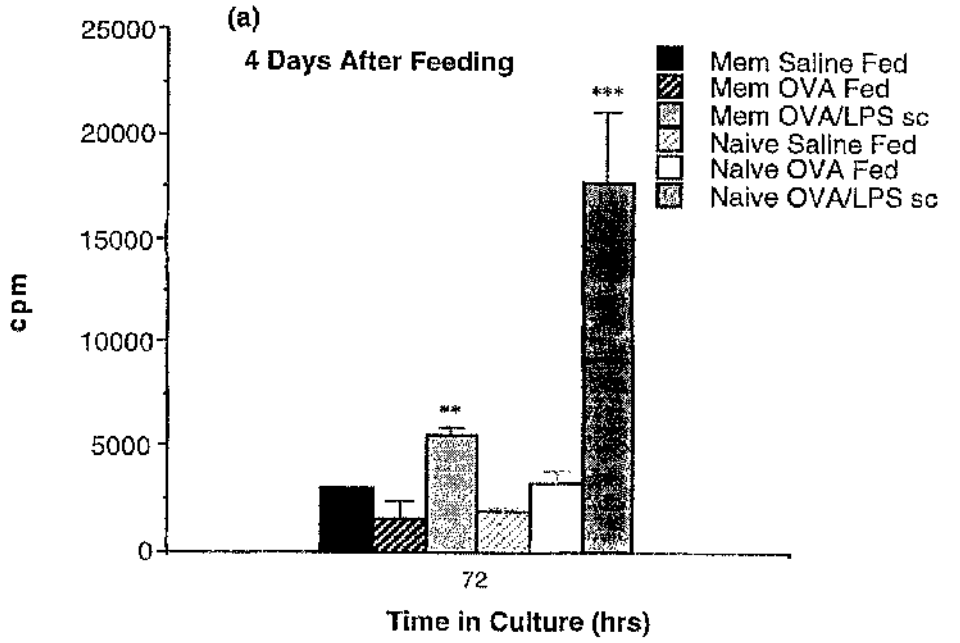
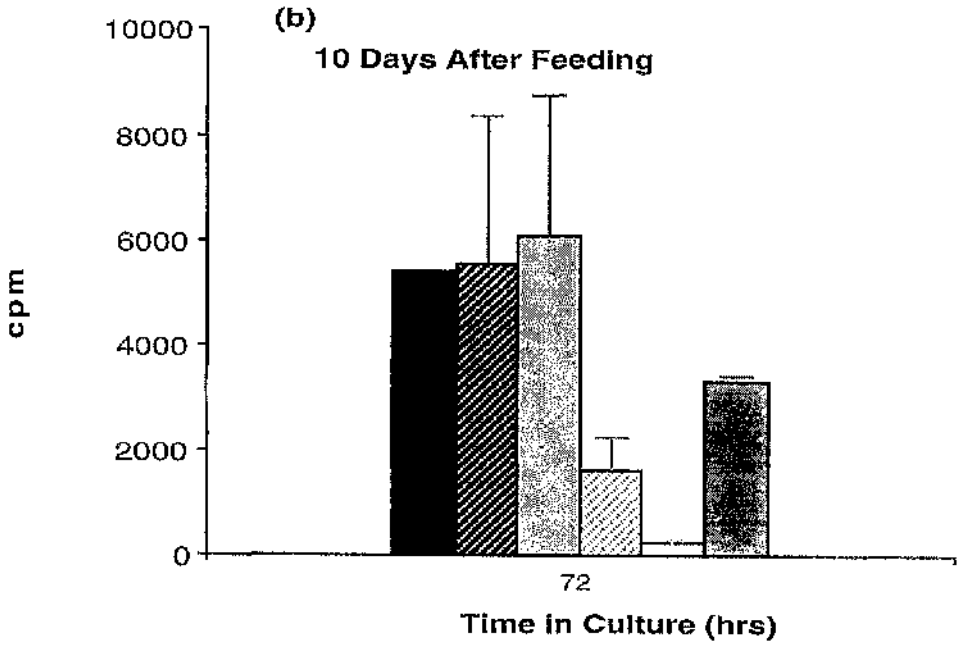


Figure 8.13 Effects of Feeding Antigen to Mice Adoptively Transferred with Memory or Naive Tg T Cells.

Expansion of OVA-specific transgenic naive or memory T cells in response to tolerogenic or immunogenic challenge with OVA *in vivo*. Mice were transferred with 0.5 x 10^6 memory or naive KJ1-26$^+$CD4$^+$ cells and 1 day later fed 200mg OVA or 0.2ml saline, or immunised with 10mg OVA + 50μg LPS subcutaneously. The results shown are **(a)** the mean percentage of KJ1-26$^+$CD4$^+$ cells or **(b)** the total number of KJ1-26$^+$CD4$^+$ cells in pooled lymph nodes from 2 individual mice per group ± 1 SD, 4 and 10 days after feeding or sc immunisation. (*$p<0.01$ versus naive saline fed control, **$p<0.005$ versus mem saline fed control)


(a)
4 Days After Feeding
Mem Saline Fed
Mem OVA Fed
Mem OVA/LPS sc
Naive Saline Fed
Naive OVA Fed
Naive OVA/LPS sc
25000
20000
15000
10000
5000
0
cpm
***
**
72
Time in Culture (hrs)


(b)
10 Days After Feeding
10000
8000
6000
4000
2000
0
cpm
72
Time in Culture (hrs)

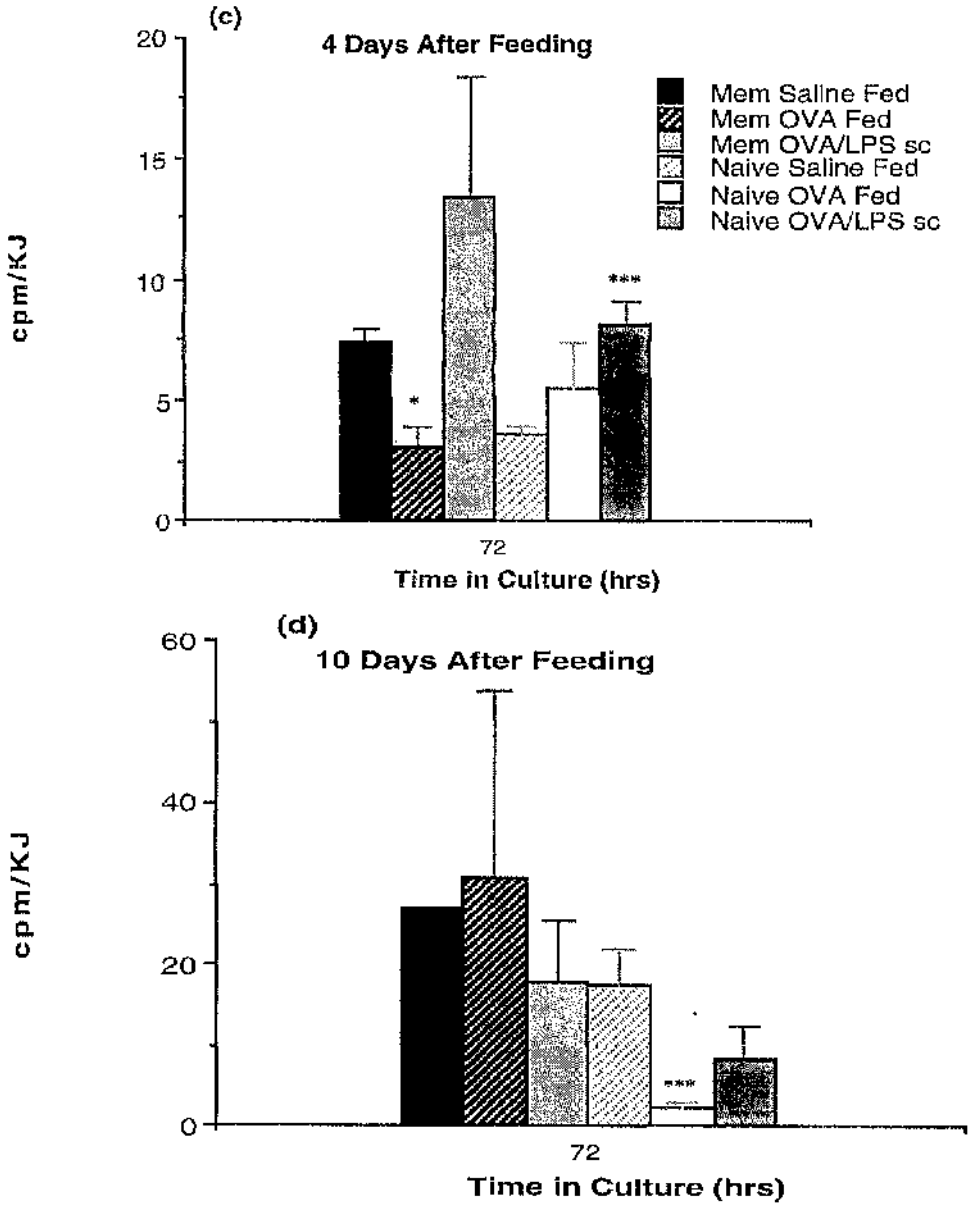


Figure 8.14 Effects of Feeding Antigen to Mice Adoptively Transferred with Memory or Naive Tg T Cells.

In vitro immune responsiveness of OVA-specific transgenic naive or memory T cells in response to tolerogenic or immunogenic challenge with OVA *in vivo*. Mice were transferred with 0.5 x 10^6 memory or naive KJ1-26$^+$CD4$^+$ cells and 1 day later fed 200mg OVA or 0.2ml saline, or immunised with 10mg OVA + 50µg LPS subcutaneously. The results shown are the mean OVA-specific proliferative responses in lymph nodes pooled from 2 mice/group shown as **(a,b)** mean uptake of ^{3}H-TdR ± 1 SD and **(c,d)** the uptake of ^{3}H-TdR corrected for the numbers of Tg cells ± 1 SD 4 **(a,c)** and 10 **(b,d)** days after feeding. (*p<0.05 versus memory saline fed control, **p<0.02 versus memory saline fed control, ***p<0.05 versus naive saline fed control)

Chapter 9 Discussion

9.1 Introduction

The results presented in this thesis have confirmed and extended previous findings on the induction of oral tolerance by feeding antigen after priming. In addition, they have highlighted several important features of inducing oral tolerance in primed mice, many of which differ from the equivalent state in naive mice. My results have implications not only for the use of oral tolerance in the treatment of inflammatory diseases, but also for understanding the regulation of immune responses to protein antigens *in vivo*.

9.2 Effects of Feeding Regime on Oral Tolerance in Primed Mice

The usual result of feeding protein antigens to naive animals is the induction of profound immunological unresponsiveness and this is currently being exploited to treat inflammatory disease. As the most useful therapeutic application of feeding antigen would be to suppress established disease, the aim of this study was to compare the immunological basis of oral tolerance induced by feeding a model antigen to naive and primed animals. By applying recent insights into immunoregulation and oral tolerance in naive animals, the experiments described in Chapter 3 were designed to establish a model for inducing oral tolerance to OVA in mice with an established systemic immune response. Thus I examined a variety of antigen doses administered at different times after priming with OVA/CFA and investigated the scope of responses influenced by the tolerance. The results in Chapter 3 showed that feeding 2-200mg ovalbumin (OVA) 7 days after immunisation suppressed a wide range of specific immune responses *in vivo* and *in vitro*. The scope of this tolerance was similar to that found when antigen was fed before immunisation, encompassing DTH *in vivo*, as well as proliferation and the production of both T_H1 and T_H2 cytokines *in vitro*. However, there was little or no effect on IgG antibody production and the extent of the tolerance in primed mice depended markedly on the dose of antigen fed. These results are similar

to the limited work that had been done previously, which also found that feeding OVA to systemically primed mice suppressed subsequent DTH responses better than humoral immunity (124,125).

The ability of systemic injection of antigen to induce effective antigen-specific hyporesponsiveness in the treatment of established organ-specific autoimmune diseases has been reviewed recently (226). Passively induced EAE has been successfully treated with IV injections of MBP after the induction of disease, but before symptoms appeared. The presumed mechanism of protection was peripheral T-cell apoptosis (227). Conversely ip injections of MBP peptide altered peptide ligand (APL) resulted in a shift in cytokine profile to IL4 and active suppression of the TH1 mediated disease (228). MBP or APL given iv has also been shown to suppress actively induced EAE after clinical signs of disease have appeared, but the mechanism of protection was not determined (229). Peripheral T cell apoptosis has been proposed to be responsible for the protection from actively and passively induced neuritis induced by P2 myelin protein given iv after disease induction (230). It is interesting to note that parenterally induced protection from experimental autoimmune disease in these studies required multiple injections iv or ip of antigen early after the induction of disease. This contrasts with the single feeds used in earlier oral tolerance work (124).

As I also found that tolerance was induced better by high doses of antigen given soon after immunisation (124,125), I decided to examine in more detail the effects of feeding a very high single dose of antigen and of using regimes of multiple feeding. Feeding a single dose of 400mg OVA 7 days after immunisation produced tolerance of DTH, proliferation and cytokine production, but also revealed some tolerance of total OVA-specific IgG production, which was not found using lower doses of fed antigen. However, IgG1 and IgG2a antibody isotypes were still not tolerised by feeding 400mg OVA. In an attempt to enhance tolerance, I increased the frequency of feeding. However, multiple feeds of 5x5mg or 5x25mg OVA did not radically alter the scope of tolerance, with DTH, proliferation, and IL5 and IFNγ production still being tolerised, but antibody responses remained generally resistant. Thus, in contrast to other

studies (131), I found that multiple feeds of antigen did not extend significantly the scope of tolerance in primed mice, although those responses which were tolerised were often suppressed to a more significant degree under these conditions. My findings in primed mice also differ markedly from those obtained using naive mice which are easier to tolerise by feeding antigen. Even very high single doses of antigen or regimes of multiple feeding produced less tolerance in primed mice than in mice fed before priming and I would suggest that the most appropriate regime for inducing oral tolerance in primed mice might be multiple feeds of greater than 200mg OVA early after immunisation.

The resistance of all classes of the antibody responses to tolerance in primed mice contrasted with their susceptibility in naive mice in my own and other studies. However, it has often been reported that humoral immunity can be more difficult to tolerise by feeding antigen than T cell functions (54,64). The reasons for this dichotomy remain controversial, but one suggestion in naive mice is that preferential upregulation of T_H2 cell activity inhibits T_H1 activity. However I generally found efficient suppression of both T_H1 and T_H2 dependent cytokine production in both naive and primed mice. An alternative possibility to explain the poor tolerance of antibody responses is that in primed mice, only T cells and not B cells can be rendered tolerant by feeding antigen. This could occur if B cells in immunised mice have already been primed by antigen and been exposed to T cell derived cytokines and will therefore undergo further expansion when the appropriate antigen is fed. It has been reported that B cells are not readily tolerised by feeding antigen and indeed, one report on oral tolerance in humans concludes that feeding KLH induced B cell priming but systemic T cell tolerance (232). However, an older study has reported that B cells can be tolerised by feeding antigen, although this is more difficult than tolerising T cells by the same protocol (50).

An important finding of my study was that the effects of feeding after immunisation were highly time dependent. Thus DTH, proliferation and cytokine production were all suppressed more readily when antigen was fed in the first 2-4 days

after immunisation. This is consistent with previous reports, although the window of susceptibility to induction of tolerance I found (around 7 days) was considerably narrower than the 14 days suggested in earlier studies (124,125). In further contrast with previous reports, I found only a slight improvement in the tolerance of antibody responses if antigen was fed 2 days rather than 8 days after immunisation. This may have been due to differences in the dose of antigen and feeding regime used in the different studies.

There could be a number of possibilities why tolerance is more difficult to induce in primed mice. One is that in primed animals, the relevant T and B cells have already localised in the microenvironment of the germinal centres where they may be inherently resistant to tolerance because of their advanced differentiation status, or because here they are inaccessible to the tolerogenic antigen derived from the gut. This idea is consistent with the fact that germinal centre formation takes 7 days or more after primary immunisation (231), the time at which I found mice became resistant to the induction of tolerance. In addition, I found that the loss of tolerance coincided with the appearance of primary antibody, supporting previous evidence that serum antibody inhibits the induction of oral tolerance by reducing the levels of circulating antigen found after intestinal absorption (123). Mopping up antigen in this way may result in very low overall amounts of antigen being available to the immune system, thus blunting the effects of feeding different amounts of antigen. This may also explain the fact that I did not find a clear dose response when antigen was fed after priming. This was surprising in view of the marked dose dependence of oral tolerance in naive animals (92,231), but is consistent with recent work on oral tolerance in established experimental acute encephalomyelitits (EAE), where the frequency of feeding, rather than the dose of antigen was the most important factor in determining the efficacy of the tolerising regime (131,132). In view of the impact this phenomenon could have on designing therapeutic regimes, it would be important to compare directly the amounts of antigen which gain access to the serum and lymphoid tissues of naive and primed mice fed different doses of protein.

Further reasons underlying the less efficient oral tolerance in primed mice may be because primed mice may simply have more antigen-specific T cells to tolerise, or because systemically primed T cells are inherently more resistant to subsequent tolerance induction. Alternatively, the less profound tolerance may reflect the fact that in primed animals, only a few naive T cells remain to be tolerised and these may have a relatively low affinity for antigen. Some of these issues were addressed later in my project.

9.3 Reasons for Relative Resistance of Oral Tolerance in Primed Mice

To investigate some of these reasons, I first explored the effects of persisting antigen after initial priming and examined the inherent susceptibility of Ag-specific memory T cells to tolerance induction.

I first examined whether my original protocol of systemic priming and tolerance induction was ineffective because I fed antigen at a time when one would assume that the systemic response was in its most active phase of development, with rapid clonal expansion of T and B cells, formation of germinal centres and migration of activated lymphocytes through the lymphoid tissues and to effector sites. Indeed, using the adoptive transfer system in which transgenic $CD4^+$ T cells are transferred into normal syngeneic recipients and tracked by flow cytometry using the anti-clonotypic mAb (143), it has been shown that the peak of clonal expansion of T cells is around 5 days after immunisation (225). Thus I hypothesised that the immune system might be too dynamic to be modified by tolerogenic antigen, or that the relevant T cells were sequestered in microenvironments that were inaccessible to fed antigen.

To address this possibility I fed antigen six weeks after systemic immunisation, at which time clonal expansion would have ceased and I considered that the primary systemic immune response would have stabilised fully. However, this did not extend the scope or degree of oral tolerance. Indeed, only IFNγ production was suppressed at all in OVA fed mice, while OVA-specific proliferation and IL5 production were actually enhanced by feeding antigen. These results are consistent with previous studies

showing that it is increasingly difficult to induce oral tolerance as time elapses after systemic priming (124,125), suggesting that the immune system of the fully primed animal is no longer susceptible to signals which are normally tolerogenic. One explanation I considered for this was that the priming inoculation of OVA in CFA acted as a depot for antigen release in the context of continued inflammation induced by the adjuvant. In this way, there may be chronic upregulation of costimulatory molecules on antigen presenting cells so that antigen emenating from the gut will be presented in the periphery of a primed animal in an immunogenic, rather than a tolerogenic manner.

To address the depot effect of adjuvant on the induction of tolerance I primed mice with OVA in LPS, an immunomodulator which is only retained in significant quantities at the site of sc injection for a short time, with only 28% of the amount injected being found at the sc injection site 7 days after the injection of LPS (233). In contrast, OVA/CFA persists at the site of sc injection 6 months after the time of injection. Nevertheless, as I found when mice were primed with OVA/CFA, it was easier to induce tolerance by feeding OVA soon after immunisation with OVA/LPS, with more profound suppression 2 days, rather than 7 days after priming. In addition the pattern of tolerance was the same irrespective of the adjuvant used to prime systemically, with no tolerance of antibody responses in either case. There was also no tolerance when OVA/LPS primed mice were fed OVA 6 weeks after immunisation, suggesting that the depot effects of CFA did not influence the limited scope of oral tolerance in primed animals. Although these findings probably eliminate a possible role of adjuvant induced chronic inflammation in producing resistance to tolerance soon after systemic priming, I cannot exclude the possibility that persistence of antigen itself may be important in this respect. Staining sections of the draining lymph node or site of injection with monoclonal antibodies to peptide loaded MHC molecules would allow investigation of this possible persistence of antigen.

A further possibility is that the presence of memory cells was the cause of lack of tolerance at six weeks after priming with OVA/LPS. Memory cells are reputedly

difficult to tolerise and to date no-one has shown that it is possible to tolerise an established immune response. The reason why memory cells might be more difficult to tolerise is probably because they are much more readily activated than naive T cells due to their expression of increased levels of adhesion molecules which allow them to interact effectively with an APC bearing much lower levels of antigen-MHC complexes. Memory T cells are also less dependent on certain costimulatory signals provided by APCs, meaning that presentation of fed antigen by APC lacking costimulatory molecules may induce activation rather than the tolerance found with naive T cells (234-238).

To investigate further how the expansion and activation of antigen-specific T cells influenced the induction of oral tolerance in primed mice, I exploited a recently described adoptive transfer system (143) in which transgenic $CD4^+$ T cells are transferred into normal syngeneic recipients. The first of these systems to be described was that using DO11.10 transgenic T cells specific for the OVA323-339 immunodominant peptide and $I\text{-}A^d$ which are recognised by KJ1-26 antibody (143, 180, 225). This model has now been widely used to study peripheral immune responses and also more recently oral tolerance in naive mice (114,179). The advantages of this system are the availability of the clonotypic antibody to track antigen-specific T cells directly and analyse their functions by intracellular cytokine production and apoptosis. In my experiments I transferred naive OVA-TCR transgenic (Tg) cells into naive mice and 2 days later immunised sc with OVA/CFA. Then I fed antigen at various time-points after immunisation and followed the number and function of the transgenic T cells.

In the first experiments, I found that feeding soluble OVA after priming led to a small but significant reduction in the proportion and absolute numbers of transgenic T cells in the draining lymph nodes compared with saline fed controls. However, this alteration in the kinetics of T cell expansion only occurred if feeding was delayed until 10 days after immunisation. This effect did not simply appear to be due to re-exposure of primed Ag-specific T cells to antigen, as there was no equivalent effect of feeding

OVA in an immunogenic manner with CT. The inhibited expansion of T cells when antigen was fed 10 days after priming was accompanied by suppressed proliferative responses to OVA *in vitro*. Again these responses were not inhibited when antigen was fed earlier after priming. These results contrasted with my earlier findings in normal mice, where tolerance could be induced early, but not late after priming. To try and investigate why this might be the case, I fed a higher dose of OVA, as I thought the higher proportion of Ag-specific T cells found in adoptive transfer recipients compared with naive mice might complicate the consequences of feeding antigen. This approach confirmed that it was possible to induce tolerance late after priming, although the scope of this tolerance was somewhat variable, in that early after feeding antigen, proliferation was significantly reduced compared with controls whereas cytokine production was normal. In this experiment, feeding a high dose of antigen earlier (5 days) after priming also produced some evidence of tolerance, as shown by significantly decreased proliferation 7 days after feeding. However, T cell expansion and cytokine production were normal under these conditions. Feeding high doses of OVA in an immunogenic form with CT also had variable effects, as the transgenic T cells did not proliferate well compared with the controls when given this secondary immunogenic challenge 10 days after systemic priming. However, feeding a high dose of antigen with CT 5 days after priming adoptively transferred mice resulted in significantly increased proliferation 7 days after feeding. It is surprising that OVA/CT did not always stimulate expansion of transgenic T cells and cytokine production as one would expect from a secondary response. This refractory state of antigen-experienced T cells has been reported in other systems and could reflect differences in TCR mediated signalling which render them unresponsive to antigen for a period of time after initial exposure (239).

It therefore remains unclear why I obtained different patterns of tolerance induction in intact mice compared with adoptively transferred primed mice, raising the question of the physiological relevance of the transfer model. It could be that transgenic T cells behave differently from normal T cells as they all have a highly specific TCR for a single immunodominant epitope and are in a microenvironment surrounded by

many similar cells. This may alter the amount and nature of expansion T cells undergo under these conditions. To clarify these discrepancies it would be necessary to look more directly at the functions of individual transgenic T cells e.g. by using intracellular cytokine staining.

I next used the adoptive transfer model to examine the susceptibility of "memory" T cells themselves to tolerance induction. These "memory" cells were defined as having been previously exposed to antigen in adjuvant. In this experiment, I did find evidence that OVA-specific "memory" cells could be tolerised by feeding antigen, as there was a reduction in the Ag-specific proliferative response 4 days after feeding. However there was no reduction in the percentage or numbers of Tg cells in the lymph nodes after feeding. As the naive control T cell transfer that was fed OVA did reveal some evidence of oral tolerance in terms of Ag-specific proliferation responses, this suggests that it is fair to interpret from this experiment that memory cells are not inherently difficult to tolerise by feeding antigen. I conclude that their apparent resistance in the intact animal is more likely due to persistence of antigen and thus their Ag-specific T cells being exposed to antigen in the context of costimulation. Thus oral tolerance may only be induced effectively when memory cells are not in an environment where antigen is presented with costimulation. Interestingly, my results from mice transferred with "memory" cells and immunised with the immunogenic challenge of OVA/LPS showed that these cells could only respond poorly to antigen *in vitro* probably because this is the third occasion they have been presented with antigen further highlighting the refractory state of these cells to restimulation. Finally, one feature of these transfer experiments was that very few "memory" T cells were found after adoptive cell transfer into normal recipients fed saline. This could reflect the absence of antigen under these conditions, as it is thought that persistence of antigen is required for antigen-specific memory cells to survive (237).

Together my findings show that oral tolerance is defective in primed mice unless used early after priming. This is not influenced by the stage of the developing systemic immune response but by factors present in the microenvironment of the

primed mouse such as the persistence of antigen or presence of costimulation. These findings have implications for the use of oral tolerance in the therapy for autoimmune diseases as they suggest that oral tolerance would probably only be effective if applied at the very early stages of disease.

9.4 Longevity of Oral Tolerance in Primed Mice

A further practical requirement of oral tolerance as a therapy is that the antigen specific suppressive effects will need to be long lasting. Previous studies in naive mice found that several parameters of the systemic immune responses remained tolerant for up to 17 months after feeding antigen (178) and so I investigated how long tolerance persisted after a single feed of 25mg OVA given 7 days after priming.

The results presented in chapter 4 indicated that the oral tolerance induced when antigen is fed after immunisation is not as long lasting as that reported in naive mice. *In vivo* tolerance was only found early after feeding, when antigen specific DTH responses were suppressed compared with controls. DTH responses were normal at later time points and antibody responses were not tolerised at any time after feeding, confirming my initial findings in Chapter 3. Some aspects of the *in vitro* response showed longer lasting tolerance however. Thus IFNγ production remained tolerant in OVA fed mice for 12 months after feeding, although the interpretation of these findings is complicated by the fact this function was not inhibited at the earliest time after feeding in contrast to my other experiments. The reason for this discrepancy is unknown and time did not permit me to repeat the experiment. Similarly, the experiments performed in chapter 4 indicated that IL5 production was primed early after feeding, whereas it had been unaffected or reduced in my first experiments. This underlines the possible resistance of T_H2 responses to feeding antigen, but it should be noted that IL5 production was then tolerised at 6 months after feeding before returning to normal at 12 months. OVA-specific proliferation was found to be tolerised at the first time point but then returned to control levels by the 6 month time point.

Surprisingly, the proliferative response was enhanced in the group fed OVA at the 12 month time point.

Overall, these results suggest that only some aspects of the established immune response can be tolerised for any length of time by a single feed of antigen. It seems that as in other forms of oral tolerance (90-92), IFNγ production is particularly sensitive to feeding in primed mice, although my results suggets that other aspects such as IL5 might also be tolerised at different times. Thus oral tolerance is a dynamic phenomenon, but there is surprisingly little correlation between those aspects which can be tolerised at different times. Although this is consistent with previous results in the lab using naive mice (178), it emphasises the unpredictability of using oral tolerance as a blanket therapy strategy particularly in primed animals. Further, the fact that oral tolerance in primed mice was not long lasting suggests that the mechanisms of tolerance in primed mice are short lived. This could reflect the replacement of anergised/deleted T cells by responsive naive T cells exiting the thymus, an idea which could be tested by thymectomising mice after feeding antigen and investigating if the mice remain tolerant. Alternatively, it could reflect the recovery of anergic cells or their escape from active suppression to become fully responsive T cells. To address whether functionally impaired $CD4^+$ T cells persist *in vivo* following the induction of oral tolerance, a small population of $CD4^+$ OVA-specific TCR transgenic T cells could be tracked following the induction of oral tolerance by soluble OVA. Indeed this has been done following the induction of peripheral tolerance iv (180). Here they found that the unresponsive state was not associated with immune deviation or suppression. Instead, they found that the population of antigen-specific T cells were initially impaired in their ability to proliferate and produce cytokines *in vivo* and survived for several months, after which they recovered from their unresponsive state.

A possible technical issue which could complicate the interpretation of my experiments could be that I had to give the mice a secondary boost/immunisation of OVA/CFA in order to obtain a measurable response in the saline fed group at the later times. Thus, it may be that any mechanism of tolerance induced by the feed of antigen

could not overcome this secondary immunisation with OVA/CFA. Although tolerance is not usually easy to break with repeated immunisations of this kind (123) in repeating this experiment, it might be better to challenge with a less immunogenic protocol such as soluble OVA.

This experiment showed that oral tolerance induced in primed mice is not long lasting and this has implications for its use in clinical therapy in that it may not be a stable long lasting therapy. Repeated administration of oral antigen may therefore be required to induce long term therapeutic effects.

9.5 Role of IL4 and IL12 in Oral Tolerance in Primed Mice

The experiments in chapters 3 and 4 showed that feeding antigen after systemic priming results in *in vivo* and *in vitro* tolerance of several aspects of the systemic immune response. In these experiments it was impossible to tolerise antibody responses and in some cases, IL5 production was more difficult to tolerise than IFNγ. This contrasts with the findings in naive mice from our laboratory (57) and could suggest that T_H2 responses are unusually resistant to oral tolerance induction in primed mice.

A number of mechanisms have been implicated in oral tolerance in naive animals including clonal anergy, clonal deletion or active suppression. Of particular note, cross regulation of T_H1 cells by activated T_H2 cells is often believed to be responsible for models of tolerance in which there is a dichotomy of effects on humoral

and CMI responses (169,240). It is interesting to note here that in naive mice, the conditions for generating T_H2 lymphocyte tolerance may be different from those required to generate tolerance of T_H1 lymphocytes. These include extended continuous exposure to high doses of antigen, rather than the single or intermittent feeding regimens which are usually sufficient to induce tolerance in T_H1 lymphocytes. Some workers have reported that T_H2 dependent responses may also be relatively preserved

in orally tolerised naive mice and have suggested that IL4 dependent T_H2 cells may act as active suppressor cells under their conditions (77,91,92).

My results do not support the idea that IL4 dependent T_H2 cells may act as active suppressor cells in mice tolerised by feeding after immunisation, as there was no preferential increase in the production of T_H2 cytokines in my mice with selective tolerance of CMI responses. However as the tolerance of IL-5 production was occassionally less dramatic, I decided to investigate formally whether T_H2 cells were necessary for oral tolerance in primed mice by examining the effects of feeding OVA to $IL4^{-/-}$ mice which had been immunised with OVA/CFA. The results presented in chapter 5 confirmed previous findings from the laboratory that feeding OVA to naive $IL4^{-/-}$ mice results in oral tolerance. I then extended these studies by showing that primed $IL4^{-/-}$ mice fed 2-200mg OVA 7 days after immunisation developed a pattern of oral tolerance similar to that found in normal animals, with suppression of DTH *in vivo* and antigen-specific proliferation *in vitro*, as well as some suppression of IFNγ and IL5 production *in vitro*, but no effects on serum antibodies. As in naive mice (57), these findings argue against a role for regulatory T_H2 cells in oral tolerance in primed mice. I propose that my results are more consistent with the hypothesis that the predominant mechanism of T cell tolerance after feeding high doses of antigen to primed mice is clonal anergy/deletion or an alternative active suppressor mechanism. That anergy may be the critical mechanism for oral tolerance in primed mice is consistent with previous findings that this model of tolerance can not be prevented by 2'-deoxyguanosine, an agent which inhibits the induction of oral tolerance in naive mice and which is believed to act by depleting regulatory T cells (125). The presence of anergy could be investigated further by adding IL2 to cultures of orally tolerised T cells to see if they regain their ability to proliferate in response to antigen *in vitro*.

One suppressor mechanism which I attempted to investigate was TGFβ. As mentioned above, release of TGFβ by a discrete subset of T cells has been implicated as a mechanism of oral tolerance in naive animals. This is proposed to operate primarily when oral tolerance has been induced using multiple low doses of antigen

(190,200,205) and it has been suggested that TGFβ and IFNγ play opposing roles in the regulation of mucosal immune responses (214,215). The idea that TGFβ may play a role in oral tolerance is supported by the fact that IFNγ-dependent gut inflammation caused by TNBS or by transfer of $CD45RB^{hi}CD4^{+}$ T cells to *scid* mice can be prevented by TGFβ-secreting T cells (171,172). Further, anti-IL12 antibody enhances both TGFβ production and high dose oral tolerance in OVA-specific TCR transgenic mice (214,215). Although the presence of bystander suppression and other mechanisms of active regulation have not yet been examined directly in oral tolerance in primed mice, it has been reported that TGF-β production is not enhanced in mice tolerised by feeding myelin basic protein after priming (131).

I decided to investigate if I could exploit this proposed regulatory axis to improve oral tolerance in primed mice, by feeding antigen to mice lacking IL12 ($IL12^{-/-}$). This has not been examined previously, but as these mice have deficient production of IFNγ, I predicted this might allow the development of enhanced TGFβ responses and so increase susceptibility to oral tolerance compared with wild type mice. I therefore examined oral tolerance in naive $IL12^{-/-}$ mice using protocols which might be likely to involve TGFβ-dependent mechanisms, such as feeding 5x1mg OVA starting 10 days before immunisation. I also used my standard protocol of feeding 25mg OVA once 7 days after immunisation.

Certain aspects of tolerance were enhanced in $IL12^{-/-}$ mice, namely IgG1 antibody levels. However, other components of tolerance were normal or reduced, namely OVA-specific DTH levels, and no clear overall pattern emerged. Therefore, it is probably true to conclude that the absence of IL12 has no overall effect on the induction of tolerance induced either before or after immunisation. In particular, the absence of IL12 does not seem to reproducibly enhance tolerance in primed animals, although these experiments need to be repeated. It would now be useful to measure TGFβ production directly in such animals. To examine directly the role of TGFβ in oral tolerance in primed mice it would also be useful to deplete TGFβ *in vivo* with monoclonal antibodies or to use $TGF\beta^{-/-}$ mice. Recently it has been shown using

TGFβ$^{-/-}$ mice that active suppression mediated by TGFβ is not the main mechanism of oral tolerance in naive mice, although there did appear to be some contribution from this cytokine (217). Taking the results of this report and my experiment in IL12$^{-/-}$ mice together, it seems unlikely that the main mechanism of oral tolerance in naive or primed mice is active suppression via TGFβ.

Other possible regulatory mediators could be tested for their role in oral tolerance in primed animals. For example, IL10$^{-/-}$ mice could be used to further investigate the role of T_H2 cells in oral tolerance in primed mice as these cytokines are also released by T_H2 cells but are not dependent on them. The role of IL10 is particularly important as it is known to suppress T_H1 cells (241) by acting on macrophages by blocking macrophage IL12 synthesis. This is because IL12 secretion by macrophages can activate natural killer (NK) cells to produce IFNγ and naive CD4 T cells activated in the presence of IL12 and IFNγ are committed to differentiate into T_H1 cells. Thus if IL10 production is enhanced as a result of feeding antigen then this may suppress the T_H1 cells that are causing the pathology in autoimmune diseases. Further, IL10 is a growth factor for a population of regulatory T cells in the gut that release TGFβ (171,172).

If it is confirmed that active suppression is not involved in oral tolerance in primed mice, my findings would suggest that it may be difficult to induce "bystander suppression" in primed mice. This phenomenon occurs when an orally tolerised animal is challenged both with the original antigen and an unrelated antigen and it is believed to be mediated by antigen-non-specific cytokines, such as TGF-β, released by regulatory T cells (173,174). Bystander suppression is therefore the basis for most therapeutic uses of oral tolerance, which employ antigens that are not normally the original antigen inducing the immunopathology. My results indicate that there could be a problem in inducing "bystander suppression" by oral tolerance to treat established disease.

9.6 Attempts to Enhance Oral Tolerance in Primed Mice with Flt3L

As the oral tolerance in primed mice was not as profound or wide ranging as that found when equivalent amounts of antigen are fed to naive mice, I was interested in finding an alternative strategy for enhancing tolerance in primed animals.

The haemopoietic growth factor Flt3ligand (FLT3L) increases the numbers of dendritic cells (DCs) when it is given *in vivo* and recent work has shown that administration of Flt3L to mice enhances the induction of oral tolerance in naive mice (114). This affected all aspects of the immune response and allowed normally non-tolerogenic doses of OVA to induce significant tolerance. The rationale for this appears to be that Flt3L expands the numbers of resting dendritic cells in the gut which then present fed antigen without costimulation to T cells, resulting in more profound tolerance. Thus, I decided to test the idea that expansion of DCs by Flt3L might also extend the scope of oral tolerance in primed mice.

My results showed that expanding DC with Flt3L did not interfere with the induction of oral tolerance in primed mice and enhanced the induction of tolerance in some instances, as well as allowing tolerance of some responses not normally susceptible to tolerance, such as antibody production. However, the effects of Flt3L were quite variable and Flt3L seemed to reveal early T cell priming after feeding OVA, perhaps due to the fact that Flt3L was being administered around the time of systemic immunisation. It proved difficult to determine precisely the best time course and protocol for examining the effects of Flt3L on oral tolerance and I did not have time to extend these studies to obtain definitive results. This is most likely due to Flt3L causing an increase in the kinetics of the response to the fed antigen. This is because in mice treated with Flt3L there appeared to be an early increase in the response of PLN cells from mice fed OVA compared with mice fed saline. As this did not occur in saline treated mice it could be that Flt3L was enhancing the numbers of DCs presenting fed antigen and so increasing the chance of T cells coming into contact with fed antigen compared with saline treated mice fed antigen. This would mean that T cells from mice treated with Flt3L and fed antigen would come into contact with more DCs presenting

antigen more quickly compared with saline treated mice fed antigen and hence showed greater initial responses.

Although the initial immune response of mice fed OVA and treated with Flt3L is higher compared with saline fed controls, the outcome is slightly enhanced tolerance when compared with saline treated mice fed OVA. This has recently been confirmed in a TCR Tg adoptive transfer model (Williamson, E and Viney, JC. Personal Communication) and could possibly be explained by Flt3L promoting greater activation induced cell death (AICD) in tolerised T cells. This needs to be studied directly, but is consistent with my earlier conclusions that direct inactivation of T cells may be a major mechanism of oral tolerance in primed mice. Therefore, as Flt3L has been shown to enhance oral tolerance in mice, it could potentially be used to enhance the induction of oral tolerance in patients already presenting symptoms of disease.

9.7 Conclusions

In conclusion, the results presented in this thesis have confirmed and extended previous findings on the induction of oral tolerance by feeding antigen after priming. In addition, they have highlighted several differences between oral tolerance in naive and primed mice. These include the fact that antibody responses are not readily tolerised in primed mice and that higher doses of antigen have to be fed to obtain tolerance equivalent to that found after feeding antigen to naive mice. Although my study does not allow me to make firm conclusions whether similar mechanisms underly the oral tolerance induced by feeding antigen before or after systemic immunisation, I would propose that anergy and/or clonal deletion is the main mechanism of oral tolerance in primed mice. However this needs to be proven directly and the mechanisms remain unclear. There is also a need for careful definition of the effects of different dose regimes and timing of feeding regimes, if this approach is to be used therapeutically. My results have implications not only for the use of oral tolerance in the treatment of inflammatory diseases, but also for understanding the regulation of immune responses

to protein antigens *in vivo* in that they highlight the differences in the response of a naive and a primed immune system to antigen given via a tolerogenic route.

A number of new techniques have been described for studying the immune system since the start of my studies. One new experimental system that could be used to understand the mechanisms of oral tolerance would be the use of antibody to peptide on MHC Class I or II antibodies allowing the fate of antigen to be followed directly. In addition, by combining this with adoptive transfer of Tg T cells or the use of peptide-class II MHC tetramers, it should be possible to assess where antigen is presented to Ag-specific T cells and their subsequent activation could be monitored *in vivo*. Together with labelling of APC, these strategies would allow the interactions between antigen, T cells and APCs to be tracked during the induction of oral tolerance. These studies would be useful as they may reveal the best specific APCs to target that would present antigen in a tolerogenic manner thus enhancing the tolerance induced by feeding antigen. By employing these and other, new advances in immunological research techniques, the immunological consequences of feeding protein antigens to primed mice could be better understood. This would hopefully lead to the successful therapeutic use of oral tolerance to treat inflammatory diseases.

9.8 Prospects for Clinical Uses of Oral Tolerance

As I have noted, very little is known about the mechanisms of oral tolerance in primed mice and if treatment of humans is to become a therapeutic reality, it will be necessary to re-establish tolerance in a previously sensitized host. My studies showing that feeding antigen can suppress an ongoing immune response confirm that oral tolerance may be exploited for suppressing established immune responses *in vivo*. This confirms and extends results from experimental models of autoimmune disease. However, my studies have also highlighted several aspects which indicate that caution is necessary before clinical use can be considered routinely. Also, they may help explain the poor results from human trials. The main drawbacks are the limited scope of tolerance and that it is relatively transient. My results would suggest that feeding

multiple high doses of antigen very soon after disease initiation will probably be required to induce a therapeutic level of systemic tolerance. Next, it may be necessary to use the exact antigen responsible for the disease, as my evidence supported a role for direct inactivation of T cells, rather than active suppression and therefore bystander effects. This has implications for the production of enough antigen to be fed in large doses which would be both difficult and expensive. It may also be necessary to explore ways of modulating or enhancing tolerance which might allow oral tolerance to be reinduced in patients already presenting symptoms of disease. As described above, molecules such as Flt3L which has been shown to enhance oral tolerance in mice could be employed. Cytokines such as TGFβ and IL10 which are known to suppress harmful inflammatory T_H1 responses may also be used to enhance the suppression induced by feeding antigen. If these cytokines were given at the same time antigen was fed they might also allow the development of a population of antigen specific active suppressor cells which may provide a longer lasting therapy. Blocking antibody against inflammatory cytokines given while antigen was fed might also promote the development of antigen specific suppressor cells. Targetting the antigen to specific APCs that would present antigen in a tolerogenic manner would also be useful. Thus a combined approach may be necessary to achieve reproducible therapeutic tolerance by feeding antigen.

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