THE BASIS OF THE ORAL AND PARENTERAL ADJUVANT PROPERTIES OF IMMUNE STIMULATING COMPLEXES (ISCOMS)

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SUMMARY

There is currently a great deal of interest in the development of vaccines using purified recombinant protein antigens. For practical and scientific reasons, it would be advantageous if these vaccines could be administered orally. This is esssential for stimulating widespread immunity at the mucosal surfaces where most pathogens are encountered and, as the success of the oral polio vaccine shows, this can also protect against systemic infection. Furthermore, oral immunisation is one of the few effective means allowing passive transfer of immunity from mother to infant via milk. The poor immunogenicity of purified soluble proteins, their inability to induce class I MHC-restricted CTL responses and the systemic tolerance induced by their oral administration are major obstacles to the development of synthetic oral vaccines. Thus, there is a need for well characterised vaccine vectors which will circumvent these difficulties and allow soluble protein antigens to be immunogenic when administered orally.

Immune stimulating complexes (ISCOMS) are lipophilic, cage-like particles composed of cholesterol, phospholipid and the saponin adjuvant Quil A. Parenteral immunisation with ISCOMS is known to induce humoral and cell-mediated immune responses as well as protective immunity against a number of infections. However, their efficacy by mucosal routes and their use with non-hydrophobic proteins had received little attention. My studies exploited a recently established method for incorporating purified OVA into ISCOMS and aimed to extend preliminary work in the laboratory showing that ISCOMS-OVA were immunogenic by oral and parenteral routes. I therefore explored the full range of responses

induced and investigated the immunological basis of the adjuvant properties of ISCOMS when used by the oral and parenteral routes.

A single parenteral immunisation with as little as 5-10μg ISCOMS-OVA primed potent OVA-specific systemic DTH and IgG responses and high levels of class I MHC-restricted OVA-specific CTL activity were detected in the spleen. These findings indicate that ISCOMS allow presentation of incorporated protein to the class II MHC-restricted T cells required for antibody and DTH responses, as well as permitting efficient entry of the protein into the endogenous antigen processing pathway required for stimulation of class I MHC-restricted CTL responses. A multiple dose oral immunisation schedule induced the same range of systemic immune responses as parenteral immunisation and also stimulated the production of secretory IgA antibodies in the intestine and local CTL precursors in MLN. These findings indicate that ISCOMS may allow protein antigen to subvert the regulatory mechanisms which normally cause protein antigens to induce tolerance at mucosal surfaces.

One of the most novel properties of ISCOMS was their ability to stimulate potent class I MHC-restricted CTL responses to exogenous protein antigens *in vivo*. As this would be important for a vaccine capable of stimulating protection against many viral infections, I characterised the CTL responses primed by ISCOMS-OVA and investigated the mechanisms involved in their induction. My results showed that the CTL primed by ISCOMS-OVA were $CD8^+$ T cells which recognised the immunodominant epitope contained within the endogenously processed peptide OVA 258-276, containing the minimal motif for binding the H-$2K^b$ class I MHC molecule. This is the epitope recognised by OVA-specific CTL in H-

2^b mice following physiological processing of OVA. OVA-specific precursor CTL appeared rapidly in the draining lymph node after subcutaneous immunisation with ISCOMS-OVA and these pCTL differentiated into effector cells when cultured merely with IL-2, indicating the efficiency of priming *in vivo*.

Phagocytic accessory cells (Mϕ) were required for the priming of a large proportion of the OVA-specific CTL, as paralysis or depletion of these cells produced a marked decrease in the priming of OVA-specific CTL *in vivo*. However, detectable CTL activity remained in the absence of Mϕ, suggesting that there may be more than one pathway of CTL induction utilised by ISCOMS *in vivo*. The CTL responses were entirely dependent on helper T cells, as depletion of $CD4^+$ T cells completely abolished priming of CTL responses by ISCOMS-OVA. These findings suggest that the induction of CTL by ISCOMS is a complicated process, involving both phagocytic accessory cells and $CD4^+$ T helper cells.

The dependence of the CTL responses on $CD4^+$ T cells, together with the potent DTH and humoral responses elicited, suggested that the activation of $CD4^+$ T_h cells was a critical event in the induction of immune responses by ISCOMS. I therefore examined directly the priming of antigen-specific $CD4^+$ T cells by ISCOMS-OVA and determined if there was any preferential induction of the T_h subsets. A single subcutaneous immunisation with ISCOMS primed strong antigen-specific proliferative responses in the draining lymph node. These were entirely dependent on $CD4^+$ T cells and purified $CD8^+$ T cells appeared to be unable to proliferate, even in the presence of the epitope peptide OVA 257-264 and exogenous IL-2. MLN cells proliferated after oral immunisation, but antigen-specific proliferative responses could not be detected in ISCOMS

primed spleen cell populations after either oral or parenteral immunisation. However, parenteral or oral immunisation with ISCOMS primed for substantial amounts of antigen-specific cytokine production by spleen cells following restimulation with antigen *in vitro*, indicating efficient dissemination of the initially localised response after immunisation with ISCOMS.

ISCOMS primed lymphoid cells produced a characteristic pattern of cytokines when restimulated with antigen *in vitro*, with a transient IL-2 response, followed by the later accumulation of both IFN-γ and IL-5. This evidence that both T_h1 and T_h2 type $CD4^+$ T cells were activated by immunisation with ISCOMS correlates with the wide range of both humoral and cell-mediated immune responses primed by ISCOMS *in vivo* and is supported by the fact that antigen-specific IgG1 and IgG2a antibody responses were both primed by ISCOMS. The pattern of proliferative and cytokine responses induced by feeding ISCOMS was similar to that primed by parenteral immunisation, indicating that ISCOMS were able to prime a wide range of local and systemic responses, regardless of the route of immunisation.

As a vital attribute of any potential vaccine vector is the ability to elicit long-lasting specific immunological memory, I examined the persistence of immune responses for up to one year after primary immunisation with ISCOMS-OVA. A single subcutaneous immunisation with 10μg protein antigen in ISCOMS primed long-lasting memory responses, which included antigen-specific DTH and IgG antibody responses *in vivo*, as well as antigen-specific proliferation and cytokine production *in vitro*. These persisted for at least 8-12 months after primary immunisation and included both T_h1 and T_h2 type responses, as IL-2 and IL-5

responses were both present. The kinetics of all these responses were similar, with a marked reduction in magnitude observed 12 months after priming. Thus, ISCOMS are potent inducers of long-lasting immunological memory, which encompasses both B cells and T_h1 and T_h2 $CD4^+$ T cells.

I next went on to examine the properties of the ISCOMS themselves which contribute to their adjuvanticity. As the adjuvant properties of saponins have been known for some time, it seemed likely that Quil A played a crucial role in the activity of ISCOMS. However, the range of responses involved had not been studied in detail and there had been no comparative studies of Quil A and intact ISCOMS. I therefore compared directly the adjuvant effects of Quil A and ISCOMS on a range of local and systemic OVA-specific immune responses. Parenteral immunisation with OVA and Quil A primed systemic antigen-specific DTH, CTL and IgG antibody responses *in vivo*, as well as T cell proliferative activity and T_h1 and T_h2-type cytokine production *in vitro*. In addition, oral immunisation with OVA and Quil A induced local antigen-specific intestinal IgA production, together with systemic IgG and CTL responses. These findings indicate that, like intact ISCOMS, free Quil A allows antigen to gain access to both the exogenous and endogenous pathways of antigen processing and presentation *in vivo*. In addition to its effects on the presentation of exogenous protein antigen to specific lymphocytes, Quil A also had marked effects on innate immune responses including the activation of $M\phi$ and NK cells, as well as the non-specific stimulation of regulatory cytokines such as IFN-γ. However, as 5-10 fold more free Quil A was required to elicit the same magnitude of responses as those

elicited by Quil A in ISCOMS, factors additional to Quil A must be important for the potent adjuvanticity of intact ISCOMS.

I therefore compared ISCOMS-OVA with OVA incorporated in a particulate vector lacking Quil A, biodegradable microparticles composed of poly(lactide-co-glycolide) (PLG-OVA). Oral or parenteral immunisation with PLG-OVA microparticles primed a wide range of humoral and cell-mediated immune responses, both locally and systemically, including antigen-specific CTL activity. However, the CTL responses induced by ISCOMS were usually higher than those induced by PLG microparticles, requiring fewer or lower doses of antigen. It was also impossible to detect CTL activity in PLG-OVA immunised mice without *in vitro* restimulation with antigen. Interestingly, PLG seemed relatively more efficient when given by the oral route and induced good antigen-specific IgA responses in the small intestine itself. These findings indicate that only part of the adjuvant effect of ISCOMS can be ascribed to their particulate nature, supporting my observations on the adjuvant properties of free Quil A.

My results thus extend previous observations on the induction of immune responses by ISCOMS and highlight their potential as oral vaccine vectors of the future. ISCOMS have several advantages over existing oral adjuvants, as they combine the benefits of a stable particulate structure with a built-in adjuvant, Quil A. In addition, they induce an unparalleled range of local and systemic immune responses by both parenteral and oral routes and if concerns over their safety for admininstration to humans can be satisfied, then they may form the basis of future oral vaccines against almost any class of pathogenic organism.

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DECLARATION

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

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PUBLICATIONS

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

1. Mowat AMcI, Maloy KJ, Donachie AM. Immune stimulating complexes as adjuvants for inducing local and systemic immunity after oral immunization with protein antigens. Immunology 1993;80:527-534.

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3. Maloy KJ, Donachie AM, O'Hagan DT, Mowat AMcI. Induction of mucosal and systemic immune responses by immunization with ovalbumin entrapped in poly(lactide-co-glycolide) microparticles. Immunology 1994;81:661-667.

4. Maloy KJ, Donachie AM, Mowat AMcI. Induction of Th1 and Th2 $CD4^+$ T cell responses by oral or parenteral immunization with ISCOMS. Eur J Immunol 1995;25: 2835-2841.

ABBREVIATIONS

APC	Antigen Presenting Cell(s)
BALT	Bronchial-Associated Lymphoid Tissue
Bio	Biotin
CFA	Complete Freund's Adjuvant
Cl_2MDP	Dichloromethylene Diphosphonate
CMI	Cell-Mediated Immuninty
Con A	Concanavalin A
CT	Cholera Toxin
CTL	Cytotoxic T Lymphocyte(s)
DC	Dendritic Cell(s)
DEA	Diethanolamine
dH_2O	Distilled H_2O
DTH	Delayed-Type Hypersensitivity
DMSO	Dimethylsulfoxide
EDTA	Ethylendiaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron Microscopy
FACS	Fluorescence-Activated Cell Scanner
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
GALT	Gut-Associated Lymphoid Tissue
^3H	Tritium
HAO	Heat-Aggregated OVA
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HEV	High Endothelial Venule(s)
IBD	Inflammatory Bowel Disease

IEL	Intra-Epithelial Lymphocyte
IFA	Incomplete Freund's Adjuvant
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
ISCOMS	Immune Stimulating Complexes
LP	Lamina Propria
LT	Heat-Labile Enterotoxin
Luminol	5-amino-2,3-dihydro-1,4-phthalazinedione
Mϕ	Macrophage(s)
mAb	Monoclonal Antibody
MALT	Mucosa-Associated Lymphoid Tissue
2-ME	2-mercaptoethanol
MEGA-10	Decanoyl-N-methylglucamide
MHC	Major Histocompatability Complex
MLN	Mesenteric Lymph Node
$Na_2{}^{51}CrO_4$	Sodium ^{51}Chromate
NALT	Nasopharyngeal-Associated Lymphoid Tissue
NCS	Newborn Calf Serum
NK cell	Natural Killer Cell
NPS	N-(palmitoyloxy) Succinimide
O.D.	Optical Density
OVA	Ovalbumin
PBS	Phosphate Buffered Saline
pCTL	Precursor Cytotoxic T Lymphocyte(s)
PE	Phycoerythrin
PEC	Peritoneal Exudate Cells
PEG	Polyethylene Glycol
PLG	Poly(D,L-lactide-co-glycolide)

PLN	Popliteal Lymph Node
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl Fluoride
PP	Peyer's Patch
PVA	Polyvinyl Alcohol
rhIL	Recombinant Human Interleukin
SA	Streptavidin
SB	Staining Buffer
S.D.	Standard Deviation
TcR	T Cell Receptor
TGF-β	Transforming Growth Factor-β
T_h1	T-helper 1
T_h2	T-helper 2
TMB	3,3'5,5'-tetramethylbenzidine
TNF-α	Tumour Necrosis Factor-α
TrisHCl	Tris(hydroxymethyl) Methylamine
VMD	Volume Mean Diameter

DEDICATION

This thesis is dedicated to my parents, Joe and Barbara, for their love and for encouraging my inquisitiveness in the first place.

CHAPTER 1: INTRODUCTION

Preface

Vaccination is the most cost-effective form of medical treatment and the worldwide eradication of smallpox through vaccination is considered one of modern medicine's greatest triumphs. Mass immunisation programs have also led to the virtual eradication, at least in developed countries, of several other diseases associated with significant mortality, including diphtheria, polio and measles.

Most of the highly successful vaccines currently available were developed before 1970 and, despite the considerable advances in our understanding of the immune system which have occurred during the intervening period, there are still many diseases for which we lack effective vaccines. For example, WHO estimates that 300-500 million clinical cases of malaria occur each year, resulting in up to 2.7 million deaths (1). Similarly, respiratory infections and diarrhoeal diseases remain major causes of mortality and morbidity, particularly in children.

An additional problem is that although vaccines such as measles or polio can be used effectively in developed countries, technical and economic problems impede their widespread use in developing world. For instance, the current, highly effective measles vaccine is heat-sensitive, making its use difficult in tropical countries (2). Finally, the emergence of diseases for which there is no currently available therapy, such as HIV and antibiotic-resistant tuberculosis, pose major public health risks in both developed and developing nations.

Traditional vaccine research has focussed on the induction of systemic immunity through parenteral immunisation. However, while this approach may be appropriate against diseases which gain acccess to the body through damaged or punctured skin, the majority of pathogens infect hosts via mucosal surfaces, particularly the respiratory, intestinal and urogenital tracts. Parenterally administered vaccines generally do not induce the mucosal immune responses required to prevent infection with pathogens encountered at mucosal sites (3).

Orally administered vaccines are widely favoured because, in addition to inducing local immunity in the intestine, oral immunisation may also stimulate immune responses at distal mucosal surfaces and can induce widespread systemic immunity. The potential of such an approach is illustrated by the success of the oral polio vaccine (4). Oral immunisation is also one of the few effective means of promoting vertical transfer of immunity from mother to infant, via secretory IgA antibodies contained in breast milk (5). Oral vaccines have several economical and practical advantages, including ease of administration, reduced side-effects and the potential for almost unlimited frequency of boosting without the need for trained personnel. Furthermore, the avoidance of the use of needles eliminates any risks of infection or cross-contamination and reduces the cost of vaccination. For these reasons the development of orally active vaccines was one of the major goals outlined by the Children's Vaccine Initiative (CVI) initiated by WHO in 1990 (6).

1. THE DEVELOPMENT OF EFFECTIVE VACCINES

A) The Induction of Protective Immunity

An effective vaccine must stimulate all forms of the immune response which would normally be provoked by the candidate pathogen itself. In most cases, this will require activation of more than one limb of the immune response, including local and systemic antibody responses, cytokine production, and cytotoxic T cells (CTL). In order to appreciate the magnitude of this challenge, it is first important to consider briefly the different types of immune response which may play a role in immunity against infection.

The spectrum of mechanisms employed by microorganisms to infect and persist in mammalian hosts is matched by the variety of immune effector mechanisms which have evolved to counteract such infections.

i) Extracellular infections

Pathogens which inhabit the extracellular milieu are first attacked by a combination of non-specific mechanisms, such as complement-mediated lysis and phagocytosis by polymorphonuclear cells (7). If these fail, specific antibodies can participate in clearance of infectious agents by neutralization (8, 9), opsonization (10, 11) or complement activation, thus promoting lysis and phagocytosis (12).

The majority of specific antibody responses are dependent on antigen-specific $CD4^+$ helper T cells (T_h) (13, 14). In turn, activation of these $CD4^+$ T cells requires efficient uptake and processing of antigens by professional antigen-presenting cells (APC) such as

macrophages (Mϕ) and dendritic cells (DC) (15, 16). These cells aquire exogenous antigens via an endocytic pathway, and digest and process the antigens with lysosomal enzymes in an acidic compartment, resulting in the generation of antigenic fragments of around 12-24 amino acids in length (17-20). These peptides are then bound by newly synthesized class II MHC molecules which transit through the late endosomes on their way to the cell surface (21, 22). The resulting cell surface peptide-MHC complexes are then recognised by specific $CD4^+$ T_h cells which, when activated, provide the stimuli necessary to promote the proliferation and differentiation of antigen-specific B cells (14, 23). These factors include cell-surface molecules such as CD40-ligand (24, 25), as well as secreted cytokines like IL-4, IL-5 and IL-6 (26-29). A schematic representation of the processing and presentation of exogenous antigens is shown in Figure 1.1.

ii) Intracellular infections

The intracellular habitat occupied by most viruses, many parasites and some bacteria allows them to evade the humoral arm of the immune response. Under these circumstances, specific antibody responses are of little use in eradicating primary infection and cell-mediated immune (CMI) responses are required. Two main effector mechanisms have evolved for this purpose.

Infectious agents which replicate in the cytoplasm or nucleus of host cells, such as viruses and some intracellular bacteria and parasites, are eradicated via the lysis of infected cells by cytotoxic T lymphocytes (CTL) (30-32). These $CD8^+$ T cells recognise 8-10 amino acid long antigenic peptides expressed in association with class I MHC molecules, which, unlike the peptides presented to $CD4^+$

T cells, are derived from proteins degraded in the cytosol itself, probably by the organelles known as proteasomes (20, 33-35). These peptides are then transported by specific transporters of antigen presentation (TAP)(36) into the endoplasmic reticulum (ER) where they associate with newly synthesised class I MHC molecules and the complexes are then exported to the cell surface (37). A schematic representation of the endogenous antigen processing processing and presentation pathway is shown in Figure 1.2. Upon recognition of the appropriate peptide-class I MHC complex on infected cells, the CTL delivers a 'lethal hit', resulting in the death of the infected cell (30). This can occur either by directed release of cytolytic granules from the CTL to the target cell (31, 32), or by crosslinking of the fas molecule on the surface of the target cell by its ligand on the CTL surface (fas-L) (32, 38-40). In both cases, death of the target cell occurs via the process of apoptosis, in which target cell endonucleases produce DNA fragmentation and death (30, 41). In this way, infected cells can be killed and the infection eliminated rapidly, while neighbouring uninfected cells are preserved intact.

Pathogens such as mycobacteria or leishmania, persist within cells of the reticulo-endothelial system (Mϕ and monocytes), and these infections are usually controlled by a chronic inflammatory response triggered by the release, from $CD4^+$ T cells, of cytokines such as IFN-γ and TNF-α (42, 43). These cytokines activate powerful microbicidal mechanisms within Mϕ and monocytes, leading to the destruction of the pathogen (44). As with T_h-dependent antibody production, these delayed-type hypersensitivity (DTH) responses require the presentation of antigens which have been acquired and

degraded in endocytic cellular compartments and expressed on the cell surface in association with class II MHC molecules.

In mice, the subset of CD4+ T_h cells which triggers DTH responses can be distinguished from that which provides help for antibody production, by the pattern of cytokines which each produces upon activation (27, 45). The former subset, referred to as T_h1 cells, produce predominantly IL-2, IFN-γ, and TNF-α, while the latter, designated T_h2 cells, produce IL-4, IL-5, IL-6 and IL-10. Although it is now clear that these distinct cytokine profiles can determine different immune effector functions, the factors which enable different organisms to elicit T_h1 or T_h2 responses preferentially are not clearly understood and are the focus of much current research. It is clear however, that cytokines, APC and the local microenvironment as well as the pathogens themselves may all play a role (23, 27, 45, 46).

iii) Interactions between different protective mechanisms

Although the individual responses outlined above account for protective immunity against many pathogens, it should be noted that in several infections, more than one protective mechanism may be required. This is particularly the case with large, multicellular organisms such as parasitic worms, which are too large to be phagocytosed and often occupy sites not easily accessible to the immune system, such as the lumen of the intestine. In these cases, both cell-mediated and humoral responses are involved in the destruction and/or expulsion of the parasite (47, 48). In other instances, the induction, expansion and differentiation of anti-pathogen CTL responses may be dependent on cytokines derived from CD4+ T cells (49-51). Different effector mechanisms may also

be required for eradicating primary infection and preventing reinfection with the same pathogen. This occurs in viral infections, such as influenza, where CTL are required to clear primary infection, while neutralising antibodies may be important in limiting the spread of infection and in preventing subsequent reinfection (52).

B) The 'Ideal' Vaccine

It is clear that the immune system of higher vertebrates contains a powerful and diverse armoury of protective mechanisms which have to be tailored carefully to eradicate primary infections and to prevent reinfection by individual pathogens. The ideal vaccine will trigger the appropriate protective response required. Of particular importance, the vaccine must allow antigen to be processed and presented via the appropriate pathway and in association with the appropriate class of MHC molecule, in order to elicit the necessary effector mechanism(s). The ideal vaccine would also be able to provide long-lasting protection in the genetically disparate human population after a single or limited numbers of administration. It should also contain a defined protective antigen, rather than the entire organism.

Although traditional vaccines have been derived through an empirical process of attenuation of live microorganisms, this method is not applicable to all pathogens, as it usually requires repeated passage through experimental hosts *in vivo* or long term culture in mammalian cells *in vitro*. Furthermore, such attenuated organisms are often poorly characterised and it is not always possible to be sure that they are safe for use in vaccines. An

alternative approach, which forms the background to this thesis, is to develop vectors which would allow recombinant proteins to form the basis of non-living vaccines. However, as purified protein antigens are generally poorly immunogenic (3, 53), some form of adjuvant is a vital prerequisite for such a strategy. Unfortunately, many of the traditional adjuvants that are used in experimental systems, such as Freund's adjuvant, are too toxic for use in humans. Conversely, the only adjuvants currently licensed for administration to humans are the alum salts, which generate mainly humoral immune responses and do not promote immunity at mucosal surfaces (53). Furthermore, most conventional adjuvants allow only for antigen presention via the exogenous pathway, and do not facilitate the induction of class I MHC-restricted CTL responses. Thus, there is currently a great deal of interest in the development of safe adjuvants which will promote a full range of immune responses. Additionally, such vectors should be stable under a wide range of storage conditions, should be easy and economical to manufacture and simple to administer. Finally, as I have discussed, it would also be extremely advantageous if the adjuvant vector could be used for oral vaccination.

2. ORAL IMMUNISATION AND MUCOSAL IMMUNITY

A) General Considerations

Putative oral recombinant vaccines must overcome a number of problems additional to those described above. Firstly, the vaccine must be able to withstand the harsh environments of the stomach and intestine, by being resistant to acid pH and the actions of

digestive enzymes and bile salts. Secondly, the usual result of oral administration of protein antigens is a state of profound immunologic unresponsiveness (54, 55). This oral tolerance abrogates any local or systemic primary immune response to the initial dose of antigen and prevents secondary responses to subsequent challenge with the antigen, irrespective of its route of administration (54, 55). As it is probably the homeostatic mechanism which prevents hypersensitivity to food proteins, the phenomenon of oral tolerance represents the single greatest barrier to the development of oral vaccines utilising defined protein antigens.

The last prerequisite for a new oral vaccine is that it must promote high levels of protective immunity locally and systemically, while ensuring that potentially damaging immunopathological reactions do not occur if the antigen is subsequently encountered in the small intestine. Unlike most parenteral tissues, where inflammatory responses are unlikely to cause serious health problems, immunopathological reactions in the intestine may have major consequences for the host (54, 55).

B) The Mucosa-Associated Lymphoid Tissue (MALT)

The development of effective oral vaccines must take into account our knowledge of the induction of immune responses at mucosal surfaces and the possible consequences of stimulating mucosal immunity. In man, the mucosal epithelia have a total surface area of over 400 square metres, which is more than 200 fold greater than the surface area of the skin (56). In order to protect this large and fragile epithelium, and so prevent disruption

of essential physiological functions, higher vertebrates have developed a complex local immune system.

The mucosal immune system can be divided anatomically and functionally into inductive and effector compartments, collectively known as the mucosa-associated lymphoid tissue (MALT) (57, 58). MALT consists of several components including the gut-associated lymphoid tissue (GALT), bronchial-associated lymphoid tissue (BALT), nasopharyngeal-associated lymphoid tissue (NALT), the mammary, lacrimal and salivary glands and the lymphoid tissues of the urogenital tract and inner ear. All the tissues of the MALT share similarties in their organisation and function. MALT is characterised by the predominance of local secretory IgA production (59) and by the ability of activated lymphocytes from one mucosal surface to recirculate and localise selectively in other mucosal surfaces (60). This permits immunity initiated in one part of the MALT to protect other mucosal sites, hence providing the opportunity to use oral vaccination to protect against respiratory pathogens such as influenza or RSV.

i) The Gut-Associated Lymphoid Tissue (GALT)

Most of what is known about the nature and function of MALT is based on extensive studies of the GALT as a site of induction and expression of mucosal immunity (57, 58, 61).

The GALT consists partly of organised lymphoid aggregates, represented by the Peyer's patches, appendix, mesenteric lymph nodes and solitary mucosal lymphoid nodules. In addition, there are substantial numbers of lymphoid cells scattered throughout the epithelium and lamina propria of the intestine itself.

ii) Induction of Mucosal Immune Responses

The major inductive sites for intestinal immunity are the Peyer's patches (PP), first described by Johann Conrad Peyer in 1677 (61). These clusters of lymphoid tissue are found in the wall of the small intestine, from the ileum to the colon and extend through the lamina propria and submucosa. On the mucosal surface, PP appear as mounds protruding between the intestinal villi and consist of secondary lymphoid aggregates covered by a single layer of columnar epithelial cells. The PP have a typical secondary lymphoid organ structure, with T cell areas and B cell follicles (61) (Figure 1.3).

Peyer's patches differ from secondary lymphoid organs elsewhere in the body because they lack afferent lymphatics. Instead, antigen is sampled directly from the intestinal lumen via the overlying epithelium, and thence to adjacent APC and lymphoid cells. This function is performed by specialised epithelial cells, known as M cells because of the characteristic irregular microfolds present on their luminal surface, in contrast to the microvilli found on normal enterocytes (62, 63). M cells take up materials from the intestinal lumen and transport them to lymphocytes and macrophages enfolded in pockets formed by the basolateral membranes of the M cells (64). A variety of antigenic structures can be taken up by M cells, including particles and macromolecules, as well as intact viruses, bacteria and parasites (65). Antigens transported by M cells may also pass downward through the basal lamina into the lymphoid follicles before being carried into mesenteric lymph nodes via draining lymphatics.

The PP contain discrete B cell areas, or follicles, which have germinal centres that contain the major source of of surface IgA^+ B

cell precursors, although few IgA plasma cells are found within the PP (66). The parafollicular areas of the PP are the T cell areas and are also the sites where lymphocytes enter the patch via high endothelial venules (HEV). The T cells present in the parafollicular regions are virtually all mature αβ T cells (67). $CD4^+$ T cells account for 50-60% of PP T cells, and some of these are found in the germinal centre. The PP also contain many APC, including dendritic cells and Mϕ, which are found throughout the patch in both the T and B cell areas.

Thus, the PP contain all the components required for the initiation of immune responses to antigens encountered in the lumen of the small intestine.

iii) Effector Arms of the Mucosal Immune System

Upon encountering the appropriate antigen in the PP, activated lymphocytes exit the PP via the afferent lymphatics into the draining mesenteric lymph nodes (MLN) and hence via the thoracic duct (TD) to the bloodstream. Lymphocytes which have been activated in mucosal inductive sites show a striking predominance to recirculate back to mucosal tissues, particularly those associated with expression of effector functions, such as the intestinal wall (60, 68, 69). This preferential homing is thought to be due to interactions between adhesion receptors selectively expressed on mucosal endothelia and unique ligands present on lymphocytes. One such adhesion receptor, mucosal addressin cell adhesion molecule 1 (MAdCAM-1) is expressed on endothelia in mucosal tissues, such as HEV in PP, as well as venules in the intestinal lamina propria and in the lactating mammary gland, but is not found on HEV in peripheral lymphoid tissues. MAdCAM-1

binds to the $\alpha 4\beta 7$ integrin molecule (LPAM-1) expressed on the surface of lymphocytes activated in mucosal tissues and this interaction is thought to play a key role in the homing of effector lymphocytes to mucosal tissues (70, 71).

The main effector sites for mucosal immune responses are the lamina propria of the gastrointestinal, respiratory and urogenitary tracts, as well as the parenchyma of the mammary, salivary and lacrimal glands. Both humoral and cellular immune responses can be expressed in these tissues.

iv) Secretory IgA: Humoral Immunity at Mucosal Surfaces

The lamina propria of the gut contains 40-60% T cells and 20-40% B cells, including many plasma cells (67). The majority (up to 90%) of these plasma cells synthesize IgA and are responsible for the antigen-specific IgA found in external secretions, which is the hallmark of mucosal humoral immunity (72).

Secretory IgA contains an IgA dimer held together by a small polypeptide known as the J (joining) chain, which forms disulphide bonds with the IgA monomers (72). This polymeric molecule is secreted intact by mucosal plasma cells and binds specifically to a molecule called the poly-Ig receptor expressed on the basolateral surfaces of the overlying epithelial cells (Figure 1.4). When the poly-Ig receptor has bound a molecule of dimeric IgA, the complex is internalised and transported in an endocytic vesicle through the cytoplasm of the epithelial cell to its apical surface, a process known as transcytosis. At the apical surface the poly-Ig receptor is enzymatically cleaved, releasing the dimeric IgA which retains part of the poly-Ig receptor bound to its Fc regions. This fragment of the poly-Ig receptor, called secretory component, may help to protect

dimeric IgA from proteolytic cleavage. In this way, IgA is transported across epithelia into the lumen of several organs that are in contact with the external environment (Fig. 1.4). The poly-Ig receptor is also expressed by cells of the hepatobiliary system and may contribute to the removal of immune complexes containing polymeric IgA from the circulation (73).

Secretory IgA has a number of biological properties, although its overall function remains uncertain. It can neutralize several viruses, bacteria and toxins, including cholera toxin and the heat-labile toxin produced by some strains of *E.coli*, LT (74). Protection against influenza correlates with levels of neutralising secretory IgA antibodies (52, 75). Intranasal administration of specific monoclonal IgA also protects against Sendai virus infection in mice (76) and intestinal IgA is thought to be the major protective mechanism against cholera toxin (74).

By binding to the mucus layer overlying the epithelia, secretory IgA can also inhibit the adherence of microorganisms to mucosal surfaces and promote agglutination and entrapment in mucus, thus preventing colonisation (77). It is particularly efficient at preventing bacterial attachment because of its multiple binding sites (78, 79). A similar mechanism may also reduce the absorption of other dietary and respiratory antigens (80). This function, known as immune exclusion, may explain why individuals with IgA deficiency have a higher incidence of atopic reactions to environmental antigens (81). As noted above, the ability of IgA to bind to absorbed antigen and be excreted by the hepatobiliary route may also contribute to this process (73). The agglutination, neutralization and exclusion of pathogens and antigens by IgA represent non-inflammatory mechanisms of protection. In addition,

IgA possesses poor complement activating properties (82, 83), consistent with its role in protecting the fragile epithelia while avoiding potentially harmful inflammatory reactions.

Although secretory IgA is by far the predominant Ig isotype found in mucosal secretions, there are also small amounts of other isotypes present. Polymeric IgM can bind the poly-Ig receptor and in humans with IgA deficiency, the production of secretory IgM probably compensates (84). IgG is not found in normal intestinal secretions, but is relatively abundant in respiratory tract secretions and large amounts are found in all mucosal secretions during inflammatory conditions (85). Similarly, IgE is normally absent from mucosal secretions and is usually only present during immediate type hypersensitivity reactions or parasitic infections (86).

v) Cellular Immunity at Mucosal Surfaces

Despite the predominance of IgA in mucosal secretions, selective IgA deficiency occurs in around 1 in 500 individuals in Western populations (81). As the majority of these subjects do not suffer serious consequences, additional protective mechanisms must operate at mucosal sites. Indeed, all conventional T cell subpopulations, as well as some novel ones, are represented in the inductive and effector mucosal lymphoid tissues.

The majority of T cells found in PP and lamina propria are $CD4^+$ T cells. One of their primary functions is to provide help for the induction and expression of secretory IgA responses, with the differentiation of surface IgA^+ B cells into plasma cells being dependent on factors produced by the T_h2 subset of $CD4^+$ T cells, including IL-5 and IL-6 (26, 87-89). In addition, isotype switching

of IgM^+ B cells to IgA^+ B cells is also dependent on T cells, probably via the production of TGFβ (90). Consistent with these findings, $CD4^+$ T cells secreting T_h2 cytokines are abundant in the mucosal lamina propria, glandular tissues and PP (91).

Mucosal inductive and effector sites also contain large numbers of $CD4^+$ cells of the T_h1 subset which produce IFN-γ (91). The precise role of T_h1 cells in mucosal immunity is not clear, although they are presumably important in counteracting intracellular bacterial and parasitic infections and in assisting the development of mucosal CTL responses, through production of IL-2 and IFN-γ. As immunopathological reactions in the intestine are often associated with the production of T_h1 cytokines, including IFN-γ and TNF-α (92), T_h1 cell activation must normally be tightly regulated. The relative predominance of T_h2 type cells in mucosal effector sites, together with oral tolerance, may reflect the need to avoid inflammatory reactions at these surfaces.

$CD8^+$ T cell responses can also be mounted by the mucosal immune system. Antigen-specific CTL have been observed in the PP and in lamina propria after oral immunisation with rotavirus or reovirus (93, 94) and these protocols also induced CTL activity in the systemic lymphoid tissues. Although it is believed that mucosally induced CTL are derived from precursor CTL (pCTL) in the PP, the pathways of mucosal CTL induction have not been elucidated clearly. A better understanding of the induction and functional characteristics of CTL in mucosal tissues is necessary in order to understand their role in protection against infection.

vi) Intra-Epithelial Lymphocytes (IEL)

The mucosal immune system also contains a unique population of T lymphocytes, known as intraepithelial lymphocytes (IEL). IEL are found interspersed between the columnar epithelial cells of the villi in the small and large intestine (95) and hence may constitute the first line of defense against luminal antigens.

IEL have a number of unique phenotypic characteristics which distinguish them from other T cells. The majority (≈90%) are $CD8^+$ T cells, which can be divided into distinct subpopulations based on the surface molecules that they express (95-97). In rodents and some other species, a high proportion express the γδTcR and also express a homodimeric αα form of the CD8 molecule, not found on peripheral $CD8^+$ T cells. A substantial proportion of murine $\gamma\delta TcR^+$ IEL are extrathymically derived (98).

IEL also contain $CD8^+$ cells which express the αβTcR and these can also be subdivided depending on the form of CD8 expressed and whether they are of thymic or extrathymic origin. There are also a number of other minor populations including $CD4^+8^-$, $CD4^+8^+$ and $CD4^-8^-$ T cells, each accounting for <10% of $\alpha\beta TcR^+$ IEL.

The exact function of IEL is not known, but one suggestion is that they constitute a primitive population specialised for immune surveillance of epithelial surfaces (99). In support of this hypothesis, it has been shown that IEL exhibit restricted usage of particular TcR V region genes, suggestive of a locally expanded oligoclonal T cell population (100). In addition, IEL exhibit constitutive cytotoxic activity *in vitro* (101) and there is some evidence that they may recognise non classical MHC IB molecules or heat shock proteins, which may be expressed by infected epithelial cells (102). Other suggestions are that IEL may play a role in oral

tolerance or in other aspects of local immunoregulation, as they produce a variety of cytokines including IL-2, IFN-γ, IL-5 and TGF-β (91, 103).

vii) Immune Regulation in the Intestine

The mucosal immune system has the task of protecting the epithelium, while at the same time avoiding harmful inflammatory reactions which may have serious consequences for homeostasis. It is continually exposed to a diverse antigenic load and it must distinguish food antigens and harmless normal gut flora from potentially dangerous pathogens.

In addition to the specific immune mechanisms discussed above, mucosal surfaces are also rich in non-specific inflammatory cells, such as Mϕ and mucosal mast cells. Although these mucosal inflammatory responses are designed for protective immunity, inappropriate expression of their activities has the potential to cause immunopathology. Aberrant inflammatory responses of this type are associated with a number of human pathological conditions including coeliac disease, Crohn's disease and ulcerative colitis (85, 92). Recent studies in transgenic mice have confirmed the importance of a tight immunoregulatory system to prevent immunopathology in normal animals, as targeted depletion of cytokines such as IL-2 or IL-10, or of αβTcR$^+$ T cells all lead to inflammatory bowel-like disease (104-106). Furthermore, other animal models make it clear that different subsets of CD4$^+$ T cells may normally interact to maintain mucosal homeostasis. Thus CD4$^+$ T cells expressing high levels of CD45RB molecule can mediate IBD when transferred in isolation into naive immunodeficient recipients, while T cells expressing low levels of CD45RB can

prevent this activity (107). Hence the intestinal epithelium and immune system operate in a delicate equilibrium, where alterations of one may have profound effects on the other.

The precise factors which determine whether a given antigen will induce tolerance or immunity when administered orally are not clear, but they remain an important consideration for oral vaccines. The nature of the antigen is obviously important, since oral administration of soluble ovalbumin induces tolerance, while OVA coated onto polyglycoside microparticles, or expressed in live bacteria, stimulates active immunity by the oral route (108, 109). Antigen dose is also important, as feeding very low amounts (1-50μg) of soluble OVA may actually promote systemic priming, while high doses (>1-2mg) induce tolerance (55). Other factors, including the age and genetic background of the host, the presence of normal intestinal flora and the permeability of the intestine, also contribute to the uptake and processing of intestinal antigens (55).

3. MODERN APPROACHES TO ORAL VACCINATION

In view of the complex factors underlying the induction and expression of mucosal immunity, it is unsurprising that a number of different strategies have been proposed as oral vaccines. These include live vectors which have been genetically altered to attenuate their pathogenicity and to express antigens derived from other pathogens in order to elicit protective immunity, as well as a variety of non-replicating adjuvant vectors. I shall now review some of the main strategies currently employed in this rapidly developing field.

A) Live Vectors for Oral Vaccines

Immune responses to live organisms are generally of greater magnitude and of longer duration than those produced by non-replicating immunogens and are particularly efficacious at eliciting cell-mediated immune responses. As noted above, the efficacy of the attenuated oral polio vaccine provides a precedent for the use of such vectors in mucosal immunisation.

i) Attenuated Recombinant Bacteria

Many different bacteria have been investigated for their potential as vaccine vectors. Attenuated *Salmonella* are the most extensively studied and results obtained from these mutants highlight many of the benefits and problems associated with live vaccine vectors, particularly those administered orally.

Oral immunisation with avirulent *Salmonella* strains which have been attenuated by mutating genes essential for growth and survival *in vivo* and/or *in vitro*, such as *aroA*, *purA* or *crp*, can elicit secretory IgA production, as well as systemic IgG responses and CMI responses (110-112). Recombinant *Salmonella* mutants have also had a number of successes as vectors for inducing immunity to heterologous antigens from bacteria, viruses and parasites. Oral immunisation of mice with such mutants induced protective immunity against tetanus, *Streptococcus pyogenes* and *Leishmania major* (113-115).

A major advantage of enteric pathogens such as *Salmonella* is that they can be administered by the natural route of infection (peroral) and interact directly with mucosal associated lymphoid

tissue (MALT). *Salmonellae* persist and replicate in the Peyer's patches and are also taken up by macrophages in the liver, spleen and regional lymph nodes, thus presenting and retaining antigens at inductive sites of both mucosal and systemic lymphoid tissues (116).

Although attenuated *Salmonellae* have excellent potential as oral vaccine vectors, they are also associated with a number of problems. Firstly, the possibility of reversion to virulence *in vivo* must be avoided absolutely and, unfortunately, over-attenuation can reduce immunogenicity *in vivo*. Secondly, it is often difficult to obtain stable expression of foreign genes at sufficient levels in recombinant *Salmonellae* and the plasmids are often quickly lost *in vivo* (111). A further disadvatage is that the way in which the immune response is polarised by *Salmonella* itself influences the nature of the immune response to the heterologously expressed antigen. Although they elicit secretory IgA responses, *Salmonellae* are most effective at promoting systemic T_h1 type responses, thus limiting the range of potential pathogens for which attenuated *Salmonella* vaccines could be adapted (56, 115). Furthermore, the ability of *Salmonellae* to induce antigen-specific CTL responses has not yet been clearly demonstrated. In addition, the strong responses induced by *Salmonellae* to themselves may limit the use of booster doses. Finally, like all live vectors, attenuated *Salmonellae* may not be sufficiently temperature stable or able to survive lyophilization to make them practical vectors in the field.

Although other bacteria are also being investigated as possible vaccine vectors, including BCG, *Listeria*, *Shigella* and *Vibrio*, most of these show the same disadvantages as *Salmonella* based

vaccines (56). Most have not been studied to the same extent and thus their potential as oral vaccine vectors remains uncertain.

ii) Recombinant Viral Vaccines

Mucosal vaccine delivery may also be accomplished by viral agents and these have the added advantage that they should allow antigen to be presented to $CD8^+$ T cells. However, very few have been examined for their ability to induce mucosal immunity.

Of these, vaccinia virus is one of the best studied and recombinant vaccinia viruses containing the rabies virus glycoprotein gene (VRG) can immunize a variety of wild animal species when adminstered orally (117, 118). This approach is likely to be an important future strategy for eradicating rabies from wild animal reservoirs. Intranasal or oral immunisation with recombinant vaccinia virus expressing the F glycoprotein of respiratory syncytial virus (RSV F-Gp) also induces protective immunity in rats (119). However, the major drawback with the current vaccinia vectors is their reactogenicity and the risk of disseminated disease in immunocompromised individuals (120).

Oral or intranasal immunisation with a number of recombinant adenovirus vaccines has also been shown to induce protection against rabies glycoprotein, vesicular stomatitis virus (VSV) Gp and hepatitis B virus surface antigen (121-123). However, there are concerns over the potential oncogenicity of adenoviruses, while pre-existing immunity within human populations may limit their effectiveness (124). Not surprisingly, poliovirus has also been explored as a vaccine vector. Despite the small size of its genome limiting the size of foreign genes which can be inserted, heterologous antigen fragments of up to 400bp can be expressed in

poliovirus (125). Mucosal immunisation of a primate with one such recombinant was able to induce secretory IgA and systemic IgG responses specific for the heterologous antigen (125). Aside from these isolated reports, the use of viral vectors for mucosal immunisation is still in its infancy and even less is known about other potentially appropriate mucosal virus systems, such as rotaviruses and herpesviruses.

B) Non-Replicating Vectors for Oral Immunisation

The disadvantages of live vaccine vectors outlined above, together with technological advances permitting the identification and manufacture of microbial antigens which are the target of successful immune responses, have enhanced interest in the use of non-viable antigen delivery systems as vaccine vectors.

i) Particulate Delivery Systems for Oral Immunisation

It has been known for some time that particulate antigens are more potent inducers of immune responses than soluble antigens and this is also true of orally administered antigens (126, 127). There are a number of factors which may explain why particulate antigens are more effective oral immunogens than soluble antigens (128). Firstly, macromolecular complexes may survive better in the harsh environments encountered following ingestion. In addition, particulates may be absorbed by M cells with greater efficiency than soluble molecules, thus increasing their concentration in mucosal inductive sites and avoiding the induction of oral tolerance which occurs to soluble antigen (129).

ii) Liposomes

A variety of vectors of this type have been explored, of which liposomes are the most extensively studied. These bilayered phospholipid membranes have a number of additional theoretical advantages such as biodegradability, the ability to be lyophilized and freeze-dried and the potential for the incorporation of additional targetting agents and adjuvants (128, 130). The immunoenhancing properties of parenterally administered liposomes are probably related to their ability to promote phagocytosis by professional APC (131, 132), but the precise mechanisms by which they present antigen to host cells for the induction of immune responses requires further investigation. Similarly, the potency of liposomes as oral vaccine vectors is controversial, and although the induction of antigen-specific IgA has been reported (133), little is known on the the induction of cell-mediated immune responses after oral administration of liposomes. Finally, although liposomes are relatively easy to manufacture, it is possible that the immunogenicity of some antigens may be altered by entrapment within liposomes.

iii) Biodegradable Microparticles as Oral Vaccine Vectors

These are microspheres constructed from synthetic biodegradable polymers into which antigens can be entrapped. The best characterised are the poly(D,L-lactide-co-glycolide) (PLG) microspheres which degrade slowly *in vivo*, via hydrolysis, to yield the biocompatible products, lactic acid and glycolic acid (134). The degradation rate of PLG copolymers can be adjusted by altering their size and/or lactide to glycolide ratio (135). They have been used safely in humans for over 30 years as resorbable sutures and

surgical clips and recently attention has focussed on their potential as drug and antigen delivery systems.

Parenteral immunisation with OVA or staphylococcal enterotoxin B (SEB) entrapped in PLG microparticles elicited serum IgG responses equivalent in magnitude to those induced by these antigens in complete Freund's adjuvant (108, 136). Furthermore, oral immunisation with these microparticles induced both antigen-specific secretory IgA and systemic IgG responses (137, 138). Interestingly, fluorochrome-containing microparticles were detected sequentially in the PP, MLN and spleen after oral administration (137), suggesting that they can deliver antigens to both mucosal and systemic lymphoid tissues.

One possible problem with microparticle-based vaccines is that during the construction process, antigens are subjected to potentially damaging conditions including exposure to organic solvents and high shear, which may influence their immunogenicity. Finally, as with liposomes, the cell-mediated immune responses induced by PLG microparticles, particularly following oral immunisation, have not yet been well defined and this area is investigated more fully in chapter 8 of this thesis.

iv) Non-Particulate Adjuvants for Mucosal Immunisation

Despite the apparent advantages of particulate vectors, most practical immunisation regimes require use of an adjuvant which non-specifically stimulates the immune system as well as enhancing uptake or presentation. However, only a limited number of these substances have shown reproducible adjuvant activity when administered orally.

v) Cholera Toxin and *Escherichia coli* Heat-Labile Toxin

The most potent non-viable mucosal adjuvants are cholera toxin (CT), the exotoxin produced by *Vibrio cholerae*, and the closely related heat-labile enterotoxin (LT), produced by several strains of *E.coli*.

These molecules are composed of 5 binding (B) subunits which form a ring structure into which a toxic-active (A) subunit is inserted (139). The CT-B subunits bind to the GM1 ganglioside expressed on intestinal epithelial cells resulting in translocation of the CT-A subunit into the cell (140). CT-A has ADP-ribosylating activity, which activates adenylate cyclase leading to an increase in intracellular cAMP in the affected cell (141). This stimulates the electrolyte and fluid secretion responsible for the potentially fatal diarrhoea found in cholera.

Unlike most proteins, CT is highly immunogenic when given orally and can also promote secretory IgA responses to co-administered antigens (142, 143). In addition, CT can prime for antigen-specific T cell proliferative and cytokine responses, both locally and systemically after oral administration and is effective by the intranasal route (142-145). The responses primed by CT are long-lasting, persisting for up to 18 months after a single oral immunisation (146).

Toxicity is the major obstacle to the use of CT as an adjuvant in humans, with oral administration of as little as 1-5μg CT able to cause severe diarrhoea (147). Although considerable effort has been devoted into evaluating the mucosal adjuvant potential of the non-toxic CT-B subunit, conflicting results have been reported (143, 145) and current evidence suggests that this agent has no inherent adjuvant effect (148). Although this problem may be overcome by

covalently linking CTB to antigen, this technology is complex and may not be appropriate to many antigens (148).

The mechanisms responsible for the adjuvant effects of CT are also controversial and complicated by the fact that CT may directly affect a number of cell types including B and T cells, Mϕ and enterocytes as well as increasing gut permeability (149-152). Furthermore, the spectrum of immune responses primed by CT may be biased toward Th2 types (153-155). Together, these problems indicate that the use of CT as an oral adjuvant must remain questionable.

vi) Other Soluble Mucosal Adjuvant Molecules

Few other compounds with systemic adjuvant activity have been examined as oral adjuvants. Oral administration of muramyl dipeptide (MDP), the smallest structural component of the mycobacterial cell wall that retains adjuvanticity, has been shown to stimulate non-specific immune mechanisms and also to augment specific mucosal immune responses to fed antigens (156, 157). Avridine is a synthetic lipoidal amine which is able to augment secretory IgA responses when given intraduodenally (158), but its ability to stimulate systemic immunity when administered mucosally is unclear.

In summary, although there appear to be many living and non-viable vectors with the potential to function as mucosal adjuvants, much further work is required to elucidate those with realistic vaccine potential and no toxicity. For these reasons the search continues for novel alternative oral vaccine vectors.

4. ISCOMS AS VACCINE VECTORS

A) Definintion and Discovery of ISCOMS

Immunostimulating complexes (ISCOMS) were first described by Morein and colleagues in 1984 (159). Upon mixing solublised membrane viral glycoproteins with saponin (Quil A), in the presence of detergent, they observed the formation of hydrophobic matrices of around 30-40nm in diameter with a characteristic cage-like structure (159), see also Figure 2.1. Initial studies showed that these ISCOMS were far more potent inducers of antigen-specific IgG antibodies than micelles formed from the viral glycoproteins alone.

ISCOMS form spontaneously when detergent is dialysed out of a lipid mixture in the presence of Quil A at or above its critical micellar concentration (0.03%) (160). Cholesterol is an essential component of such ISCOMS and most preparations also contain a less rigid lipid such as phosphatidyl choline or phosphatidyl ethanolamine. The constituents of the ISCOM are held together by hydrophobic interactions, with the binding between cholesterol and Quil A crucial for ISCOM formation (161, 162). Electron microscopy shows that ISCOMS are composed of 20 ring-like subunits of about 12nm in diameter which form an icosahedral structure (163). The resulting honeycomb-shaped micelles have hydrophilic pores, through which small water-soluble molecules may diffuse into the interior of the ISCOMS particle (163).

Most early studies of ISCOMS used viral or bacterial membrane glycoproteins which interacted readily with the other hydrophobic constituents of ISCOMS. However, more recently, strategies have been developed to allow construction of ISCOMS

containing a variety of non-hydrophobic protein antigens. For example, acidification has been used to expose hydrophobic groups on BSA, allowing its successful incorporation into ISCOMS (164). The approach used by our laboratory has been to render globular proteins, such as OVA, hydrophobic by addition of palmitic acid residues, thus facilitating its incorporation into ISCOMS (165).

Once formed, ISCOMS are very stable structures, which withstand low pH conditions and are not degradable by bile salts (166). Furthermore, they are amenable to lyophilization and freezing and can be produced in large quantities (167), making them good candidates for use as potential vaccine vectors which act by the oral route.

B) Induction of Immune Responses by Parenteral Immunisation with ISCOMS

As noted above, initial experiments using ISCOMS concentrated on viral membrane proteins as antigens and their immunogenicity was monitored only by examining their ability to induce serum antibody responses (159). However, as ISCOM technology has advanced over the last decade, a great many different types of antigens have been incorporated into ISCOMS and evaluated for their ability to provoke a range of different protective immune responses.

Many studies have demonstrated the induction of specific antibodies after parenteral immunisation with ISCOMS containing antigens from many viruses, including FeLV (168), HSV-1 (169), BHV-1 (170, 171), influenza (172, 173) and CDV (174), as well as surface antigens from *E. coli* and *B. abortus* (175, 176) and parasite

antigens from *Toxoplasma* and *Trypanosoma cruzi* (177, 178). The biological relevance of ISCOMS-induced antibodies has been confirmed by their virus neutralising capabilities *in vitro* and/or by the demonstration of protection against subsequent challenge with the intact pathogen *in vivo* (159, 168-172, 174, 176, 178). In addition, ISCOMS incorporated antigens induce the production of all antibody isotypes, including IgM, IgG subclasses and IgA (179, 180).

In contrast to the numerous reports on humoral immunity, the induction of cellular immune responses by ISCOMS has been less well characterised, although those studies which have examined CMI have reported positive findings. Mice immunised with ISCOMS containing the fusion (F) protein from measles virus exhibited specific DTH responses *in vivo* and virus-specific T_h cell clones could be generated from these mice (181). Similarly, splenocytes isolated from mice primed with influenza virus proteins in ISCOMS secreted IL-2 and mounted antigen-specific proliferative responses when restimulated with the same ISCOMS *in vitro* (182). Together with the range of antibody isotypes, these findings suggest that ISCOMS are able to prime $CD4^+$ T cells *in vivo*.

One unusual property of ISCOMS is their ability to elicit antigen-specific CTL responses against incorporated protein antigens. This was first demonstrated in studies in mice primed twice intranasally with influenza virus proteins in ISCOMS, which showed a 10-fold increase in antigen-specific CTL precursor frequency being observed in lung cells (183). More recently, it has been shown that parenteral immunisation of mice with ISCOMS containing HIV-1 gp160 or influenza haemagglutinin (HA) elicits

antigen-specific CTL responses and these were confirmed to be due to MHC class I-restricted $CD8^+$ T cells (184).

Thus ISCOMS provoke a wide range of humoral and cellular immune responses *in vivo*, including class I MHC-restricted $CD8^+$ CTL activity.

C) Experimental Models of Protection Using ISCOM-Based Vaccines

The potential relevance of these findings to the development of successful vaccines has been highlighted by studies in mice, showing that parenterally administered ISCOMS-based vaccines elicit protection against many experimental viral infections, including rabies (185), influenza virus (172, 173), herpes simplex virus 1 (169) and measles (181, 186). Protection has also been achieved with antigens derived from other types of pathogens, with ISCOMS containing the porin and O-polysaccharide antigens from *Brucella abortus* protecting against challenge with virulent *B. abortus* (176). Furthermore, ISCOMS incorporating antigens from the extracellular parasite *Trypanosoma cruzi*, completely protect mice against a lethal challenge with virulent trypanosomes (178).

ISCOMS induce protective immunity in a range of different mammalian species, including rabbits (170), cats (168), dogs (174, 185), seals (187), sheep (188), pigs (189) and cattle (171), while there is now a commercially available ISCOMS-based vaccine against equine influenza virus (167, 172). Of particular note, ISCOMS have been found to induce protective immunity against experimental retroviral infections. Thus, ISCOMS containing the gp70/85 envelope protein of feline leukaemia virus (FeLV) induce

protection in cats (168), while rhesus monkeys immunised with a SIV-ISCOM preparation were protected against challenge with live SIV (190).

Taken together, these studies show that parenteral immunisation with ISCOMS can induce protective immunity against a variety of pathogenic agents in a range of mammalian hosts and have raised interest that ISCOMS-based vaccines could be used to vaccinate against lentivirus infections in primates.

D) Rationale for Using ISCOMS as Oral Vaccine Vectors

Although the results above highlighted the potential of ISCOMS as vaccine vectors, most studies only assessed their immunogenicity when administered parenterally.

However, when I began this study a number of factors suggested that ISCOMS might also be effective oral vaccine vectors. Firstly, the oral adjuvant effects of Quillaja saponins had been known for some time (191-194). Mice fed an inactivated rabies vaccine mixed with saponin had enhanced serum neutralizing antibody production, lymphocyte proliferation and CTL activity, compared with mice fed the vaccine alone (191, 192, 194). Furthermore, feeding saponin increased the ability of spleen cells to transfer resistance to intracerebral challenge with rabies virus (192) and greatly potentiated the efficacy of an intraperitoneally administered inactivated rabies vaccine (193).

ISCOMS themselves had a number of properties that suggested they might be effective oral immunogens. As noted above, they are extremely stable structures, resistant to both the low pH conditions and to the degradative actions of bile salts that

they would encounter in the gastro-intestinal tract (166). Furthermore, their particulate nature may facilitate their uptake by M cells in the Peyer's patches, thus enhancing the induction of mucosal immune responses (128). Finally, ISCOMS had been shown to prime local and systemic humoral and cell-mediated immune responses when administered intranasally (179, 183) or intravaginally (180)

The final consideration which suggested the oral route might be appropriate for delivery of ISCOMS was that this may decrease the toxicity commonly associated with saponin-based adjuvants. Saponins are surface active compounds which bind to free or membrane-bound cholesterol (162, 195). As a result they can be strongly haemolytic, creating pores of around 8nm diameter in biological membranes (162, 196). However, the toxicity of saponins is much reduced when given orally (194, 197) and indeed saponins occur naturally in many food plants and are widely used as food additives (198).

For these reasons, we thought it important to investigate directly the ability of ISCOMS to act as oral vaccine vectors.

E) Mechanisms of Adjuvanticity of ISCOMS

For vectors which have proved to be so efficacious in such a wide variety of animal models, surprisingly little is known about the cellular and chemical basis of their adjuvant activities or how they induce such a range of immune responses *in vivo*. The potent humoral responses described above indirectly suggest that ISCOMS allow for processing of incorporated antigens via the endocytic pathway, leading to the induction of $CD4^+$ T cells and subsequently,

the activation and differentiation of antigen-specific B cells. However, aside from the few isolated reports describing spleen cell proliferation and IL-2 production (182) and generation of virus-specific CD4+ T cell clones (181), there has been little direct analysis of how ISCOMS can prime CD4+ T cells *in vivo.*

Similarly, the novel ability of ISCOMS to promote the induction of CTL responses directed against exogenous protein antigens (183, 184) suggests that incorporation of protein antigens within ISCOMS facilitates their entry into the class I MHC processing and presentation pathway, but how this occurs is not yet known. Therefore the processing and presentation of proteins incorporated within ISCOMS is clearly an area which merits further study.

In addition to host factors, there is also little known about the properties of the ISCOMS themselves which are responsible for their adjuvant effects. The precise roles of Quil A and of the physical structure of the ISCOMS in adjuvanticity is not clear. As outlined above, the adjuvant properties of saponins have been known for some time but the mechanisms responsible have not been well defined. In addition to the adjuvant effects of orally administered saponins, it has also been reported that parenteral administration of Quil A can enhance antibody responses to both T-dependent and T-independent antigens (199, 200). Interestingly, the augmentation of antibody responses to T-independent antigens was also observed in nude mice (200), suggesting that Quil A may have direct adjuvant effects on B cells.

Whether the inherent toxicity of Quil A can be completely separated from it's adjuvanticity is also not yet clear. However it is encouraging to note that Quil A can be separated by reverse phase chromatography into over 20 distinct fractions (201), two of which,

QS-7 and QS-21, augmented antibody responses to a nominal protein antigen to the same degree as conventional adjuvants such as IFA and CFA (201). Furthermore, no significant toxicity was observed after parenteral administration of the purified fractions. Whether these fractions are able to enhance cell-mediated immune responses, retain the ability to form ISCOMS, or are effective when given orally are all areas requiring further attention in order to assess their suitability as oral vaccine vectors.

7. AIMS OF THIS STUDY

The first aim of this study was to examine the efficacy of ISCOMS as oral vaccine vectors, by assessing the induction of local and systemic immune responses after oral or parenteral immunisation.

ISCOMS containing the protein antigen ovalbumin (ISCOMS-OVA) were used for this purpose for the following reasons. Firstly, OVA is an immunologically well characterised protein antigen which was inexpensive and readily available from commercial sources. Secondly, it had been extensively utilised in this laboratory as an oral antigen and the techniques for assessing local and systemic OVA-specific humoral and cellular immune responses were well established. Lastly, the availability of an OVA-transfected H-2^b cell line (EG7.OVA)(202) made it possible to examine antigen-specific CTL responses induced by ISCOMS-OVA. The immune responses induced by oral or parenteral administration of ISCOMS-OVA form the basis of Chapter 3 of this thesis.

The second important aim of this thesis was to look at the mechanisms involved in the induction of immune responses by ISCOMS. In particular, I wished to examine the effects of ISCOMS on the induction of T cells of both the helper ($CD4^+$) and cytotoxic ($CD8^+$) phenotype, as this area had received little previous attention. By virtue of their ability to prime antigen-specific CTL responses, ISCOMS offered an ideal model to study the induction of CTL responses to an exogenous protein antigen. The characterization of the CTL primed by ISCOMS-OVA, and of the mechanisms involved in their induction, is described in Chapter 4.

As it is now well established that $CD4^+$ T_h cells play a key role in the induction and regulation of many immune responses, I also examined whether ISCOMS were able to prime these cells directly. Furthermore, as the actions of activated $CD4^+$ T_h cells are largely governed by the pattern of cytokines which they secrete, I also explored the nature of the $CD4^+$ T cell response in terms of both T_h1 and T_h2 subpopulations of these cells. Chapter 5 details the priming of $CD4^+$ T cells by ISCOMS.

In the second part of this study, I explored additional aspects of ISCOMS, which although not directly concerned with the nature of immune responses induced by ISCOMS, were relevant to vaccine development. A vital prerequisite for any successful vaccine vector is that it is able to induce long lasting immune responses. As previous studies had not examined whether ISCOMS were capable of this, I decided to look at the longevity of immune responses primed by ISCOMS. The results of these experiments are described in Chapter 6.

As noted above, the factors of the ISCOMS themselves which are important for their adjuvant properties are not yet clear.

However, it appeared possible that many of these activities could have been contributed by the saponin adjuvant Quil A, which is an essential constituent of ISCOMS. Therefore, in an attempt to examine whether the physical structure of the ISCOMS played a key role in their adjuvanticity, I analysed the role of Quil A in the adjuvanticity of ISCOMS. This is described in Chapter 7.

Finally, ISCOMS are one of many different vectors which are currently under evaluation as potential oral vaccine vectors. It is therefore important to compare these putative vectors in order that the best can be selected for further development. Chapter 8 of this thesis describes a study in which I compared the oral adjuvant properties of ISCOMS, with another oral vaccine vector which is attracting much current interest, poly-lactide-co-glycolide (PLG) microparticles.

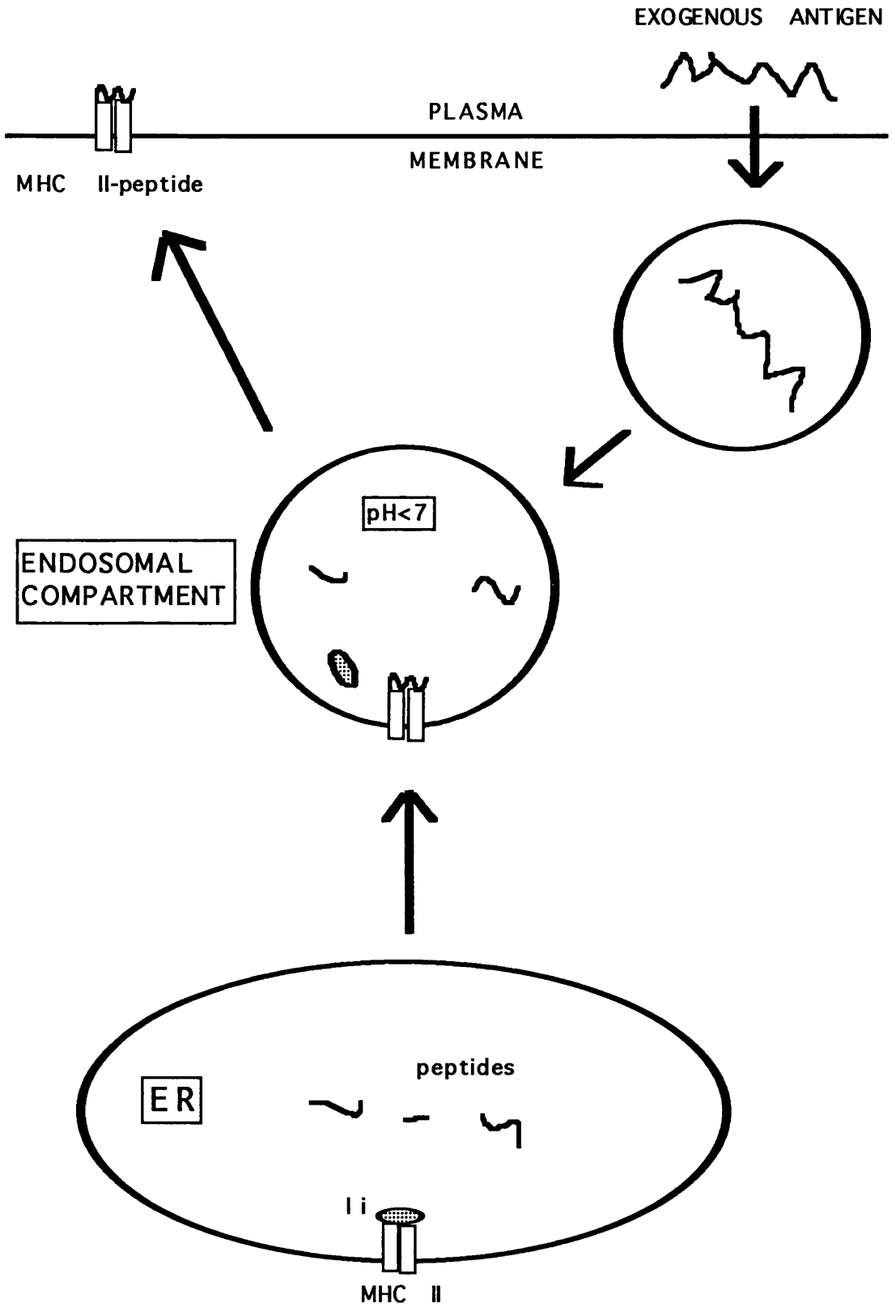

Figure 1.1.

Schematic representation of class II MHC processing and presentation. ER = Endoplasmic Reticulum.

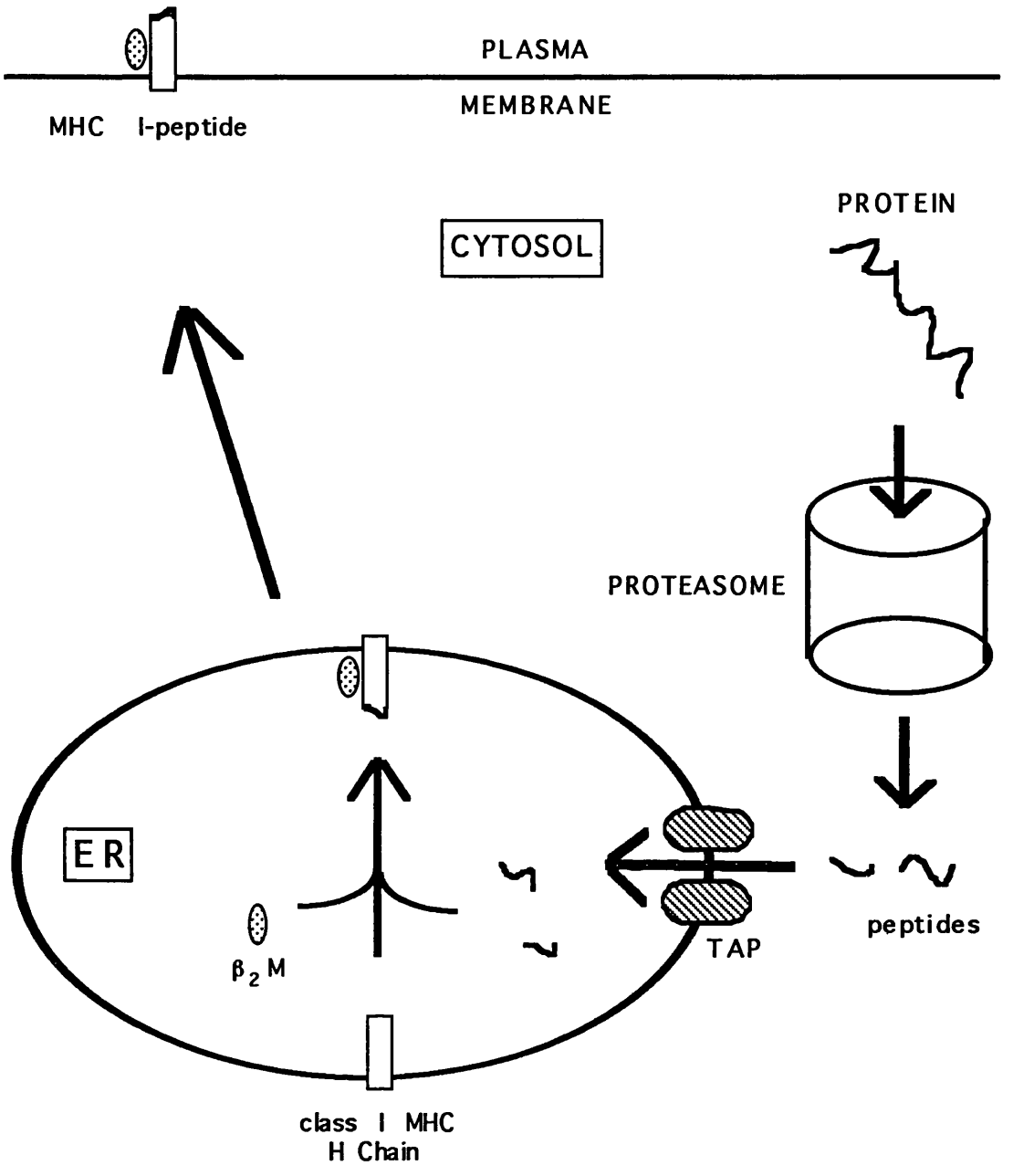

Figure 1.2

Schematic representation of class I MHC processing and presentation.

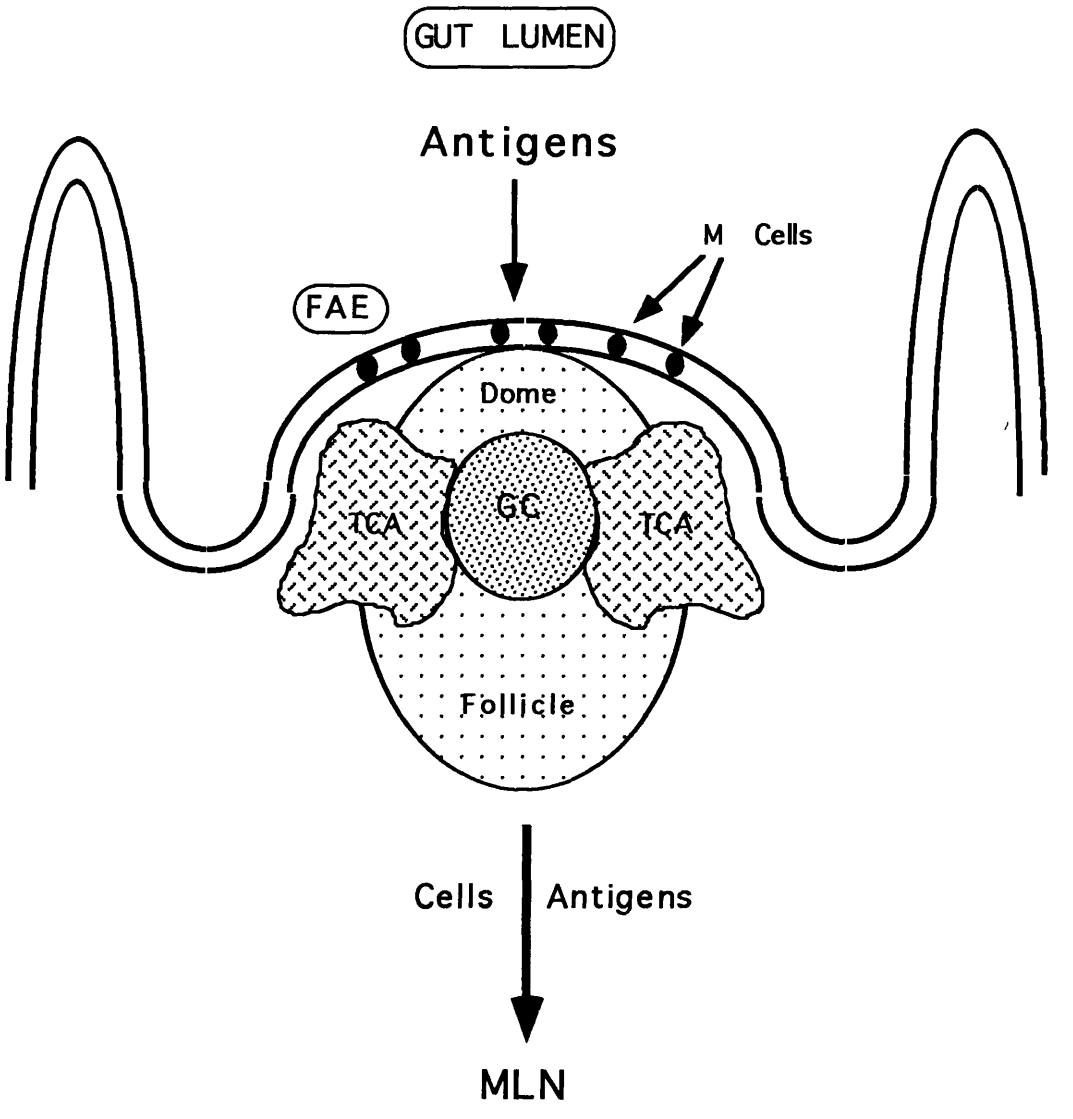

Figure 1.3

Schematic view of a typical Peyer's Patch. Antigen is sampled from the gut lumen by M cells, passed down through the Peyer's patch and via the draining lymphatics to the MLN.

FAE = Follicle-associated epithelium.

GC = Germinal centre.

TCA = T cell area.

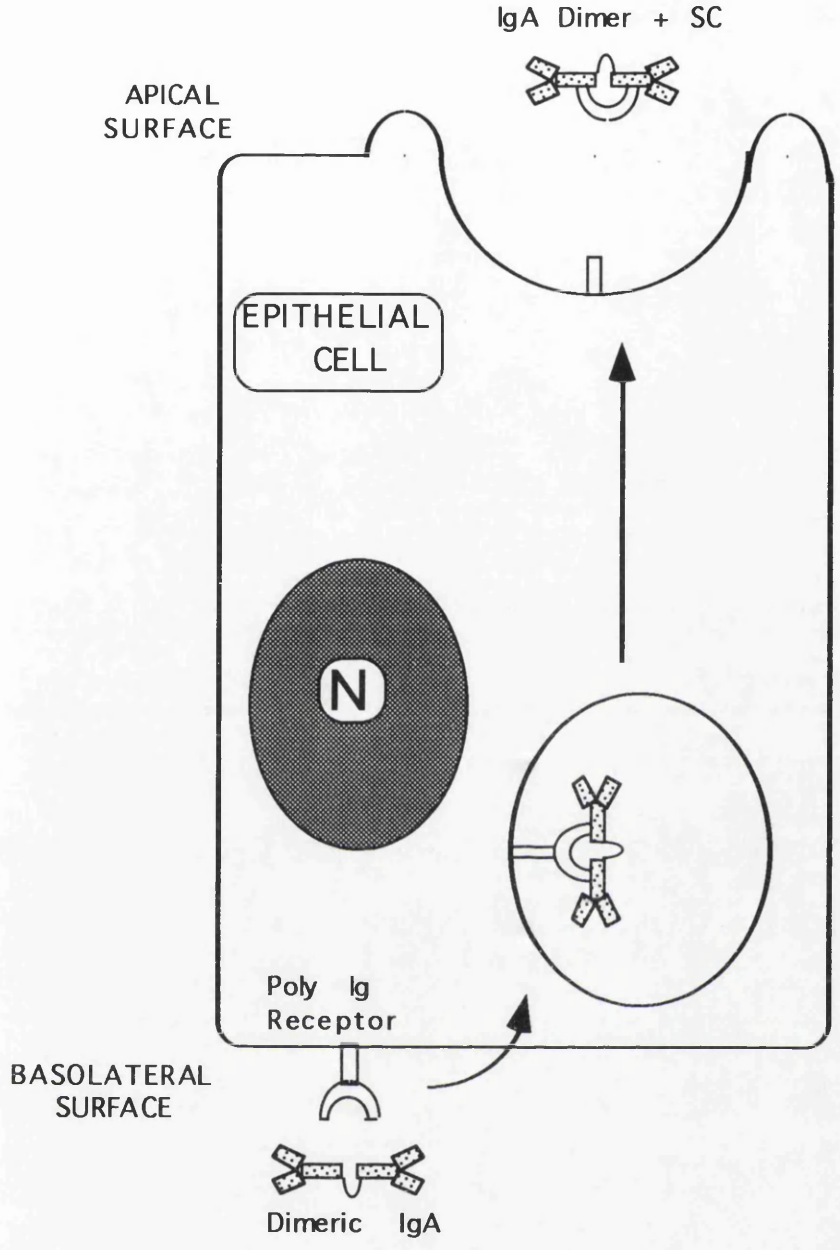

Figure1.4

Transcytosis of IgA antibody across epithelia.

N = Nucleus.

SC = Secretory Component.

CHAPTER 2: MATERIALS AND METHODS

Animals

Female BALB/c (H-2^d) and C57Bl/6 (H-2^b) mice obtained from Harlan Olac Ltd (Bicester, Oxon) were maintained in the Joint Animal Facility, University of Glasgow and were normally used when 6-10 weeks old.

All animals had access to standard rodent pellets containing no OVA and water *ad libitum.*

Anaesthesia

Procedures such as footpad injection and eye bleeding were carried out under light anaesthesia using 5% Halothane BP (Rhone Merieux Ltd, Harlow, Essex).

Antigens and Mitogens

Ovalbumin (OVA, Grade V) and Concanavalin A (Con A) were obtained from Sigma Chemical Co. (Poole, Dorset).

The ovalbumin peptides OVA 111-122 and OVA 258-276 were purchased from Multi Peptide Systems (San Diego, U.S.A.), while the octameric peptide OVA 257-264, was purchased from Medprobe A.S. (Oslo, Norway). Peptides were diluted to 1mM in dH_2O and stored at -70°C until required.

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 60min in a water bath. After centrifuging, the

supernatant was then removed and the precipitated OVA washed with cold (4°C) saline at 450g for 10min. The HAO was resuspended at 20mg/ml in saline and stored at -20°C until required. Before challenge of mice *in vivo*, the HAO was diluted to 2mg/ml in saline and sonicated for 20min to give a colloidal suspension.

Adjuvants

Quil A ('Spikoside', Iscotec AB, Lulea, Sweden) was kindly provided by Prof. Bror Morein (Dept. of Vet. Med. Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden). Quil A was stored at 4°C and dissolved in saline before being mixed with OVA solution immediately prior to immunisation.

Complete Freund's adjuvant (CFA) was purchased from Difco (Detroit, Michigan, U.S.A.) and was emulisified with an equivalent volume of antigen in PBS immediately prior to immunisation.

Preparation of ISCOMS Containing OVA

ISCOMS containing OVA (ISCOMS-OVA) were prepared using a slightly modified method of that described previously (165, 203) As ISCOMS are lipophilic particles composed of lipids and Quil A, non-hydrophobic proteins such as OVA are not incorporated spontaneously. Thus, in the procedures used here, OVA was first palmitified using a succinimide esterification reaction to attach hydrophobic palmitic acid groups. This facilitates incorporation into ISCOMS, while maintaining the native structure and antigenic characteristics of the OVA. The palmitified OVA was then mixed

with the lipids cholesterol and phosphatidyl choline in the presence of Quil A allowing spontaneous formation of ISCOMS-OVA.

i) Palmitification of OVA

20mg OVA dissolved in 2.5ml of 50mM carbonate buffer (pH 9.0) was passed through a Sephadex PD10 column (Pharmacia, Uppsala, Sweden) to remove any low molecular weight contaminants. Intact OVA was then eluted with 50mM carbonate buffer and 0.2ml fractions assayed for protein content by the Bradford assay (see below). The protein-containing fractions were pooled and adjusted to 1mg/ml OVA by dilution in carbonate buffer containing 5% (w/v) sodium deoxycholate (BDH Ltd, Poole, Dorset) and 10% (v/v) dimethyl sulfoxide (DMSO, Sigma).

Purified OVA was then palmitified by the addition of 314μl of N-(palmitoyloxy) succinimide solution (NPS, Sigma, dissolved at 10mg/ml in DMSO) to 20mg OVA (1mg/ml), giving a molar ratio of 20 palmitic acid molecules to each protein molecule. The NPS solution was added slowly, in 25μl aliquots, with constant stirring and heating in order to prevent precipitation and the mixture was then incubated overnight at 37°C in a shaking water bath with a stroke rate of 100/min. 1ml of 1M Tris(hydroxymethyl) methylamine (TrisHCl, pH8.5) was then added and the mixture incubated for a further 30min at 37°C. By providing free amino groups, excess TrisHCl prevents further palmitification of the protein.

Free palmitic acid was then separated from the palmitified protein by ultracentrifugation. First, sucrose (BDH) was added to the protein/NPS mixture to a final concentration of 5% (w/v) and 4ml aliquots of this solution were then overlaid on to 6ml of 40%

sucrose in phosphate buffered saline (PBS) in Ultraclear tubes (Beckman Ltd, High Wycombe, Bucks.). 3ml of PBS containing 2% (w/v) decanoyl-N-methylglucamide (Mega-10, Sigma) was overlaid on to the top of each tube, which were then centrifuged at 100,000g for 4hr at 4°C using a SW41.14 rotor in a Beckman ultracentrifuge. The top PBS layer containing free palmitic acid was then discarded and the palmitified OVA was harvested from the 5% sucrose layer.

ii) Preparation of ISCOMS-OVA

A lipid mixture containing phosphatidylcholine and cholesterol (both Sigma) was prepared by dissolving 50mg of each lipid in 1ml chloroform (Aldrich Chemical Co., Poole, Dorset) and then adding 5ml of dH_2O containing 20% (w/v) Mega-10. The chloro form was then evaporated under a stream of N_2 until the solution cleared, giving a final concentration of 10mg/ml of each lipid. The resulting lipid mixture was stored at -20°C until required.

To prepare ISCOMS-OVA, the lipid mixture was added to the palmitified OVA solution to a final concentration of 0.5mg/ml, and Quil A was then added at a final concentration of 0.1% (w/v). The resulting mixture was sonicated for 15min before being transferred to dialysis tubing (Visking; Medicell International Ltd, London) and dialysed extensively against 50mM TrisHCl or PBS. Dialysis was performed for 4-5 days at 20°C against 4 litres of buffer, which was changed every 12hr. The formation of ISCOMS was indicated by a slightly cloudy appearance of the dialysate and was confirmed by electron microscopy (EM).

The ISCOMS particles were separated from unincorporated reaction products using a discontinous sucrose gradient, prepared in Ultraclear tubes by overlaying 4ml of 10% sucrose in PBS on to 4ml

of 40% sucrose in PBS. The mixture containing ISCOMS was then overlayed on to the 10% sucrose and centrifuged at 100,000g for 16hr at 4°C using a SW41.14 rotor and a Beckman Ultracentrifuge. The ISCOMS were harvested from the interface between the 10% and 40% sucrose layers and then dialysed for 24hr against 4 litres of PBS to remove the sucrose.

After removal of aliquots for EM analysis and protein assay, the ISCOMS were sterilised by filtration through a 0.2μm filter (Acrodisc, Gelman Sciences, Ann Arbor, U.S.A.) and stored at -20°C until use. The ISCOMS-OVA used in this study were 30-40nm in diameter (Figure 2.1) and contained OVA and Quil A at a ratio of 10:1(165, 203).

iii) Estimation of Protein Content using Bradford's Assay

The concentration of OVA in samples of purified proteins and ISCOMS-OVA was estimated using Bradford's assay (204). 20μl aliquots of test samples were added to 180μl Bradford's reagent (Coomassie Blue Dye in 55% phosphoric acid with 15% methanol, diluted 1:5 in dH_2O, Bio-Rad, Hemel Hempstead) in 96 well microtitre plates (Falcon, Becton Dickinson, Cowley, Oxford). After 10-15min incubation at room temperature, the O.D. 570nm of the samples was read in an MR5000 automatic plate reader (Dynatech Laboratories Ltd, Billingshurst, West Sussex) and the concentration of OVA was calculated by comparison with a standard curve constructed using dilutions of OVA in PBS.

Electron Microscopy

Electron microscopical (EM) analysis of ISCOMS was performed by Mrs Jane Hare (Dept. of Pathology, Western Infirmary, Glasgow). The ISCOMS were negatively stained with 3% phosphotungstic acid (pH6.6) and analysed on a Philips CM10 transmission electron microscope (TEM) at a final magnification of 73000x.

Preparation of OVA-Containing Microparticles

Microparticles were prepared in the laboratory of Dr. Derek O'Hagan (Department of Pharmaceutical Sciences, University of Nottingham) using a solvent evaporation technique as described previously (205). Briefly, a solution of poly(D,L-lactide-co-glycolide) polymer (PLG, Resomer RG503, 50:50 lactide:glycolide ratio; Boehringer, Ingleheim, Germany) in dichloromethane (HPLC Grade, May and Baker, Dagenham) was emulsified with an OVA solution in dH_2O using a Silverson homogeniser (Silverson Machines Ltd, Chesham). The resulting water-in-oil emulsion was then added to a much larger volume of an aqueous solution of polyvinyl alcohol (PVA, 88% hydrolysed, Aldrich) and homogenised to produce a stable water-in-oil-in-water emulsion. This was then stirred overnight at room temperature to allow solvent evaporation, with resultant microparticle formation. Microparticles were collected by centrifugation, washed three times to remove non-entrapped OVA and freeze dried. The protein content was determined in a bicinchonic acid assay (Sigma) after extraction of the protein as described previously (206). The mean particle size as determined

by photon correlation spectroscopy was 660nm volume mean diameter (VMD, Figure 2.2).

Maintenance of Cell Lines *in vitro*

EL4 cells (a thymoma of C57Bl/6 origin), P815 cells (a methylcholanthene-induced mastocytoma of DBA/2 origin) and YAC-1 cells (a thymoma of A strain origin) were maintained in RPMI 1640 medium containing 10% heat-inactivated foetal calf serum (FCS), 100U/ml penicillin, 100μg/ml streptomycin, 1.25μg/ml fungizone and 2mM L-glutamine, (all Gibco BRL, Paisley, Scotland; "complete RPMI") at 37°C in 5% CO_2 in air in a humidified incubator.

EG7.OVA cells were obtained from Dr M. Bevan (University of Washington, Seattle, U.S.A.). These were derived from EL4 cells transfected with a single copy of a plasmid containing a cDNA copy of the chicken OVA mRNA (202). As this plasmid also carries a neomycin resistance gene, OVA-expressing cells were selected for in culture by maintenance in complete RPMI medium, supplemented with 400μg/ml Geneticin (G418 sulphate, Gibco BRL).

All cell lines were subcultured every 2-3 days by adding 1-2ml of cell suspension to 10ml of fresh medium and were subcultured 2 days before being used as *in vitro* targets, to ensure that the majority of cells would be in the log phase of growth.

Assessment of OVA-Expression by EG7.OVA cells

Flow cytometric (FACS) analysis was used to monitor the continued expression of OVA by the EG7.OVA cells as it has been

observed that this may decline after long-term culture *in vitro* (K.Heeg, personal communication).

Aliquots of 10^6 cells in 15ml conical plastic centrifuge tubes (Falcon, Becton Dickinson) were washed in staining buffer (PBS containing 2% FCS and 0.05% sodium azide; SB) by centrifuging at 450g for 5min. The cells were then resuspended in 50μl polyclonal sheep-anti-ovalbumin antiserum (Serotec Ltd, Kidlington, Oxford; diluted 1:50 in SB), incubated on ice for 30-40min and after washing twice in SB, the cell pellets were resuspended in 50μl of fluorescein isothiocyanate (FITC) conjugated polyclonal donkey-anti-sheep IgG (Serotec; 1:50 in SB), incubated a further 30-40min on ice, washed twice in SB and resuspended in 0.5 ml PBS.

The samples were analysed using a FACscan flow cytometer as described below. Background fluorescence levels were determined using cells incubated with FITC-donkey-anti-sheep IgG in the absence of primary antibody. Non-OVA expressing EL4 cells stained as above, were used as a negative control. A typical profile of EL4 and EG7.OVA cells stained by the above procedure is shown in Figure 2.3.

Osmotic Loading of Cell Lines with OVA

OVA was introduced directly into the cytoplasm of target cell lines by the osmotic lysis of pinosomes, as described previously (202, 207). 2.5 x 10^6 cells were washed in RPMI 1640 and resuspended in 0.5ml hypertonic RPMI medium containing 20mM HEPES (Sigma), 10mg/ml OVA, 10% (w/v) polyethylene glycol (PEG, Mol. Wt. 1000, Sigma) and 0.5M sucrose. After 10min incubation at 37°C, 14.5ml of hypotonic medium comprising 60% RPMI and 40%

dH_2O was added and the cells were incubated for a further 2-3min at 37°C. After pelleting at 400g for 5min, the cells were resuspended in 5ml of their normal culture medium and incubated overnight at 37°C in 5% CO_2 in air in a humidified incubator.

Induction of Immune Responses *in vivo*

i) Parenteral Immunisation

Subcutaneous (s.c.) immunisations were performed by injection of a total volume of 50µl into one rear footpad under halothane anaesthesia.

Intraperitoneal (i.p.) immunisations were performed by injection of a total volume of 0.2ml.

ii) Oral Immunisation

Oral immunisations were performed by feeding volumes of up to 0.5ml using a rigid steel gavage tube.

Collection of Plasma

Under halothane anaethesia, mice were bled from the retro-orbital plexus using heparinised capillary tubes (Hawksley & Sons Ltd, Lancing, Sussex). A maximum of 200µl was taken on each occasion and plasma was separated by centrifuging for 10min at 500g and stored at -20°C until use.

Collection of Intestinal Secretions

Intestinal fluid for the measurement of IgA antibodies was obtained by a modification of a published technique (208). Mice were fed 0.2ml of a 16% PEG solution (Mol. Wt. 3350, Sigma) in PBS, four times over a 60min period. The entire small intestine and caecum was then removed and washed through with 6ml ice-cold PBS containing 0.1mg/ml soyabean trypsin inhibitor and 50mM ethylenediaminetetraacetic acid (EDTA) (both from Sigma). The resulting fluid was clarified by centrifugation at 1500g for 10min and 3ml of supernatant removed. 30µl of 100mM phenylmethylsulfonyl fluoride (PMSF, Sigma) in 95% ethanol (Aldrich) was then added and the supernatants were centrifuged in eppendorf tubes at 27,000g for 15min at 4°C. 1ml of clarified supernatant was removed and 10µl PMSF solution, 10µl sodium azide (1% in PBS, Sigma) and 50µl FCS were added to each supernatant which were then stored at -20°C until required.

Depletion of $CD4^+$ T Cells *in vivo*

Mice were depleted of $CD4^+$ T cells *in vivo* by i.p. injection of 500µg $(NH_4)_2SO_4$-purified anti-CD4 monoclonal antibody (YTS191.1, kindly provided by Prof F.Y. Liew) on two occasions 4 days apart. Mice were immunised four days after the final injection of antibody.

Depletion of Macrophages *in vivo*

i) Paralysis of Macrophages using Silica

Mice were injected with 1mg silica (Sigma) in 0.2ml saline i.v., followed 24 hr later by 0.5mg silica in 0.2ml saline i.p. Before injection the silica solution was sonicated for 15min to ensure an even colloidal suspension. Mice were immunised 24hr after the second treatment.

ii) Macrophage Depletion using Liposomes Containing Dichloromethylene Diphosphonate

Mice were depleted of macrophages *in vivo* as described previously (209, 210), by i.v. injection of 0.2ml liposomes containing dichloromethylene diphosphonate (Cl_2MDP), kindly provided by Dr. N van Rooijen (Free University, Amsterdam, Holland, with permission from Boehringer Ingleheim), 24hr before immunisation.

Measurement of OVA-Specific Antibody Responses

Serum IgG and intestinal IgA antibody responses were measured using an enzyme-linked immunosorbent assay (ELISA). OVA (10μg/ml in 50mM carbonate buffer) was adsorbed to microtitre plates (Immulon 4, Dynatech) by overnight incubation at 4°C and the plates were then washed three times with PBS/0.05% Tween 20 (BDH) before addition of 100μl samples. These were either sera diluted 1:400 in PBS/Tween containing 1% normal rabbit serum or neat intestinal washes. The plates were incubated with the samples for 2.5hr at room temperature and washed three

times with PBS/Tween before addition of 100μl of a 1:1000 dilution of alkaline phosphatase conjugated goat-anti-mouse IgG or goat-anti-mouse IgA (both Sigma), respectively. After a further 3hr incubation at room temperature, the plates were washed three times with PBS/Tween and 100μl of phosphatase substrate (*p*-nitrophenyl phosphate, Sigma) at 1mg/ml in dH_2O containing 10% diethanolamine (DEA, Aldrich) was then added to each well. After a 10-30min incubation at room temperature, the O.D. 405nm of the samples was measured using an MR5000 automatic microplate reader.

Samples from individual mice were assayed in triplicate and expressed as O.D. 405nm. In some experiments, OVA-specific IgG levels are expressed as a percentage of a hyperimmune standard, which was a $(NH_4)_2SO_4$-purified anti-OVA IgG preparation obtained from mice which had been immunised twice with 100μg OVA in CFA (kindly provided by Miss Margaret Steel, Dept. of Immunology, Western Infirmary).

Measurement of OVA-Specific Serum IgG Isotypes

OVA-specific serum IgG isotypes were also measured using an ELISA. After coating with OVA as above, the plates were washed three times with PBS/0.05% Tween 20 and non-specific protein binding sites were blocked with 100μl of 3% bovine serum albumin (BSA, Sigma) in PBS/Tween for 1hr at room temperature. After three washes with PBS/Tween, 50μl aliquots of test sera in doubling dilutions (1:20 to 1:2560) in PBS/Tween were added to the plates and incubated at room temperature for 90min. Following three washes with PBS/Tween, 50μl of either biotinylated rat anti-murine

IgG1 diluted 1:16,000 or biotinylated rat anti-murine IgG2a (both Pharmingen, San Diego, CA, USA) diluted 1:1000 in PBS/Tween were added and the plates incubated for 1hr at room temperature. The plates were then washed 4 times with PBS/Tween and 75µl extravidin-peroxidase (Sigma, 2mg/ml in PBS/10% FCS) was added. Following a final incubation for 1hr at room temperature, the plates were washed 6 times, before 100µl 3,3'5,5'-tetramethylbenzidine (TMB) peroxidase substrate (Dynatech) was added to each well. The plates were read at 630nm using an MR5000 automatic microplate reader.

Individual samples were assayed in triplicate and their titres expressed as the reciprocal of the lowest dilution which gave an O.D. 630nm value of ≥20% of the maximal O.D. obtained with the hyperimmune standard anti-OVA IgG.

Measurement of Systemic Delayed-Type Hypersensitivity (DTH) Responses

Systemic DTH responses were determined by measuring the increment in footpad thickness 24hr after subcutaneous challenge with 100µg heat-aggregated OVA (211). Footpad thicknesses were measured before and after challenge using calipers (Kroplin Limited, Kingston upon Thames, Surrey).

Preparation of Lymphoid Cells

Spleens, Peyer's patches, mesenteric and popliteal lymph nodes were removed immediately after sacrifice, placed into a Petri dish containing RPMI 1640 medium and after dissection of any

surrounding material, were teased apart using 21g needles (Becton Dickinson). Single cell suspensions were obtained by repeated pipetting with a 5ml syringe (Becton Dickinson), followed by passage through Nitex mesh (Cadisch and Sons Ltd, London) to remove any clumps or debris. After washing twice in RPMI 1640 by centrifuging for 7min at 450g, viable cells were counted with a Neubauer haemocytometer (Weber Scientific, Middlesex) using phase contrast microscopy (Nikon Labophot microscope, x40 objective) and the final cell pellet was resuspended at the required concentration in appropriate medium.

Phenotypic Analysis of Lymphocytes by Flow Cytometry

Aliquots of 5 x 10^5 cells were resuspended in 50µl SB containing 3µl of phycoerythrin (PE-) conjugated anti-CD4 (GK1.5) and FITC-anti-CD8 (53.-6.7; both from Becton Dickinson) monoclonal antibodies. The samples were incubated for 30-40min on ice and washed twice in SB for 7min at 450g. Red blood cells were removed from spleen cell suspensions by incubation in 0.5ml 1:10 Facslyse solution (Becton Dickinson) for 10min at room temperature, followed by washing in SB. After resuspending in 0.5ml PBS, the samples were analysed using a FACscan IV flow cytometer (Becton Dickinson) with a 488nm argon laser. Lymphocytes were gated on the basis of their forward and side light scatter properties and the data were analysed using Lysis II program software (Becton Dickinson). In all experiments, negative control samples were cells incubated with SB in the absence of any antibodies.

Depletion of T Cell Subsets *in vitro*

$CD4^+$ and $CD8^+$ T cell subsets were depleted *in vitro* by complement-mediated lysis. Lymphoid cells were resuspended at 10^7 cells/ml in RPMI 1640 medium/5% FCS containing 200μg/ml of $(NH_3)_2SO_4$-purified anti-CD4 mAb (YTS191.1) or anti-CD8 mAb (YTS169.4) (both kindly provided by Prof. F.Y. Liew) and incubated for 60min on ice. After two washes in RPMI/5% FCS by centrifuging for 7min at 450g, the cells were resuspended at 5 x 10^6 cells/ml in RPMI/5% FCS containing 10% (v/v) rabbit complement (Low-Tox M, Vector Laboratories, Peterborough) and incubated for 60min at 37°C. The cells were then washed twice in RPMI 1640, counted and resuspended at 2 x 10^6 cells/ml in appropriate medium. Control cells were incubated in the absence of mAb or complement.

Measurement of Antigen-Specific Proliferative Responses

Spleen or popliteal lymph node cells were plated in quadruplicate in 96 well flat-bottomed microtitre plates (Costar, Northumbria Biologicals, Cramlington, Northumberland) at 2.5 x 10^5 cells/ well in a total volume of 200μl complete RPMI medium containing 5 x 10^{-5}M 2-mercaptoethanol (2-ME, Sigma; "proliferation medium"). The cells were cultured with various concentrations of OVA or Con A, at 37°C in a humidified incubator containing 5% CO_2. To assess proliferation, the wells were pulsed with 1μCi ^{3}H-thymidine (West of Scotland Radionucleotide Dispensary, Western Infirmary, Glasgow) for 16hr before being harvested onto glass fibre filters using a 1295 Betaplate 96 well

harvester and DNA-bound ^{3}H-thymidine was counted using a 1205 Betaplate scintillation counter (all LKB Wallac, Turku, Finland).

Measurement of OVA-Specific Cytotoxic T Cell Responses

A) Restimulation of Spleen and Mesenteric Lymph Node Cells *in vitro*

OVA-specific CTL activity in primed spleen and mesenteric lymph node (MLN) cells was assayed after *in vitro* restimulation using OVA transfected EG7.OVA cells as described previously (202). EG7.OVA cells were incubated at 10^7 cells/ml in RPMI 1640 medium containing 50μg/ml mitomycin C (Sigma) for 75min at 37°C, washed 5 times in RPMI 1640 by centrifuging at 400g for 5min and then resuspended at 1.5 x 10^6 cells/ml in complete RPMI containing 5% NCTC-135 medium (Gibco) and 5 x 10^{-5}M 2-ME ("CTL medium"). 1ml of this suspension was added to 25cm^2 tissue culture flasks (Costar) containing 3 x 10^7 spleen or MLN cells in 9 ml CTL medium and cultured for 5 days at 37°C in 5% CO_2 in air in a humidified incubator.

B) Restimulation of Popliteal Lymph Node Cells *in vitro*

CTL activity in popliteal lymph nodes was determined four days after footpad immunisation by *in vitro* restimulation with IL-2 as described previously (212). Aliquots of 2 x 10^6 popliteal lymph node (PLN) cells were cultured *in vitro* for 4 days in 1ml CTL medium containing 10U/ml recombinant human IL-2 (rhIL-2; Cetus Corporation, Emeryville, U.S.A.) in 24 well tissue culture plates (Costar) at 37°C in 5% CO_2 in air in a humidified incubator.

C) Measurement of OVA-Specific Cytotoxic T Cell Activity using Microcytotoxicity Assays

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

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

ii) Microcytotoxicity Assays

Effector cells which had been restimulated with EG7.OVA cells or IL-2 were washed twice in RPMI/5% NCS and recounted, before being incubated at different effector cell:target cell (E:T) ratios in quadruplicate with ^{51}Cr-labelled target cells in a total volume of 200μl in V-bottomed microtitre plates (Costar). For spleen and MLN effector cells, 2 x 10^4 target cells were added to each well, while 5 x 10^3 target cells were used for CTL assays of PLN effector cells. After incubation for 4hr at 37°C in 5% CO_2 in air in a humidified incubator, 100μl supernatant was removed from each well for analysis of ^{51}Cr release in a 1282 Compugamma counter (LKB Wallac).

In assays in which OVA 111-122 and OVA 258-276 peptides were used to pulse target cells, peptides were added to the mixture of effector and target cells for the duration of the assay, at a final concentration of 2.3μM.

In all assays, the percent OVA-specific CTL activity was calculated by the following formula:

$$\% \text{ specific lysis} = \frac{(\text{Experimental cpm} - \text{Spontaneous cpm})}{(\text{Total cpm} - \text{Spontaneous cpm})} \times 100\%$$

Spontaneous release was obtained using cells taken from unprimed mice, which had been restimulated *in vitro* with EG7.OVA cells or rhIL-2, while total release was obtained using 10% Triton X-100 (Sigma).

Measurement of Natural Killer Cell Activity

Splenic Natural Killer (NK) cell activity was assayed using a microcytotoxicity assay identical to that described above, except that in this case ^{51}Cr-labelled YAC-1 cells were used as targets and spontaneous release was obtained using normal thymocytes.

Induction and Measurement of Cytokine Production *in vitro*

Cytokine production was induced and measured using techniques which were developed by Dr. Paul Garside in this laboratory.

i) Induction of Cytokine Production

Single cell suspensions from lymphoid organs were resuspended at a final concentration of 4 x 10^6 cells/ml and cultured in 1ml aliquots in 24 well tissue culture plates (Costar) in complete RPMI medium containing 5 x 10^{-5}M 2-ME, either alone or with 10μg/ml Con A or 1mg/ml OVA. The supernatants were harvested after 2-6 days of culture, separated from contaminating

cells by centrifuging at 13,000g for 5min and stored at -20°C until assayed.

ii) Measurement of Cytokine Levels using ELISA

Cytokine levels were quantified using sandwich ELISA techniques. Immunlon 4 96-well ELISA plates were coated overnight at 4°C with 50μl of monoclonal anti-cytokine antibody at predetermined optimal concentrations (Table 2.1) in 0.1M $NaHCO_3$ buffer. The plates were then washed twice with PBS/0.05% Tween 20, after which non-specific protein binding sites were blocked by incubation with 200μl of PBS containing 10% FCS for 1hr at 37°C. Following blocking, the plates were washed twice in PBS/Tween and culture supernatants or standard preparations of recombinant murine cytokines (Table 2.2) diluted in culture medium, were added to individual wells in a volume of 50μl and incubated at room temperature for 3hr. The plates were then washed 4 times with PBS/Tween and 50μl of predetermined concentrations of biotinylated anti-cytokine antibody (Table 2.1) diluted in PBS/10% FCS was added to each well. After incubation for 1hr at room temperature, the plates were washed 6 times with PBS/Tween and 100μl of extravidin-peroxidase (2mg/ml in PBS/10% FCS) was added to each well. Following a final incubation for 1hr at room temperature, the plates were washed 8 times with PBS/Tween, before 100μl TMB peroxidase substrate was added to each well. The plates were read at 630nm using an MR5000 automatic microplate reader and the concentrations of cytokines in test supernatants, assayed in quadruplicate, were determined with reference to a standard curve constructed using serial dilutions of the standard cytokines.

Measurement of Respiratory Burst Enzyme Activity by Chemiluminescence

i) Elicitation and Isolation of PEC

Peritoneal exudate cells (PEC) were used to examine the effects of ISCOMS on Mϕ activity. PEC were elicited in BALB/c mice by i.p. injection of either 2ml thioglycollate broth (kindly provided by Dr. Jeremy Brock, Dept. of Immunology), 1mg heat-killed *Corynebacterium parvum* (Wellcome Laboratories, Beckenham, Kent), 5μg ISCOMS-OVA or 0.5μg Quil A.

Four days after injection, mice were sacrificed and PEC isolated by injecting 2.5ml RPMI 1640 containing 25mM HEPES and 10% FCS into the peritoneum, which was then massaged gently to dislodge PEC. A small incision was then made in the peritoneum and the cell suspension harvested with a Pasteur pipette.

ii) Chemiluminescence Assay

Respiratory burst activity was assayed by chemiluminescence using a slightly modified method of that described previously (213). In this assay, reactive O_2 intermediates produced by activated Mϕ interact with luminol causing it to emit photons that are detected using a luminometer.

10^7 PEC from each group were washed in Hank's Balanced Salts Solution, without phenol red (HBSS, Gibco BRL) by centrifuging at 450g for 7min, resuspended at 5 x 10^6 cells/ml in HBSS and warmed to 37°C in a water bath. 2 x 10^6 PEC in 0.4ml were then added to 0.1ml of a 10^{-4}M solution of 5-amino-2,3-dihydro-1,4-phthalazinedione solution (luminol; Sigma), before additon of 0.5ml

of a 1μg/ml solution of phorbol 12-myristate 13-acetate (PMA, Sigma). The suspensions were mixed at 37°C in a water bath and the resultant generation of chemiluminescence was measured every 10 seconds for 5-10min using a 1250 Luminometer (LKB Wallac). The results were expressed in mV and measurement was terminated when five consecutive decreases in chemiluminescence occurred, indicating the decline of respiratory burst activity. Control samples were PEC incubated with luminol in the absence of PMA.

Statistics

Unless otherwise stated, results were expressed as means ± standard deviations (SD) and were compared by Student's t-test. In instances where the data were not normally distributed, the results were compared using Wilcoxon's Rank Sum test.

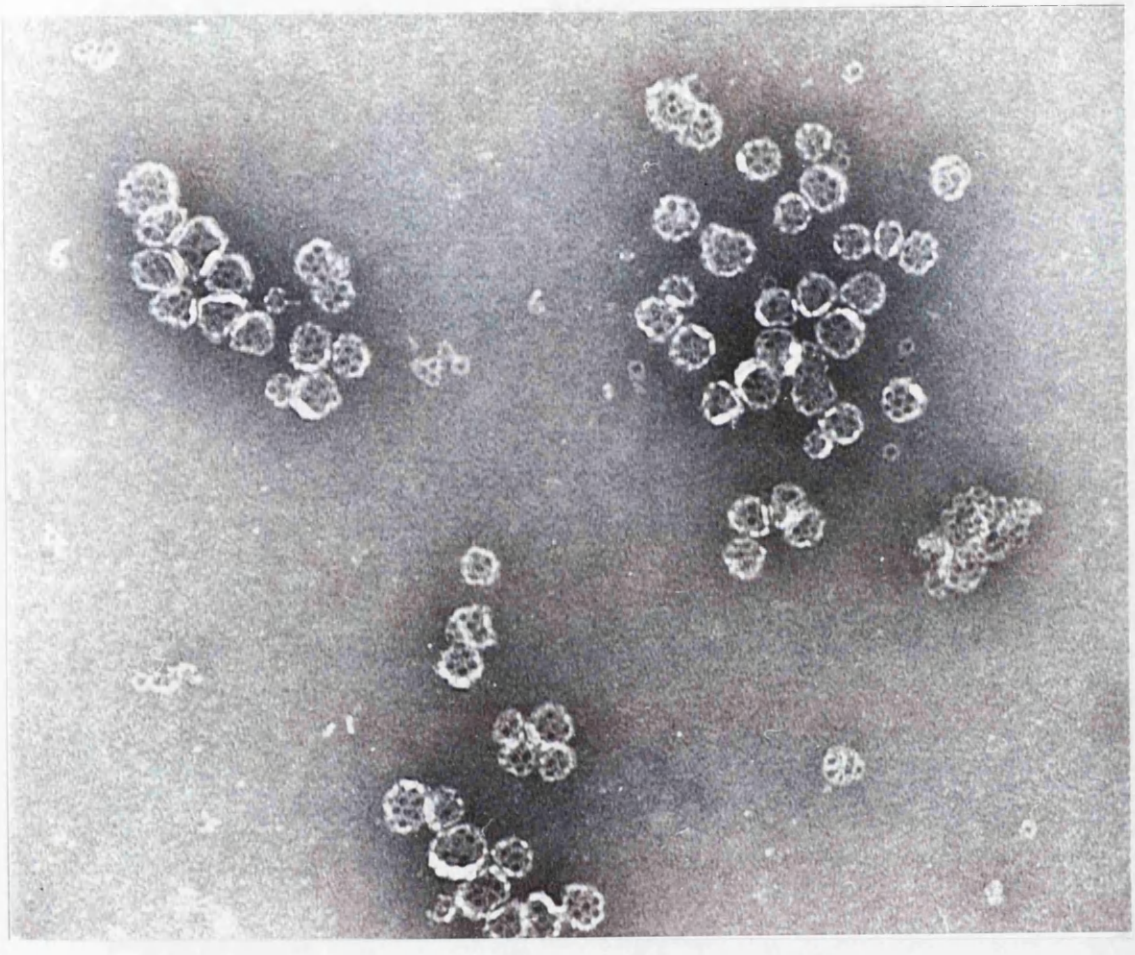

Figure 2.1

Transmission electron micrograph (TEM) of ISCOMS-OVA negatively stained with phosphotungstic acid, showing typical cage-like structure. (x73000).

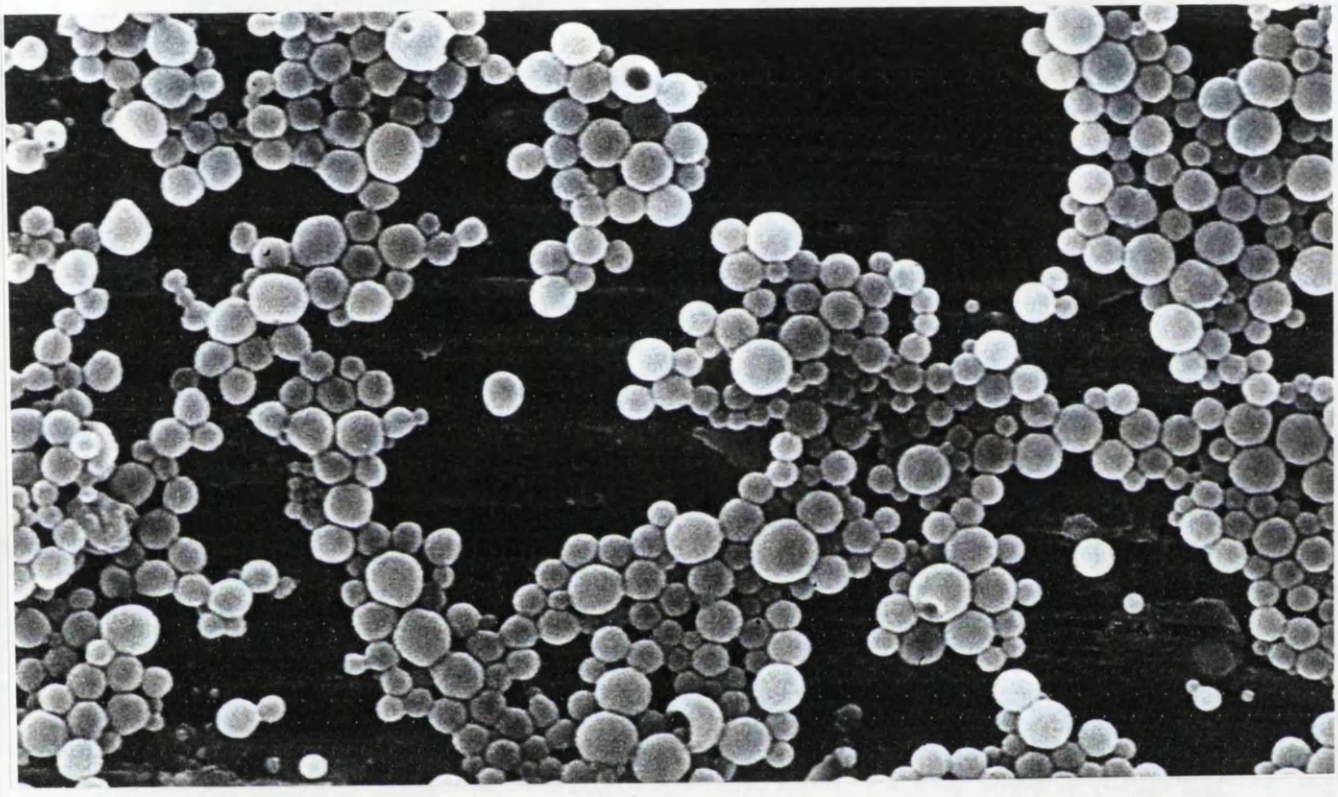

Figure 2.2

Scanning electron micrograph (SEM) of poly (lactide-co-glycolide) microparticles containing OVA (PLG-OVA). (Provided by Dr.D.T.O'Hagan).

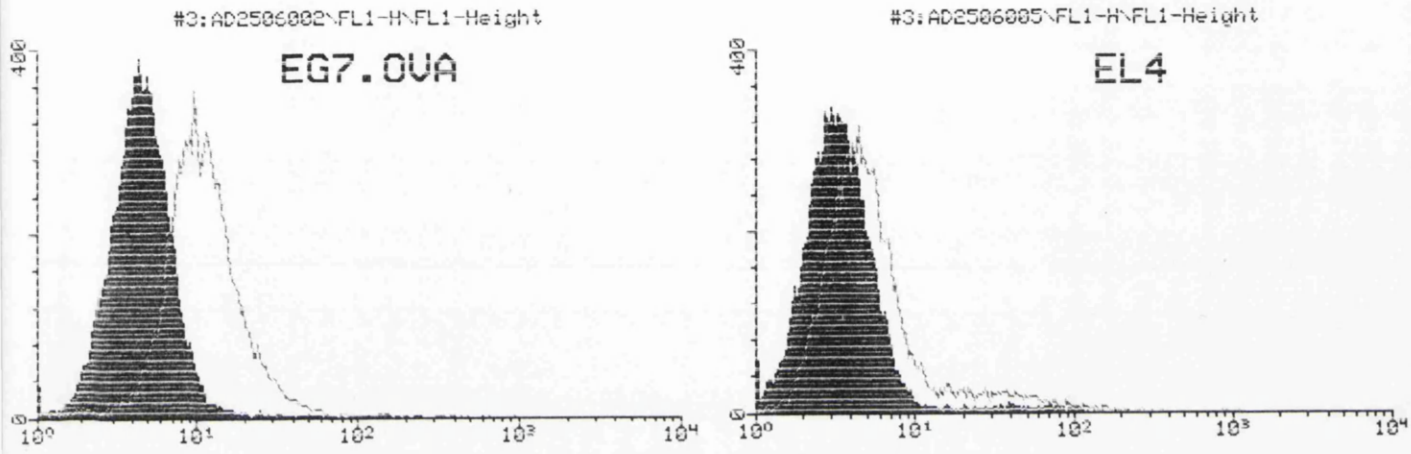

Figure 2.3

FACS analysis of OVA expression by EG7.OVA cells. Cells were stained with sheep-anti-OVA antiserum, followed by FITC-donkey-anti-sheep IgG. Non-OVA transfected EL4 cells were used as a negative control.

Table 2.1: Monoclonal Antibodies used in Cytokine Sandwich ELISAs

i) Capture Antibodies

Specificity	Clone	Isotype	Conc. (μg/ml)
Mouse IL-2	JES6-1A12	Rat IgG_{2a}	2
Mouse IL-4	BVD4-1D11	Rat IgG_{2b}	2
Mouse IL-5	TRFK5	Rat IgG_1	4
Mouse IFN-γ	R4-6A2	Rat IgG_1	2

ii) Biotinylated Detecting Antibodies

Specificity	Clone	Isotype	Conc. (μg/ml)
Mouse IL-2	JES6-5H4	Rat IgG_{2b}	1
Mouse IL-4	BVD6-24G2	Rat IgG_1	1
Mouse IL-5	TRFK4	Rat IgG_{2a}	4
Mouse IFN-γ	XMG1.2	Rat IgG_1	1

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

Table 2.2: Recombinant Murine Cytokine Standards used in Sandwich ELISAs

Cytokine	Source
IL-2	Pharmingen
IL-4	Genzyme, West Malling, Kent.
IL-5	Genzyme.
IFN-γ	Prof. F.Y. Liew, Dept. of Immunology

CHAPTER 3: PRIMING OF IMMUNE RESPONSES *in vivo* BY ISCOMS-OVA

Introduction

The principal aim of my project was to investigate the immunological basis of the adjuvant properties of ISCOMS when used by the oral and parenteral routes. This had not been addressed in previous studies, partly because immunologically well characterised protein antigens had rarely been incorporated into ISCOMS. Furthermore the oral route had not been studied extensively using ISCOMS. My studies were therefore designed to exploit a recently established method for incorporating purified OVA into ISCOMS (165). Preliminary work in this laboratory had shown that ISCOMS-OVA were immunogenic *in vivo* and had indicated that these ISCOMS could induce class I MHC-restricted cytotoxic T lymphocyte responses and were immunogenic when given orally (165).

My first series of experiments aimed to repeat and extend these studies by examining in parallel the full range of immune responses induced by oral and parenteral immunisation with ISCOMS-OVA.

Experimental Protocol

To induce OVA-specific systemic antibody and CMI responses BALB/c mice were primed s.c. with a single dose of 10μg ISCOMS-OVA into the right hind footpad. Primary antibody responses were measured 7 days after priming and mice were challenged with

100μg of heat-aggregated OVA on day14 post-priming. Systemic DTH responses were measured 24-48hr later and secondary IgG responses assayed one week later.

To induce systemic CTL responses C57Bl/6 mice were primed with a single dose of 5μg ISCOMS-OVA i.p. and spleen cells taken 7-10 days later were restimulated *in vitro* for 5 days with EG7.OVA cells (202).

For oral immunisation, mice were fed 10-100μg doses of ISCOMS-OVA on one or more occasions over a ten day period. Systemic IgG, secretory IgA and CTL responses were assayed 7-10 days after the final feed.

In all experiments control mice received identical priming doses of either soluble OVA, palmitified OVA (palm-OVA), soluble OVA in complete Freund's adjuvant or saline only.

Results

i) Induction of Systemic Antibody and DTH Responses by Parenteral Immunisation with ISCOMS-OVA

Consistent with previous studies in our laboratory (165), I found that mice which had been immunised s.c. 14 days previously with 10μg ISCOMS-OVA exhibited significant OVA-specific DTH responses when challenged in the opposite footpad with heat-aggregated OVA (Fig. 3.1). These DTH responses were comparable in magnitude to those elicited by immunisation with OVA in CFA (165).

In addition, measurement of OVA-specific IgG levels in the ISCOMS-OVA primed mice revealed that, while only modest primary antibody responses were observed 7 days after priming,

highly significant levels of OVA-specific IgG antibodies were present in secondary sera taken after challenge with heat-aggregated OVA (Fig. 3.2). These results confirmed that ISCOMS containing small amounts of protein antigen primed both humoral and cell-mediated immune responses *in vivo*.

ii) Priming of Class I MHC-restricted CTL by Parenteral Immunisation with ISCOMS-OVA

I next attempted to confirm that ISCOMS were also able to induce antigen-specific CTL responses when administered parenterally (165). Spleen cells from C57Bl/6 mice primed seven days previously with 5μg ISCOMS-OVA i.p. showed marked OVA-specific CTL activity against OVA-transfected EG7.OVA target cells (Fig.3.3). The antigen specificity of the CTL was indicated by the fact that they did not lyse the non-transfected EL4 parental cell line (Fig. 3.3). Furthermore, spleen cells isolated from mice primed with saline showed no OVA-specific CTL activity after stimulation *in vitro* with EG7.OVA cells (Fig. 3.3).

In order to characterise the phenotype of the CTL induced by ISCOMS-OVA, I used monoclonal antibodies and complement treatment to remove $CD4^+$ or $CD8^+$ T cells from the effector spleen cell population. The absence of $CD4^+$ or $CD8^+$ T cells was confirmed by FACS analysis (Fig. 3.4). Depletion of $CD4^+$ T cells had no effect on the level of OVA-specific CTL activity, but removal of $CD8^+$ T cells completely abrogated the cytotoxic response (Fig. 3.5) As the EG7.OVA cells do not express class II MHC antigens (202), these results confirm that incorporation of a protein antigen in ISCOMS leads to highly efficient priming of $CD8^+$ class I MHC-restricted T cells *in vivo*.

iii) Induction of Systemic Antibody and DTH Responses by Oral Immunisation with ISCOMS-OVA

I next investigated the immunogenicity of ISCOMS-OVA when administered orally. Previous work in our laboratory had shown that mice fed a single dose of 100μg ISCOMS-OVA developed strong OVA-specific DTH responses, but had only low levels of OVA-specific IgG antibodies (165).

To attempt to improve the generation of serum antibody responses I decided to investigate the effects of multiple feeding, an immunisation strategy which had been employed effectively with other oral adjuvants, such as cholera toxin (143). When mice were fed ISCOMS-OVA on six occasions over a ten day period, they developed highly significant levels of OVA-specific IgG antibodies in serum (Fig. 3.6). These responses were maximal using an immunising dose of 100μg ISCOMS-OVA, where the OVA-specific IgG levels were comparable to those found in mice immunised parenterally with 100μg OVA in CFA, but significant responses were also elicited with doses containing as little as 10μg ISCOMS-OVA (Fig. 3.6). Feeding palmitified OVA alone was not sufficient to induce a systemic IgG response (Fig. 3.6).

iv) Induction of Intestinal IgA Antibody Responses by Feeding ISCOMS-OVA

The ability of ISCOMS-OVA to induce such potent systemic immune responses when administered orally made it important to determine whether they could also stimulate local antibody responses in the intestine itself.

Again, mice fed a single dose of 100μg ISCOMS-OVA had very low levels of OVA-specific IgA antibodies in intestinal washes, but those receiving three or six feeds had increasingly significant levels of OVA-specific IgA in the intestine (Fig. 3.7). The magnitude of the IgA response was primarily dependent on the number of feeds, rather than immunising dose, as mice receiving six feeds of 10μg ISCOMS-OVA had similar levels to those fed 100μg on six occasions (Fig. 3.8). As before, feeding of palmitified OVA alone did not provoke OVA-specific IgA production (Fig. 3.8). Thus, repeated oral immunisation with ISCOMS provokes both strong systemic IgG responses and local secretory IgA responses to incorporated protein antigen.

v) Priming of OVA-specific CTL Responses by Oral Immunisation with ISCOMS-OVA

Lastly, I examined whether ISCOMS-OVA were able to prime class I MHC-restricted CTL responses when administered orally. Spleen cells from mice fed six times with 100μg ISCOMS-OVA had high levels of OVA-specific CTL activity (Fig. 3.9), comparable to that induced by a single parenteral immunisation with ISCOMS-OVA (Fig. 3.3). Marked OVA-specific CTL activity was also present in the mesenteric lymph node (MLN) cells taken from ISCOMS-OVA fed mice (Fig. 3.9), indicating that oral immunisation with ISCOMS primed both local and systemic CTL precursors. Feeding palmitified OVA alone did not induce any detectable CTL activity (Fig. 3.9). Hence ISCOMS allow incorporated protein antigen access to the endogenous pathway of antigen processing and presentation, even when adminstered orally.

Conclusions

The results presented in this chapter confirm and extend previous findings (165) on the potent adjuvant effects of ISCOMS and indicate that ISCOMS have several unique properties as oral vaccine vectors.

A single parenteral immunisation with as little as 5-10μg ISCOMS-OVA primed potent OVA-specific systemic DTH and IgG responses and high levels of class I MHC-restricted OVA-specific CTL were detected in the spleen. Together these findings indicate that ISCOMS allow presentation of incorporated protein to the class II MHC-restricted T cells required for antibody and DTH responses, while also permitting efficient entry of the protein into the endogenous antigen processing pathway required for stimulation of class I MHC-restricted CTL responses.

Perhaps the most novel adjuvant property of ISCOMS is their ability to induce a wide range of systemic and local immune responses when administered orally. These findings contrast with the profound immunological tolerance normally observed in animals given proteins orally (54) and highlight the potential of ISCOMS to form the basis of future oral vaccine development. As observed with other oral adjuvants (143), multiple feeds of ISCOMS were required to induce the same range of systemic immune responses as parenteral immunisation, including DTH, IgG and class I MHC-restricted CTL responses. In addition, oral immunisation with ISCOMS stimulated the production of secretory IgA antibodies in the intestine and local CTL precursors in MLN. These findings indicate that ISCOMS may allow protein antigen to subvert the

regulatory mechanisms responsible for the induction of oral tolerance, which normally operate at mucosal surfaces.

In summary, this study has shown that ISCOMS possess a number of unique and potent adjuvant effects when administered either parenterally or orally and using ISCOMS-OVA as a model system it should be possible to analyse the mechanisms underlying the basis of their adjuvanticity.

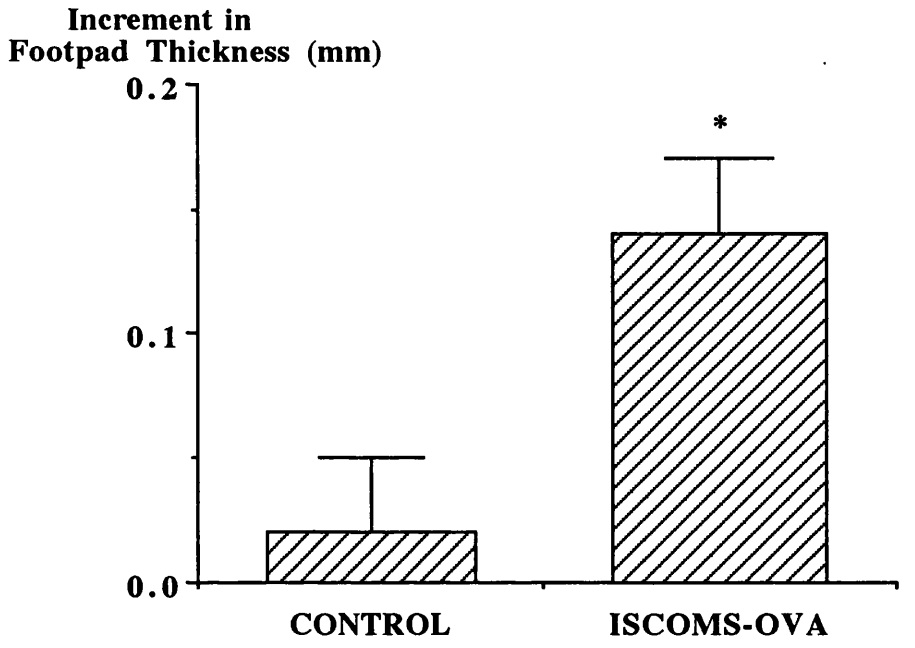

Figure 3.1.

Primary systemic DTH responses after parenteral immunisation with ISCOMS-OVA. DTH responses were measured 24hr after footpad challenge with 100μg heat-aggregated OVA in BALB/c mice which had been primed 14 days previously with 10μg ISCOMS-OVA or saline only s.c. Results are means ± 1 S.D. for four to six mice per group.

*$p<0.001$ v. CONTROL.

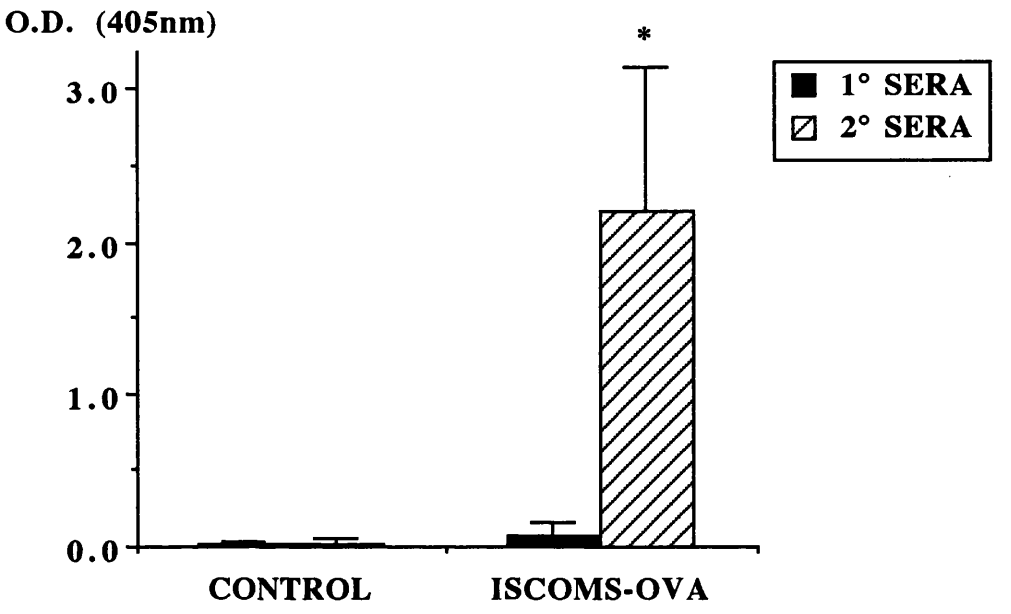

Figure 3.2.

Priming of OVA-specific humoral immunity by parenteral immunisation with ISCOMS-OVA. Primary OVA-specific IgG responses of BALB/c mice were measured one week after priming with 10μg ISCOMS-OVA or saline only s.c., while secondary IgG responses were assayed one week after footpad challenge of primed mice with 100μg heat-aggregated OVA. Mean O.D.(405nm) values ± 1S.D. are shown for six mice per group.

*$p<0.001$ v. CONTROL.

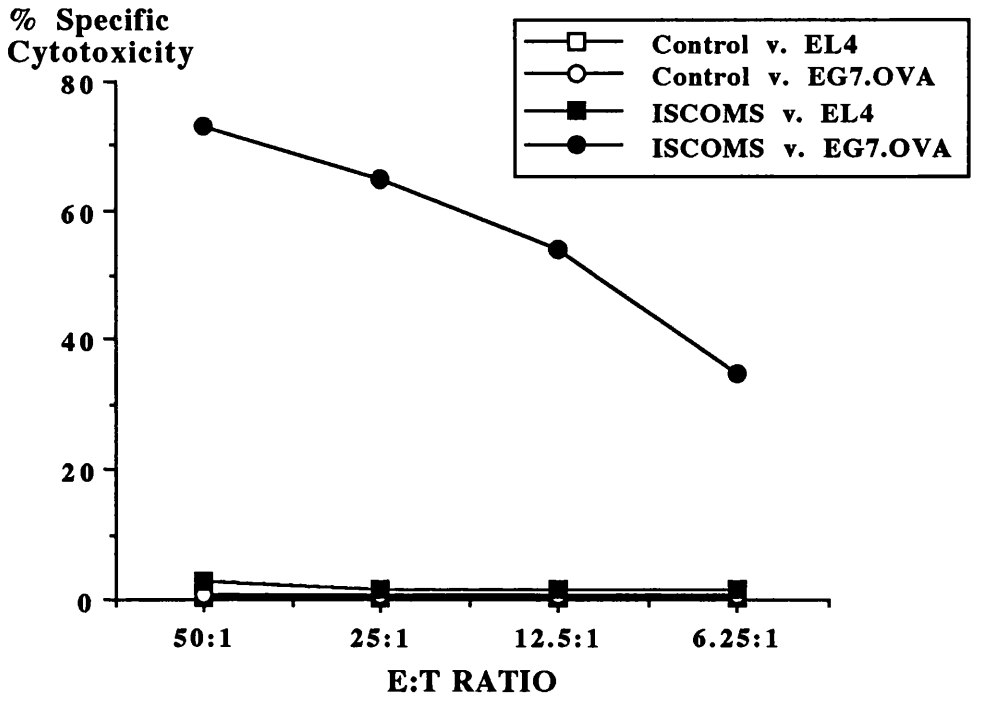

Figure 3.3.

Priming of OVA-specific CTL responses by parenteral immunisation with ISCOMS-OVA. Splenic CTL activity was assayed in C57Bl/6 mice immunised one week before with 5μg ISCOMS-OVA or saline only i.p. after subsequent restimulation *in vitro* with EG7.OVA cells. Results shown are percent specific cytotoxicity using spleen cells pooled from four mice per group.

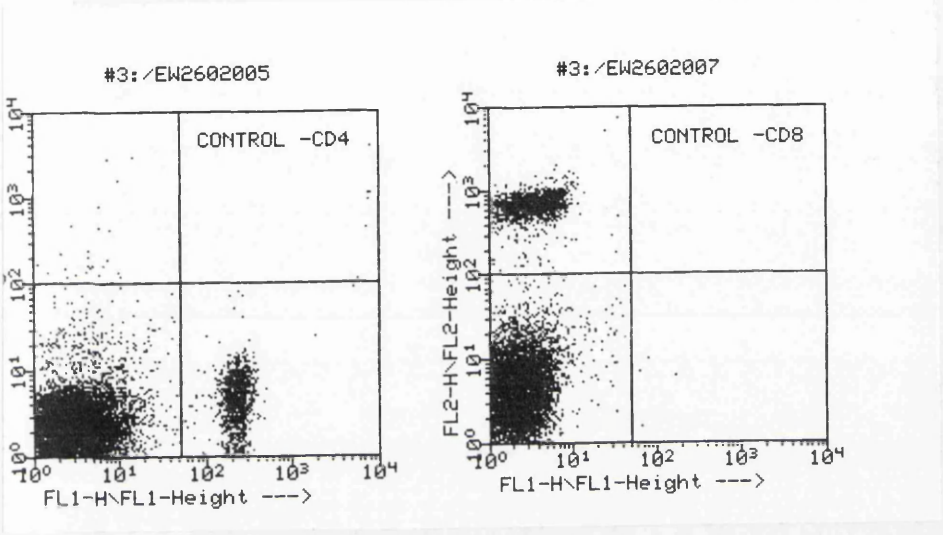

Figure 3.4.

FACS analysis of effector spleen cells after depletion of CD4+ or CD8+ Tcells. After *in vitro* restimulation with EG7.OVA cells, ISCOMS-OVA primed spleen cells from C57Bl/6 mice were depleted of CD4+ or CD8+ T cells by monoclonal antibody plus complement treatment, before being stained with PE-anti-CD4 and FITC-anti-CD8 mAbs.

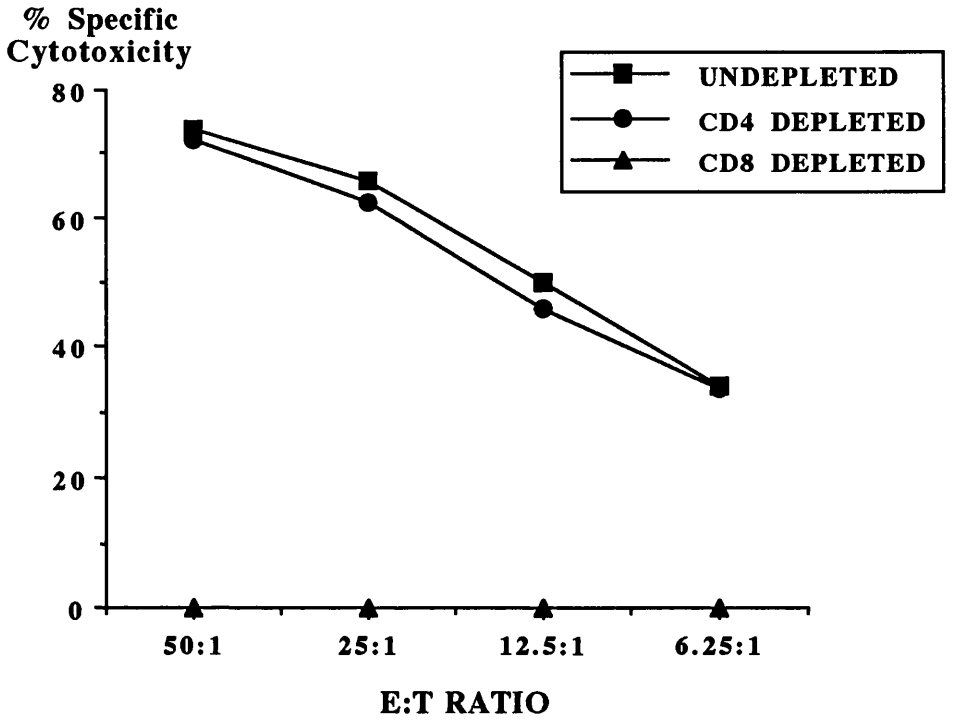

Figure 3.5.

Phenotype of CTL primed by immunisation with ISCOMS-OVA. After *in vitro* restimulation with EG7.OVA cells, ISCOMS-OVA primed spleen cells from C57Bl/6 mice were depleted of $CD4^+$ or $CD8^+$ T cells by monoclonal antibody plus complement treatment, before OVA-specific CTL activity was measured by ^{51}Cr release assay. The results shown are percent specific cytotoxicity against EG7.OVA target cells using spleen cells pooled from four immunised mice. There was no lysis of EL4 cells by any of the effector populations

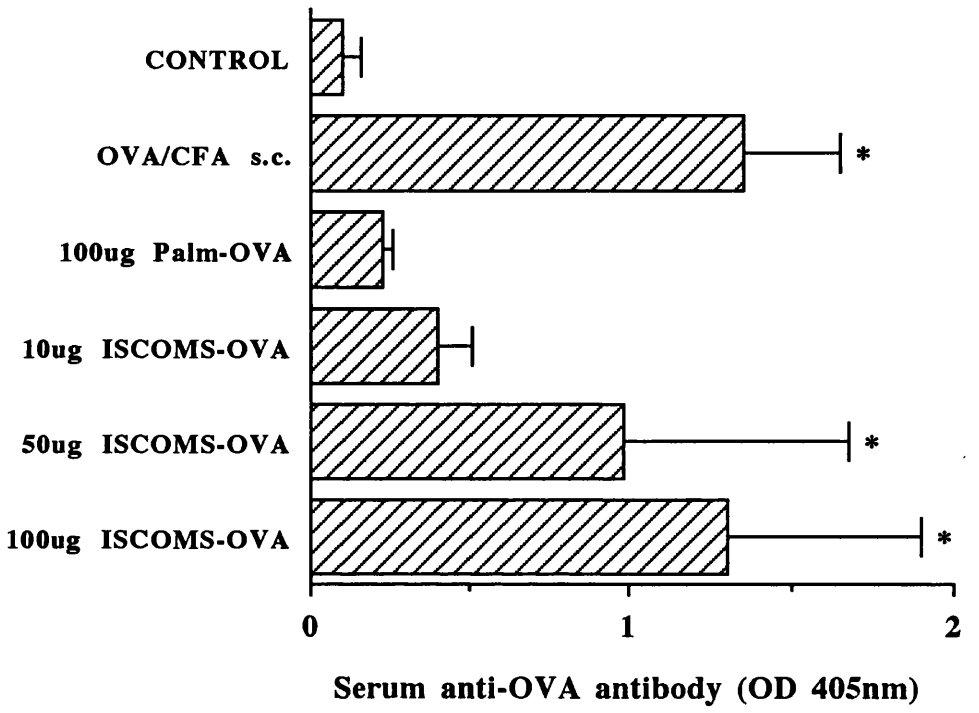

Figure 3.6.

Induction of systemic OVA-specific IgG responses by oral immunisation with ISCOMS-OVA. Sera were obtained 9 days after the last immunisation in BALB/c mice fed 10, 50 or 100μg ISCOMS-OVA or 100μg palmitified OVA (Palm-OVA) or saline only on six occasions and in mice immunised s.c. with 100μg OVA in CFA. Results shown are means ± 1S.D. from 4 or 5 mice per group.

* $p<0.05$ v. CONTROL.

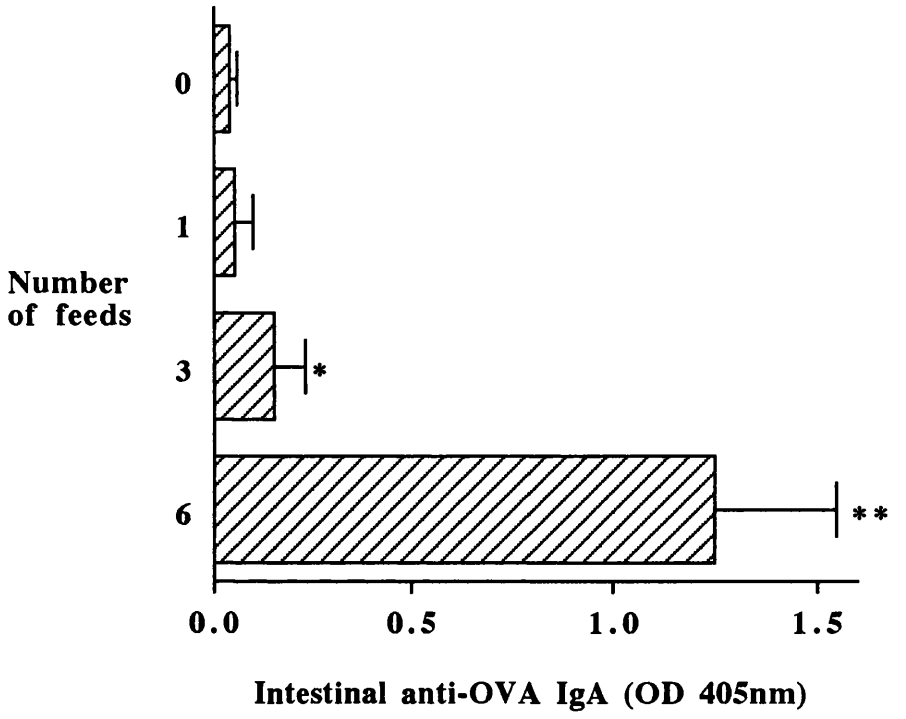

Figure 3.7.

Induction of OVA-specific secretory IgA responses by feeding ISCOMS-OVA. BALB/c mice were fed 100μg ISCOMS-OVA on one or more occasions over a ten day period and intestinal washes were obtained one week after the final feed. Results shown represent means ± 1S.D. from six mice per group.

* $p<0.05$ v. CONTROLS.

** $p<0.001$ v. CONTROLS.

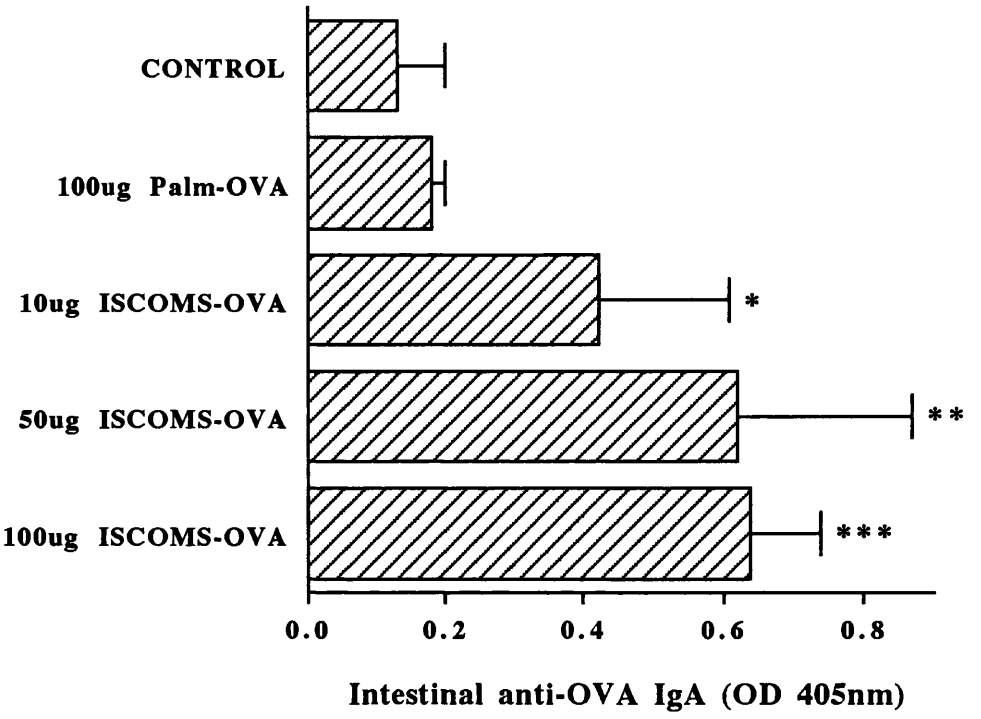

Figure 3.8.

Effect of antigen dose on the induction of secretory IgA responses by feeding ISCOMS-OVA. BALB/c mice were fed six times over a ten day period with10-100μg ISCOMS-OVA, 100μg palmitified OVA (Palm-OVA) or saline only and intestinal washes were obtained one week later. Results shown are means ± 1S.D. from six mice per group.

* $p<0.05$ v. CONTROL.

** $p<0.01$ v. CONTROL.

*** $p<0.001$ v. CONTROL.

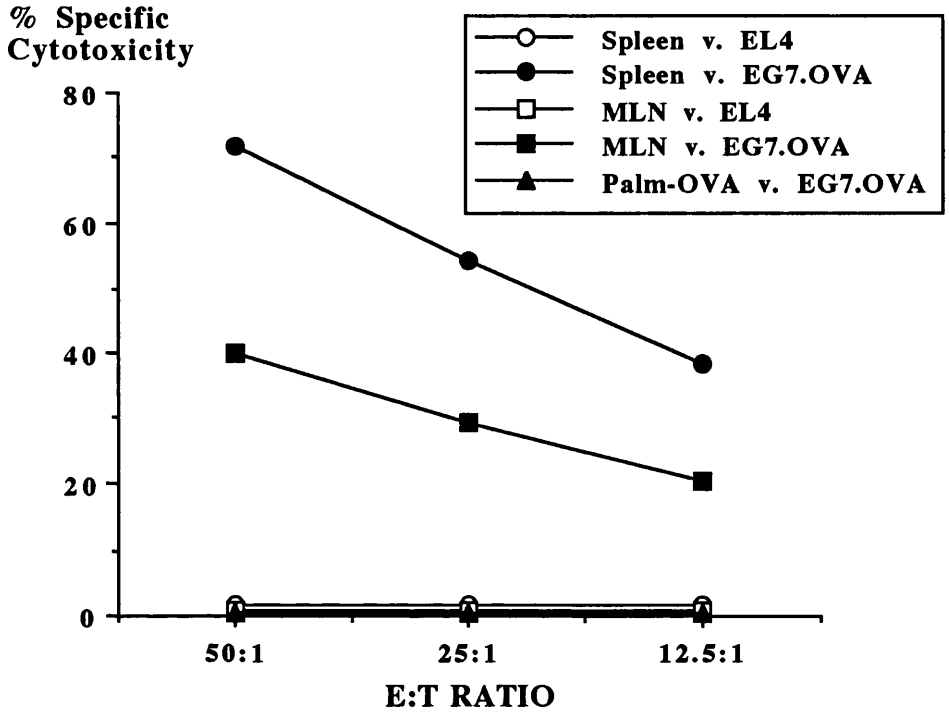

Figure 3.9.

Priming of OVA-specific CTL responses by oral immunisation with ISCOMS-OVA. Splenic or mesenteric lymph node (MLN) CTL activity was assayed in C57Bl/6 mice 10 days after the last of six feeds of 100μg ISCOMS-OVA or 100μg palmitified OVA (Palm-OVA), after restimulation *in vitro* for 5 days with EG7.OVA cells. Results shown are percent specific cytotoxicity using spleen cells pooled from four mice per group.

CHAPTER 4: IMMUNOBIOLOGY OF CLASS I MHC-RESTRICTED CTL RESPONSES INDUCED BY ISCOMS-OVA

Introduction

One of the most novel properties of ISCOMS highlighted in the previous chapter is their ability to stimulate potent class I MHC-restricted CTL responses to exogenous protein antigens *in vivo*. As this property would be important for a vaccine capable of stimulating protection against many viral infections, I thought it important to investigate the mechanisms involved.

Over the last few years it has become clear that class I MHC-restricted $CD8^+$ T cells recognise small peptides of 8-10 amino acids in length, which are generated by intracellular degradation of cytosolic antigen and are presented in the binding cleft of class I MHC molecules (20, 33-35, 214). However, the cellular mechanisms involved in the induction of CTL responses *in vivo* are still not clear and different pathways may operate depending on the system under study. In particular, there is considerable variability in the nature of antigen presenting cells involved and in the dependency on $CD4^+$ T cells in the induction of CTL responses (50, 215-219).

In this chapter, I have characterised the CTL responses primed by ISCOMS-OVA, by examining their phenotype, the nature of their recognition of antigen and the requirement for accessory cells and $CD4^+$ T cells in their priming *in vivo*.

Experimental Protocol

OVA-specific systemic CTL responses were assayed in spleen of C57Bl/6 mice primed with a single dose of 5μg ISCOMS-OVA i.p. or with 3 to 6 feeds of 50-100μg ISCOMS-OVA, as described in chapter 3.

CTL responses were also assayed in the popliteal lymph node of C57Bl/6 mice primed the hind footpad with 10μg ISCOMS-OVA 4 days previously. In this case primed cells were stimulated *in vitro* with 10U/ml rhIL-2 for 4 days before cytotoxicity was assayed.

Mϕ were depleted or paralysed *in vivo* by injecting silica or liposomes containing Cl_2MDP (209), 24-48hr before priming with ISCOMS-OVA i.p.

$CD4^+$ T cells were depleted *in vivo* by injecting 500μg anti-CD4 mAb i.p. on day-7 and day-3 before priming with ISCOMS-OVA i.p. on day0.

Results

i) Phenotype and Specificity of CTL Responses Primed by ISCOMS-OVA *in vivo*

The results in chapter 3 show that parenteral immunisation with ISCOMS-OVA primed OVA-specific $CD8^+$ CTL which lysed the OVA-transfected EG7.OVA cell line, but not the parental EL4 cell line. I examined whether the antigen being recognised was derived from physiological processing of OVA by the endogenous route, or from exogenous OVA secreted by the cells (202). To distinguish between these possibilities I tested the ability of ISCOMS-OVA primed CTL to lyse non-transfected EL4 cells in the presence of

native OVA, which was either added exogenously or which was introduced directly into the cytoplasm by osmotic lysis of pinosomes.

Pinocytic vesicles formed under hypertonic conditions lyse when the cells are returned to hypotonic medium, releasing their contents into the cytoplasm (202, 207). EL4 cells were cultured with hypertonic medium containing OVA, diluted to hypotonic medium and then washed and cultured overnight in normal culture medium before being used as target cells in a ^{51}Cr release assay. As shown in Fig. 4.1, these cells were lysed very efficiently by ISCOMS-OVA primed CTL. In contrast, there was no lysis of EL4 cells pulsed exogenously with soluble OVA (Fig. 4.1). Hence, the ISCOMS-OVA primed CTL recognise endogenously processed OVA.

The osmotic loading technique was exploited to examine the MHC-restriction of ISCOMS-OVA primed CTL. While syngeneic EL4 (H-2^b) cells endogenously loaded with OVA were efficiently lysed by ISCOMS-OVA primed CTL, there was no killing of allogeneic P815 (H-2^d) cells either alone or after cytoplasmic loading with OVA (Fig. 4.2). That ISCOMS-OVA primed conventional $CD8^+$ class I MHC-restricted CTL was further confirmed by the fact that they did not exhibit any cytotoxic activity against the NK-sensitive YAC-1 cell line (Fig. 4.2).

Previous studies had shown that OVA-specific CTL elicited in H-2^b mice by immunisation with EG7.OVA cells recognised a peptide epitope within residues 258-276 which was presented in association with the K^b class I MHC molecule (202). As shown in Fig. 4.3, ISCOMS-OVA primed CTL showed substantial lysis of EL4 cells pulsed with the OVA peptide 258-276, but did not lyse EL4 cells pulsed with the OVA peptide 111-122. The latter peptide is

recognised by OVA-specific H-2D^b-restricted CTL induced by *in vitro* stimulation with trypsinised OVA, but is not presented by endogenous processing of OVA *in vitro* or *in vivo* (220).

Together, these results demonstrate that the OVA-specific CTL primed by ISCOMS-OVA *in vivo* are CD8$^+$ T cells recognising an epitope within the OVA 258-276 peptide, which is derived from endogenously processing of OVA and which has been shown to contain the octamer motif (OVA 257-264) presented in association with K^b class I MHC molecules (33, 202, 221).

ii) Induction of Popliteal Lymph Node CTL Responses *in vivo* by ISCOMS-OVA

All the CTL responses described thus far were assayed following restimulation with the OVA-expressing EG7.OVA *in vitro*. As it could be argued that this does not truly reflect a primary antigen-specific CTL response *in vivo*, I explored further the ability of ISCOMS-OVA to prime CTL using an alternative assay which did not involve *in vitro* restimulation with antigen. Following s.c. priming with ISCOMS-OVA, draining popliteal lymph node (PLN) cells were isolated and cultured with IL-2 *in vitro*, then assayed for OVA-specific CTL activity as before. As illustrated in Fig. 4.4, ISCOMS-OVA primed PLN cells exhibited substantial OVA-specific CTL activity in this assay, indicating that ISCOMS are indeed able to efficiently stimulate primary CTL responses *in vivo*.

iii) The Role of Mϕ and CD4$^+$ T Cells in ISCOMS-OVA induced CTL responses *in vivo*

It has been reported that the production of growth factors such as IL-2 is required for the initial induction of CTL responses

under many conditions (222, 223). In addition, recent evidence suggests that phagocytic cells such as Mϕ may play a critical role in priming CTL (215, 224, 225). I therefore examined the effects of depletion of phagocytic cells or of CD4+ T cells on the priming of CTL by ISCOMS-OVA.

a) Role of Phagocytic Cells

Mice treated with silica in order to paralyse Mϕ *in vivo* showed markedly reduced OVA-specific CTL activity after priming with ISCOMS-OVA, to around one third of the level of lysis achieved in the ISCOMS-OVA primed group (Fig. 4.5). As not all CTL activity was abolished injecting silica, I decided to try and confirm these findings using another method to deplete Mϕ *in vivo*. Mice were therefore treated with liposomes containing dichloromethylene diphosphonate (Cl_2MDP) which are ingested selectively by Mϕ and degraded by phospholipases, releasing the toxic Cl_2MDP into the cytosol (209, 210). Mice depleted of Mϕ in this manner also showed reduced OVA-specific CTL activity (Fig. 4.6), confirming that a substantial proportion of ISCOMS primed CTL are dependent on the presence of Mϕ.

b) Role of CD4+ T cells

I next determined whether CD4+ T_h cells were necessary for *in vivo* priming of CTL by ISCOMS. Mice depleted of CD4+ T cells, by treatment with anti-CD4 mAb, had markedly reduced numbers of CD4+ T cells in the spleen as assayed by FACS (Fig. 4.7) and were unable to generate any OVA-specific CTL when primed with ISCOMS-OVA (Fig. 4.8). Thus ISCOMS prime CTL responses *in vivo* via a CD4+ T cell-dependent pathway.

iv) Characteristics of CTL Primed by Oral Immunisation with ISCOMS-OVA

The preceding experiments explored the CTL responses primed by parenteral immunisation with ISCOMS-OVA and I went on to determine whether the OVA-specific CTL primed *in vivo* by oral immunisation with ISCOMS-OVA exhibited similar characteristics.

As shown in the previous chapter, mice which had been fed six times with ISCOMS-OVA developed good splenic CTL responses. These were ablated by *in vitro* depletion of $CD8^+$ T cells from the effector population, while removal of $CD4^+$ T cells had no effect (Fig. 4.9). In addition, orally primed CTL were also able to lyse EL4 cells pulsed with OVA peptide 258-276, but not EL4 cells pulsed with exogenous intact OVA or with the OVA 111-122 peptide (Fig. 4.10). These results indicate that the systemic CTL responses primed by oral administration of ISCOMS are also $CD8^+$ T cells recognising an epitope within the OVA 258-276 peptide.

Conclusions

These results have confirmed that ISCOMS elicit conventional class I MHC-restricted CTL responses when administered either parenterally or orally. The CTL primed by ISCOMS-OVA were $CD8^+$ T cells which recognised endogenously processed OVA, the immunodominant epitope contained within the peptide OVA 258-276. Several studies have shown that this peptide contains an octamer (OVA 257-264) which comprises the minimal motif for binding the H-$2K^b$ class I MHC molecule and is the dominant

epitope recognised by OVA-specific CTL in H-2^b mice following physiological processing of OVA (33, 202, 221, 226). Orally primed CTL appeared identical to parenterally primed CTL in all of the above aspects.

Shortly after s.c. immunisation with ISCOMS-OVA, OVA-specific precursor CTL were present in the draining lymph node. These pCTL differentiated into effector cells when cultured in the absence of antigen, but in the presence of IL-2, confirming that these cells had already been primed *in vivo* by the ISCOMS-OVA.

Phagocytic accessory cells (Mϕ) were required for the priming of a large proportion of OVA-specific CTL, as paralysis or depletion of these cells produced a marked decrease in the priming of OVA-specific CTL *in vivo*. However, detectable CTL activity remained in the absence of Mϕ, suggesting that there may be more than one pathway of CTL induction utilised by ISCOMS *in vivo*.

The CTL responses were also dependent on helper T cells, as depletion of $CD4^+$ T cells completely abolished priming of CTL responses by ISCOMS-OVA. Thus, concomitant activation of $CD4^+$ T cells plays a key role in the *in vivo* priming of $CD8^+$ CTL by ISCOMS-OVA, possibly reflecting production of IL-2.

These findings suggest that the induction of CTL by ISCOMS is a complicated process, involving both phagocytic accessory cells and $CD4^+$ T helper cells. The helper-dependence of the CTL responses, together with the potent humoral responses described in chapter 3, suggested that the activation of $CD4^+ T_h$ cells was a critical event in the induction of immune responses by ISCOMS and this topic is addressed in the following chapter.

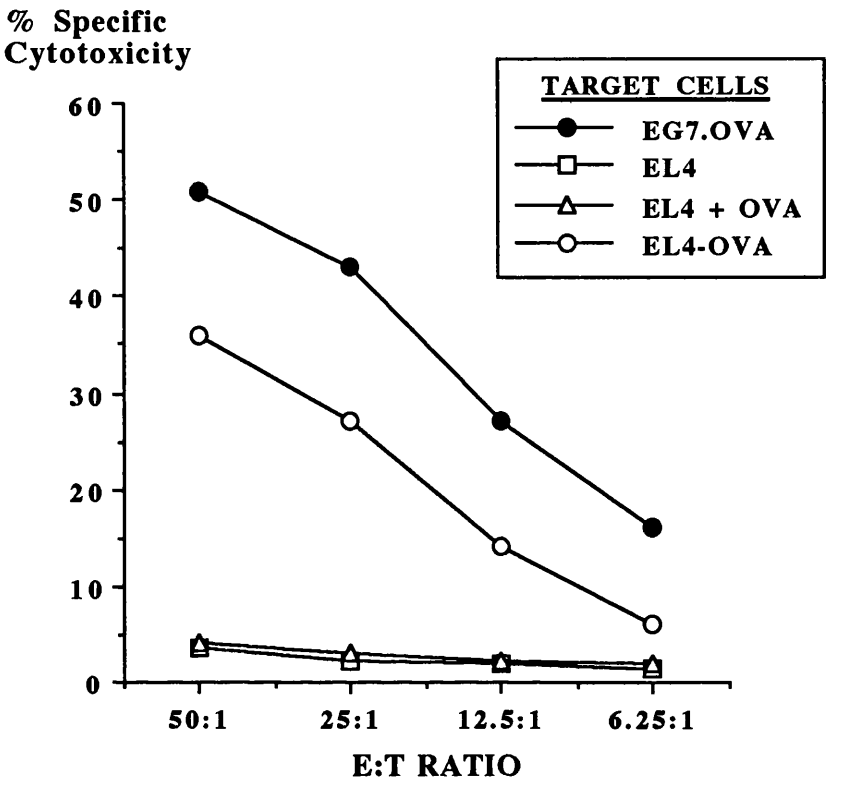

Figure 4.1.

ISCOMS-OVA induced CTL recognise endogenously processed OVA. Splenic CTL induced in C57Bl/6 mice by immunisation with ISCOMS-OVA i.p. and restimulated *in vitro* with EG7.OVA cells, were tested for their ability to lyse EG7.OVA cells, or EL4 cells pulsed with exogenous OVA (EL4 + OVA) or osmotically loaded with OVA (EL4-OVA). The results shown are percent specific cytotoxicity using pooled spleen cells from four immunised mice.

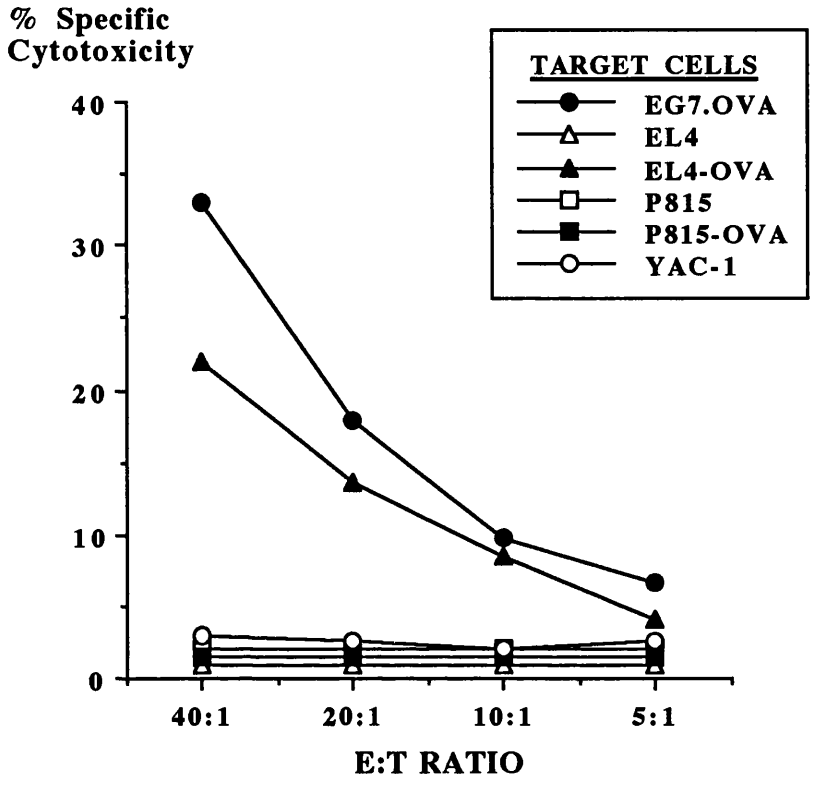

Figure 4.2.

ISCOMS-OVA primed CTL are MHC-restricted. Splenic CTL induced in C57Bl/6 mice by immunisation with ISCOMS-OVA i.p. and restimulated *in vitro* with EG7.OVA cells, were tested for their ability to lyse either EL4 (H-2^b) or allogeneic P815 (H-2^d) cells which had been osmotically loaded with OVA (EL4-OVA and P815-OVA). Control targets which had not been loaded with antigen were also included, as were EG7.OVA cells and NK-sensitive YAC-1 cells. The results shown are specific cytotoxicity using pooled spleen cells from four immunised mice.

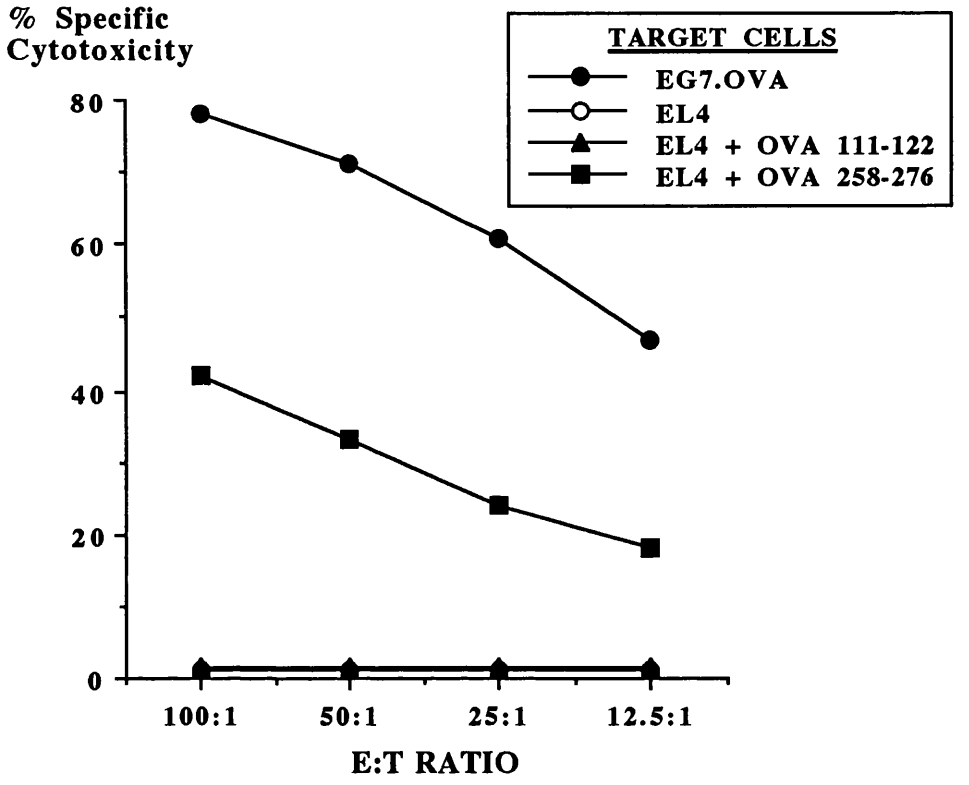

Figure 4.3.

ISCOMS-OVA primed CTL recognise an epitope within the OVA 258-276 peptide. Splenic CTL induced in C57Bl/6 mice by immunisation with ISCOMS-OVA i.p. and restimulated *in vitro* with EG7.OVA cells, were tested for their ability to lyse EL4 cells in the presence of the peptide OVA 258-276 or OVA 111-122. Peptides, which were added to the target cells before the start of the assay, remained present at a final concentration of 2.3μM throughout the ^{51}Cr release assay. The results shown are specific cytotoxicity using pooled spleen cells from four immunised mice.

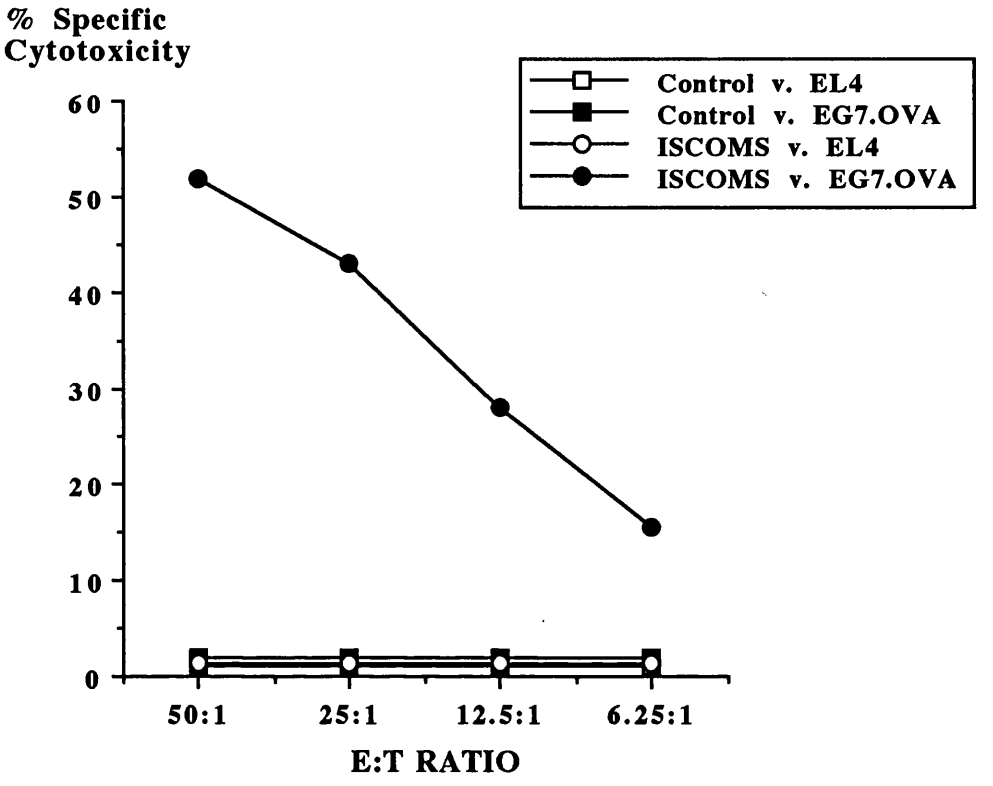

Figure 4.4.

Induction of OVA-specific CTL activity in popliteal lymph node (PLN) cells. PLN cells from C57Bl/6 mice were isolated 4 days after footpad priming with ISCOMS-OVA or saline only and cultured *in vitro* for 4 days with rhIL-2, before OVA-specific CTL activity was assayed by ^{51}Cr release assay. The results shown are specific cytotoxicity using pooled PLN cells from six mice.

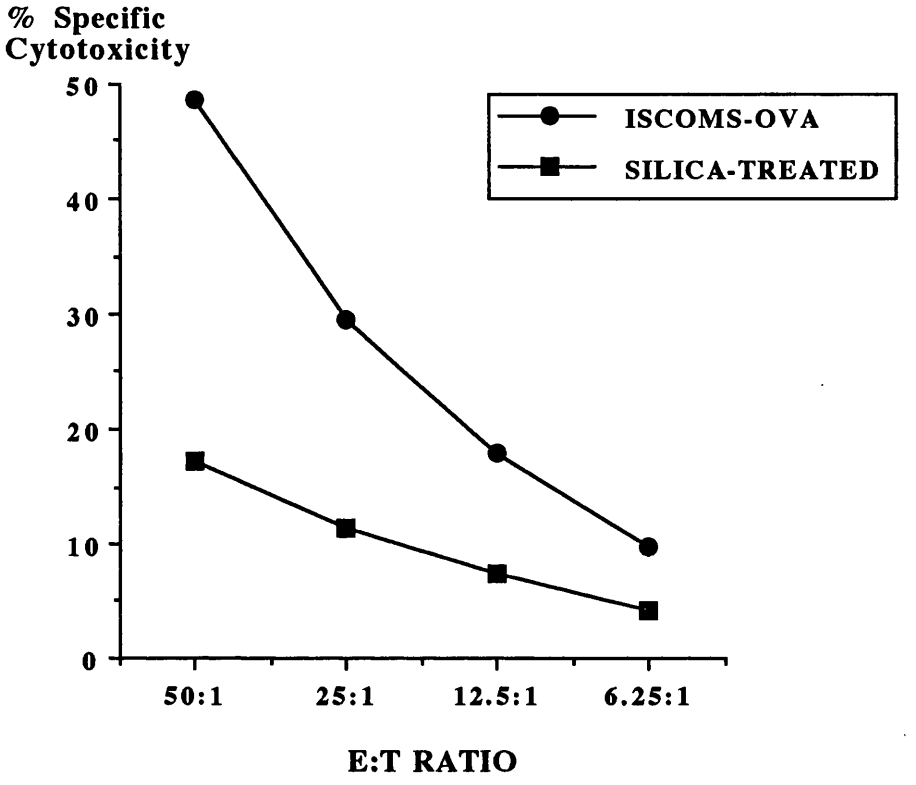

Figure 4.5.

Effect of Mϕ paralysis on the priming of CTL responses by ISCOMS-OVA. C57Bl/6 mice were injected with silica on two consecutive days, before immunisation with ISCOMS-OVA i.p. The results shown are percent specific cytotoxicity against EG7.OVA target cells using spleen cells pooled from four mice per group. There was no lysis of EL4 cells by any of the effector populations (data not shown).

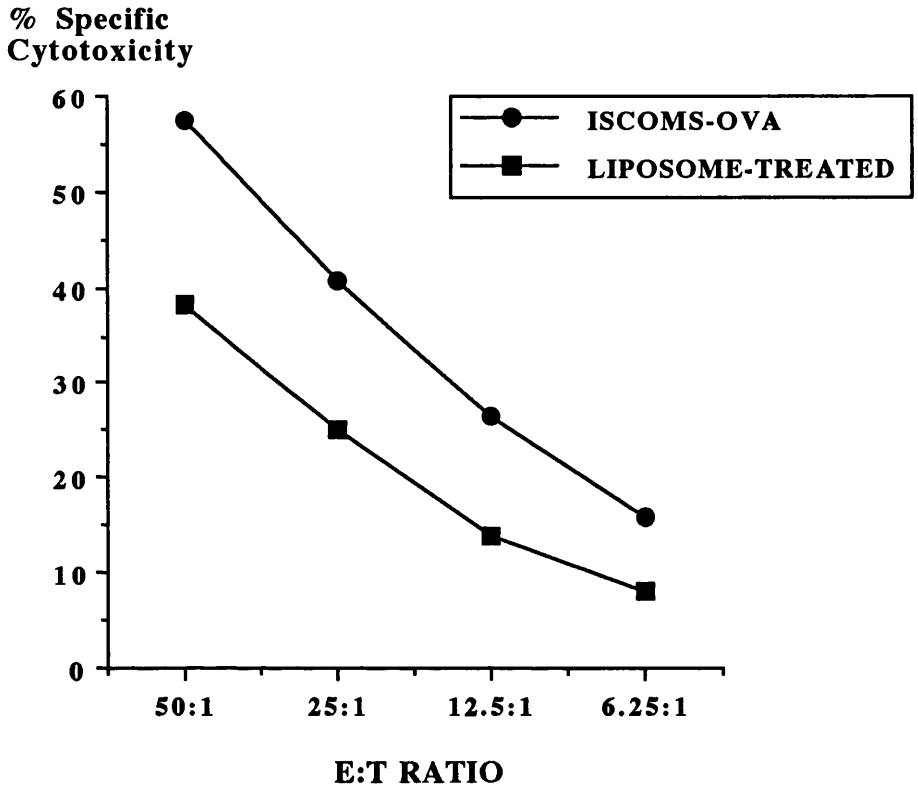

Figure 4.6.

Effect of Mϕ depletion on priming of CTL responses by ISCOMS-OVA. C57Bl/6 mice were given 0.2ml liposomes containing Cl_2MDP i.v. 24hr prior to immunisation with ISCOMS-OVA i.p. and spleen cells removed 7 days later for restimulation with EG7.OVA cells *in vitro*. The results shown are percent specific cytotoxicity against EG7.OVA target cells using spleen cells pooled from four mice per group. There was no lysis of EL4 cells by any of the effector populations.

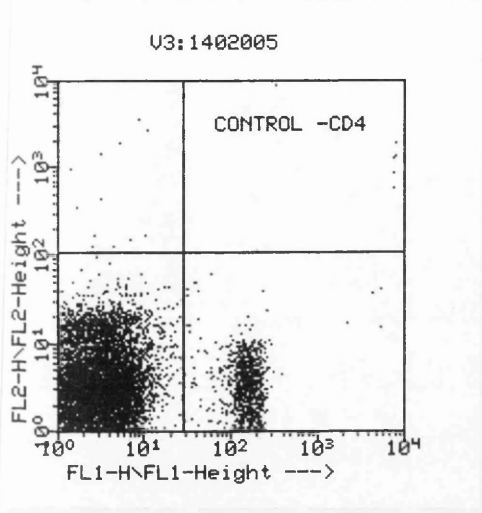

Figure 4.7.

FACS analysis of spleen cells from anti-CD4 mAb treated mice. Prior to immunisation with ISCOMS-OVA, two mice were sacrificed from the anti-CD4 mAb treated and untreated groups, spleen cells isolated and the proportion of $CD4^+$ T cells determined using FACS analysis as described in the Materials and Methods.

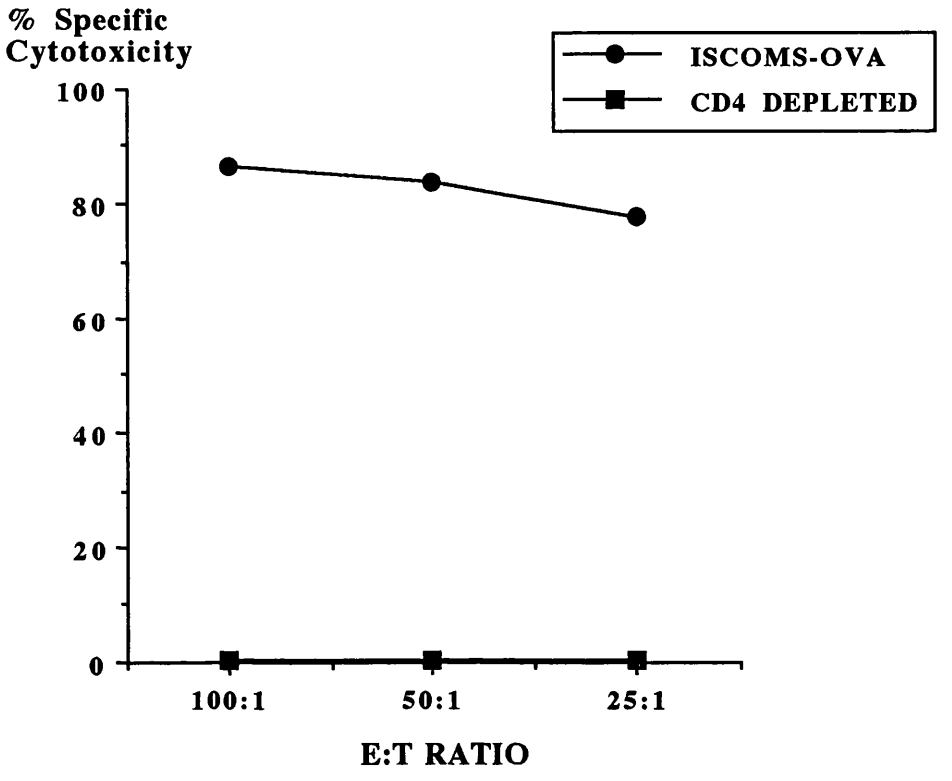

Figure 4.8

CTL responses primed by ISCOMS-OVA *in vivo* are CD4+ T cell-dependent. C57Bl/6 mice were injected with anti-CD4 mAb (YTS 191.1) on two occasions, prior to immunisation with ISCOMS-OVA i.p. The results shown are percent specific cytotoxicity against EG7.OVA target cells using spleen cells pooled from four mice per group. There was no lysis of EL4 cells by any of the effector populations.

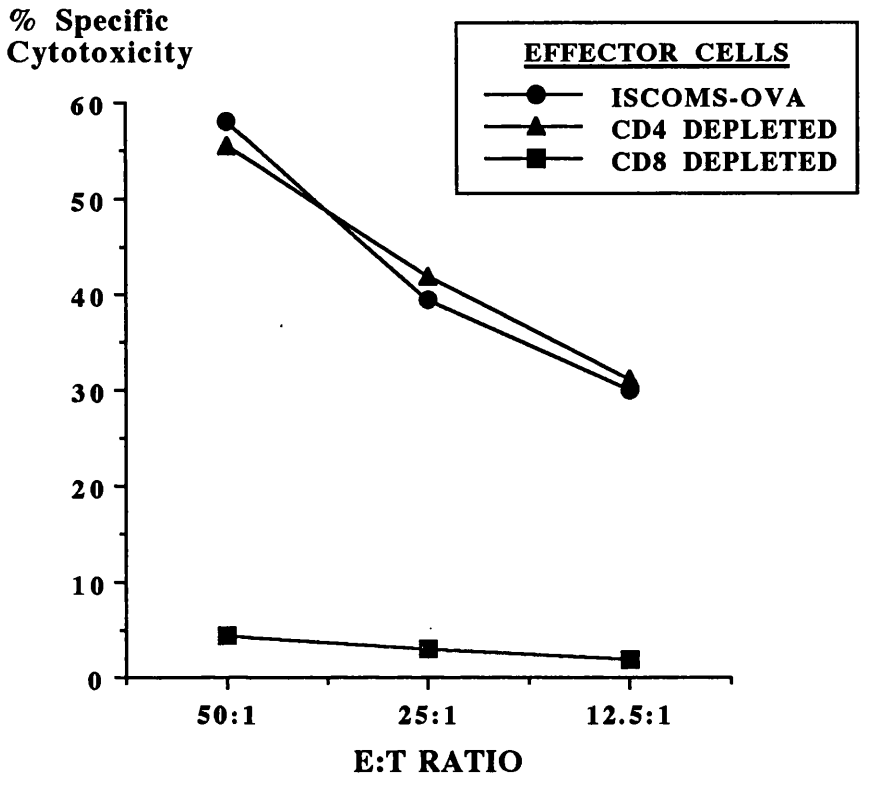

Figure 4.9.

Phenotype of OVA-specific CTL primed by feeding ISCOMS-OVA. Spleen cells isolated from C57Bl/6 mice which had been fed six times with ISCOMS-OVA were restimulated *in vitro* with EG7.OVA cells, and then depleted of $CD4^+$ or $CD8^+$ T cells by monoclonal antibody plus complement treatment, before OVA-specific CTL activity was measured by ^{51}Cr release assay. The results shown are percent specific cytotoxicity against EG7.OVA target cells using spleen cells pooled from four immunised mice. There was no lysis of EL4 cells by any of the effector populations.

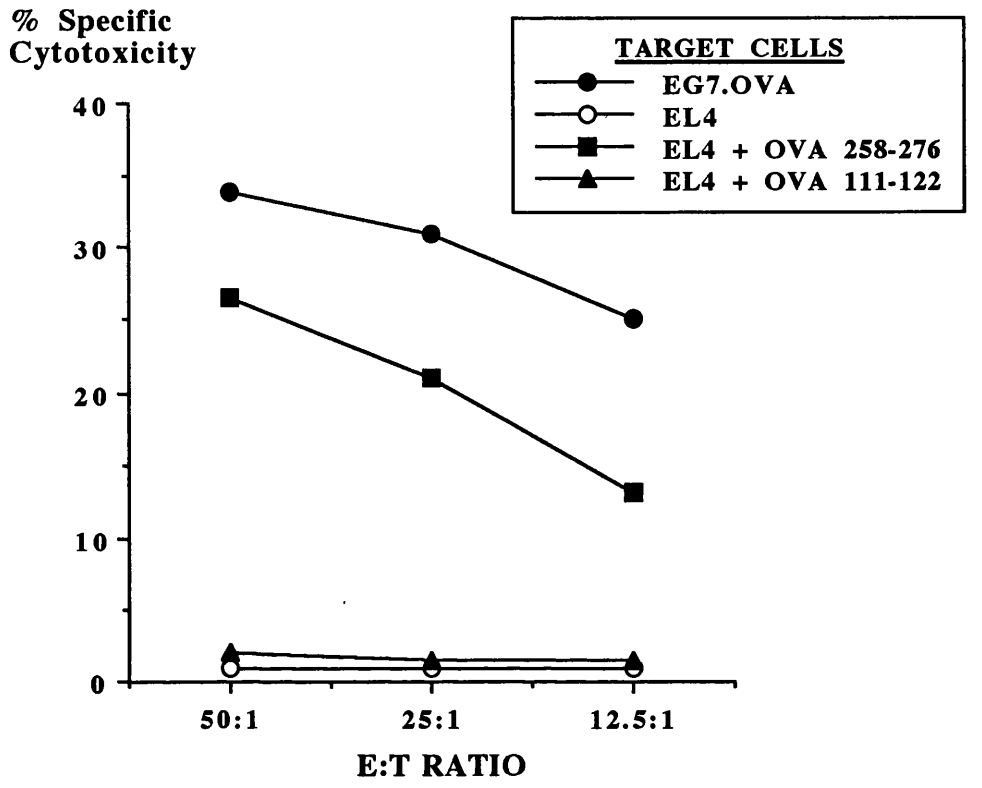

Figure 4.10.

Epitope specificity of OVA-specific CTL induced by feeding ISCOMS-OVA. Spleen cells isolated from C57Bl/6 mice which had been fed six times with ISCOMS-OVA were restimulated *in vitro* with EG7.OVA cells and tested for their ability to lyse EL4 cells in the presence of the peptide OVA 258-276 or OVA 111-122. Peptides, which were added to the target cells before the start of the assay, remained present at a final concentration of 2.3μM throughout the ^{51}Cr release assay. The results shown are specific cytotoxicity using pooled spleen cells from four immunised mice.

CHAPTER 5: PRIMING OF *in vitro* PROLIFERATIVE AND CYTOKINE RESPONSES BY ISCOMS

Introduction

The experiments I have described thus far have shown that ISCOMS induce a wide range of both humoral and cell mediated immune effector responses *in vivo*. Many of these responses, such as IgA, IgG and DTH responses are classically dependent on $CD4^+$ T cells. In addition, the induction of class I MHC-restricted CTL by ISCOMS also required $CD4^+$ T cells. It was therefore important to examine directly the priming of antigen-specific $CD4^+$ T cells by ISCOMS-OVA. Furthermore, I wished to determine if there was any preferential induction of the T_h subsets which have been described in mice, based on the pattern of cytokines which they produce (27, 45). This area had not been previously addressed following immunisation with ISCOMS and in general, there had been little investigation of ISCOMS-primed T cell functions *in vitro*.

To do this, I established the protocols necessary for inducing and assaying antigen-specific T cell proliferative responses *in vitro* in mice primed parenterally and orally with ISCOMS-OVA. In addition, I utilised ELISA techniques developed in this laboratory to assess the pattern of $CD4^+$ T cell-dependent cytokines produced by ISCOMS-OVA primed cells when restimulated *in vitro* with soluble OVA.

Experimental Protocol

Proliferative and cytokine responses were assayed in BALB/c or C57Bl/6 mice which had been immunised s.c. with 10μg ISCOMS-OVA into the rear footpad or with 5μg ISCOMS-OVA i.p. or which had been fed 50-100μg ISCOMS-OVA on 3-6 occasions. Control mice received equivalent priming volumes of saline only.

Lymphoid organs were removed at various timepoints after immunisation and proliferative and cytokine responses of pooled lymphoid cells assayed *in vitro*.

OVA-specific serum IgG isotypes were assayed in BALB/c mice primed s.c. with a single dose of 10μg ISCOMS-OVA into the right hind footpad. Primary antibody responses were measured 7 days after priming and mice were challenged with 100μg of heat-aggregated OVA on day14 post-priming and secondary IgG responses assayed one week later.

Results

A) Proliferative Responses of ISCOMS Primed Lymphocytes

i) ISCOMS Prime for Antigen-Specific Proliferative Responses *in vitro*

In the first experiments, I examined the proliferative responses of draining popliteal lymph node cells from BALB/c mice which had been immunised s.c. 7 or 14 days previously. One week after immunisation, strong OVA-specific proliferative responses were observed in ISCOMS-OVA primed PLN cells harvested after 7

days of *in vitro* culture with soluble OVA (Fig. 5.1A), even when concentrations as low as 1-10μg/ml OVA were used for restimulation. In contrast, saline-primed control PLN cells showed no proliferation in the presence of OVA (Fig. 5.1A). Both populations had strong proliferative responses to mitogenic stimulation with Con A (Fig. 5.1B). Similar antigen-specific proliferative responses were obtained using PLN cells isolated 14 days after immunisation (Fig. 5.2), confirming the ability of ISCOMS to prime potent antigen-specific proliferative responses in the draining lymph node after s.c. immunisation.

ii) Kinetics of OVA-Specific Proliferative Responses *in vitro*

The proliferative responses of ISCOMS-OVA primed PLN cells to restimulation with soluble OVA peaked between 4 and 6 days of culture *in vitro*, although significant responses remained detectable even after 8 days of culture (Fig. 5.3). At the earliest stage of the response, the level of proliferation was proportional to the concentration of OVA, but as the period of culture increased, the peak proliferative responses were observed at progressively lower concentrations of OVA (Fig. 5.3C).

Repeat experiments showed that this pattern of response was reproducible and in most subsequent studies I assayed proliferative responses 14 days after immunisation and after 6 days culture *in vitro*.

iii) Phenotype of ISCOMS-Primed Cells Proliferating in Response to Antigen *in vitro*

As ISCOMS stimulated a wide range of different T cell responses *in vivo*, I thought it important to examine the phenotype of the cells which were primed by ISCOMS-OVA to proliferate *in vitro*. PLN cells were depleted of either $CD4^+$ T cells or $CD8^+$ T cells using mAb plus complement treatment, before being cultured *in vitro* with OVA. As shown in Fig. 5.4, depletion of $CD8^+$ T cells had no effect on the subsequent proliferative response, whereas depletion of $CD4^+$ T cells completely ablated the proliferative response to OVA. Similar results were obtained irrespective of whether PLN cells were isolated 7 or 14 days after immunisation (Fig. 5.4), indicating that the proliferative responses to soluble OVA were entirely dependent on $CD4^+$ T cells.

As the soluble OVA used to restimulate *in vitro* was likely to be processed only via the exogenous antigen processing pathway, it was perhaps not surprising that the responses induced by this challenge were dependent on $CD4^+$ T cells. However, as ISCOMS induced such strong class I MHC-restricted T cell responses *in vivo*, I thought it important to examine more directly whether antigen-specific proliferation of $CD8^+$ T cells could be observed during restimulation *in vitro*.

To do this, I determined whether primed $CD8^+$ PLN cells from C57Bl/6 mice could proliferate in response to the class I MHC-restricted peptide epitope OVA 257-264, which had previously been shown to be recognised by ISCOMS-primed CTL in this strain (33, 221, 226). As in BALB/c mice, intact ISCOMS-OVA primed PLN cells from B6 mice showed significant proliferative responses to soluble OVA *in vitro*, and these were abolished by removal of $CD4^+$

T cells (Fig. 5.5). However, there was no detectable proliferative response to the peptide OVA 257-264, using either intact PLN cells or cells enriched for $CD8^+$ T cells by depletion with anti-CD4 mAb and complement (Fig. 5.5).

Addition of IL-2 to cultures containing peptide did induce significant proliferation, but this was the same in wells containing either primed or control PLN cells and occurred when cells were cultured with IL-2 in the absence of peptide (Fig. 5.5). Of particular note, CD4-depleted primed PLN cells showed no significant specific response to peptide, even when exogenous IL-2 was added. These results strongly suggest that the *in vitro* proliferative responses primed by ISCOMS are mediated entirely by $CD4^+$ T cells, even under conditions where $CD8^+$ T cells are provided with appropriate sources of antigen and IL-2.

iv) Proliferative Responses of ISCOMS Primed Spleen Cells

I next attempted to examine whether i.p. priming could also prime for antigen-specific proliferative responses in spleen cells.

As shown in Fig. 5.6, ISCOMS-OVA primed spleen cells, taken from BALB/c mice 7 days after immunisation, showed somewhat higher proliferation than control spleen cells when cultured with OVA *in vitro*. However, this was not antigen-specific, as the ISCOMS-OVA primed spleen cells showed identical incorporation of ^3H-thymidine when cultured in medium alone. The viability of the ISCOMS-primed spleen cells was shown by their ability to proliferate normally in response to the T cell mitogen Con A (Fig. 5.6C). Similar results were obtained using spleen cells taken 14 days after priming (data not shown) and I made identical findings in two futher experiments, indicating the absence of priming of

antigen-specific proliferative responses in the spleen of ISCOMS primed mice.

v) Priming of *in vitro* Proliferative Responses by Feeding ISCOMS

The results above demonstrated that ISCOMS were capable of inducing antigen-specific proliferative responses when administered parenterally. I next examined whether ISCOMS also primed similar antigen-specific proliferative responses when given by the oral route. Thus, mice were fed three times with 100μg ISCOMS-OVA, their spleen and MLN cells isolated 7 days later and *in vitro* proliferative responses assayed as before.

MLN cells isolated from ISCOMS-OVA fed BALB/c mice showed strong proliferative responses when restimulated with high concentrations of OVA *in vitro* (Fig. 5.7). However, in contrast to the PLN responses, no antigen-specific proliferation occurred with MLN cells cultured with concentrations of OVA lower than 1mg/ml. The kinetics of the proliferative responses of MLN cells were similar to those observed with PLN cells, being maximal after 6 days of culture *in vitro,* and a small, but marked, response still being detectable after 8 days (Fig. 5.7).

In contrast, spleen cells from mice primed orally with ISCOMS-OVA mounted no antigen-specific proliferative responses when restimulated with OVA *in vitro* (Fig. 5.8), with control spleen cells showing equivalent incorporation of ^{3}H-thymidine when cultured with OVA. Similar results were obtained when cultures were harvested after 4, 6 or 8 days of restimulation *in vitro* (Fig. 5.8) and were consistent with the failure of parenteral

immunisation to prime detectable proliferative responses in the spleen .

Thus, ISCOMS were able to prime antigen-specific *in vitro* proliferative responses in the draining lymph node following either oral or systemic immunisation.

vi) Ability of ISCOMS to Restimulate Proliferative Responses *in vitro*

The proliferation assays discussed thus far used native antigen to recall the responses of ISCOMS primed cells *in vitro.* It was also of interest to determine whether ISCOMS primed cells would be able to proliferate in response to restimulation with ISCOMS themselves, as this would indicate that ISCOMS could be processed and presented *in vitro.* As shown in Fig. 5.9, PLN cells isolated from BALB/c mice primed s.c. with ISCOMS-OVA exhibited strong proliferative responses when restimulated with ISCOMS-OVA *in vitro.* These responses were present in PLN cells taken 7 or 14 days after priming and were maximal using concentrations equivalent to 1-10μg/ml OVA incorporated in ISCOMS (Fig. 5.9). These results show that very low amounts of protein in ISCOMS can be processed and presented for recognition by T cells *in vitro.* I planned to make use of this finding to study whether ISCOMS could directly prime immune responses *in vitro,* but unfortunately there was not enough time for me to do this.

B) Cytokine Production by ISCOMS Primed Lymphocytes

As $CD4^+$ T cells appeared to be central to the *in vivo* and *in vitro* responses induced by ISCOMS-OVA and as the action of these

cells is determined by their production of cytokines, I next examined the pattern of antigen-specific cytokine production by ISCOMS primed cells. I chose to examine IL-2 and IFN-γ production as indicators of the T_h1 subset of $CD4^+$ T cells and IL-4 and IL-5 as markers of T_h2 cells (27, 45).

i) Cytokine Production by Parenterally Primed Popliteal Lymph Node Cells

My first experiments concentrated on PLN cells from s.c. primed mice, as this protocol had provided the strongest proliferative responses to OVA *in vitro*. In addition, the PLN cells were restimulated *in vitro* with the concentration of OVA found to be optimal for inducing proliferative responses (1mg/ml). Parallel cultures were performed using the T cell mitogen Con A as a positive control and with medium only to measure spontaneous cytokine production. After varying periods of culture, supernatants were harvested and their cytokine content assayed by ELISA.

a) IL-2

PLN cells isolated from mice 7 days after immunisation with ISCOMS-OVA produced a small, but significant, amount of IL-2 after restimulation with OVA *in vitro* (Fig. 5.10A). In contrast, PLN cells from control mice did not produce any IL-2 in the presence of OVA and immunised cells produced no IL-2 when cultured without OVA. The IL-2 response was transient, as IL-2 was only detected in supernatants collected after 2 days of culture and was not present at later timepoints (Fig. 5.10A). This pattern of IL-2 production probably reflects early activation of antigen-specific T cells, followed by its subsequent utilisation as they proliferate.

An identical pattern of IL-2 production was observed using ISCOMS-OVA primed PLN cells isolated 14 days after immunisation (Fig. 5.11A).

b) IFN-γ

In parallel, ISCOMS primed PLN cells produced IFN-γ when cultured with OVA *in vitro* (Fig. 5.10B). In contrast to IL-2, the IFN-γ response was persistent and increasing amounts of this cytokine were detected over the culture period, with the highest levels being found after 6 days.

Small amounts of IFN-γ were produced spontaneously by both ISCOMS primed and control PLN cells during culture (Fig. 5.10B), but these were much lower than the antigen-specific IFN-γ responses of ISCOMS-OVA primed PLN cells at all times (Fig. 5.10B).In addition, control cells showed no enhancement of IFN-γ production when cultured with OVA.

Similar findings were made using PLN cells isolated 14 days after immunisation, with large amounts of OVA-specific IFN-γ being produced only when ISCOMS primed cells were restimulated with OVA (Fig. 5.11B).

c) IL-4

Little or no IL-4 production could be detected at any time during culture of ISCOMS primed or control PLN cells in the presence or absence of OVA *in vitro,* either 7 or 14 days after immunisation (Table 5.1). Although these results suggest that ISCOMS do not prime antigen-specific IL-4 production in the draining lymph node, it should be noted that stimulation of both primed and control PLN cells with Con A also induced only very low

amounts of IL-4 (Table 5.1). A similar lack of IL-4 production has been observed by others in the laboratory using mice from our animal facility.

d) IL-5

In contrast to their poor production of IL-4, PLN cells isolated one week after priming with ISCOMS-OVA produced IL-5 when restimulated with OVA *in vitro* (Fig. 5.10C). The kinetics of IL-5 production were different from those found with IL-2 and IFN-γ, as IL-5 did not appear until after 4 days of culture and the highest levels were found after 6 days (Fig. 5.10C). An identical pattern of IL-5 production was found using PLN cells isolated 14 days after immunisation (Fig. 5.11C).

Together, these results indicate that a single subcutaneous immunisation with ISCOMS can prime for the production of both T_h1 and T_h2 cytokines by draining lymph node cells .

ii) Subcutaneous Immunisation with ISCOMS Primes for Splenic Cytokine Production

I next examined the ability of s.c. immunisation to prime disseminated cytokine production, by measuring the production of cytokines by spleen cells from mice primed s.c. with ISCOMS-OVA.

a) IL-2

Spleen cells isolated one week after s.c. priming with ISCOMS-OVA showed no antigen-specific IL-2 response when restimulated *in vitro* with OVA, despite the fact that spleen cells from both control and immunised mice showed marked production of IL-2 in response to Con A (Table 5.2). There was no difference in the

magnitude of the mitogen-induced IL-2 responses between the groups (Table 5.2) .

In contrast, spleen cells isolated 14 days after priming with ISCOMS-OVA produced small amounts of IL-2 when restimulated *in vitro* with OVA, whereas control spleen cells did not (Fig. 5.13A). Again the IL-2 response was observed only at the earliest timepoint, indicating that IL-2 production was rapidly triggered in primed cells upon restimulation with antigen.

b) IFN-γ

As with PLN cells, spleen cells isolated one week after s.c. priming with ISCOMS-OVA produced high amounts of IFN-γ when restimulated with OVA *in vitro* (Fig. 5.12A). The kinetics of this response were similar to those observed for PLN cells, with the level of IFN-γ increasing progressively during the culture period (Fig. 5.12A).

Although some IFN-γ was also produced by control spleen cells, particularly when cultured with OVA *in vitro* (Fig. 5.12B), the amount was markedly less than that produced by the ISCOMS-OVA primed cells. As with PLN cells, spleen cells from both naive and primed mice produced small amounts of IFN-γ when cultured in the absence of OVA.

A similar pattern of IFN-γ production was observed using spleen cells isolated 14 days post-immunisation, with high antigen-specific IFN-γ production by ISCOMS primed spleen cells and very little produced by control spleen cells (Fig. 5.13B). At this time, spontaneous IFN-γ production by both primed and control spleen cells was very low (Fig. 5.13B).

c) IL-4

As with the PLN cells, little or no IL-4 was produced by spleen cells restimulated with OVA (Table 5.3). Both control and ISCOMS-OVA primed spleen cells produced small amounts of IL-4 following stimulation with Con A and there was no difference between the groups (Table 5.3).

d) IL-5

Spleen cells taken one week after s.c. priming with ISCOMS-OVA produced IL-5 when restimulated with OVA *in vitro* (Fig. 5.12B). The pattern of IL-5 production was similar to that observed for PLN cells, first appearing after 4 days of culture and increasing thereafter (Fig. 5.12B). Control spleen cells produced little detectable IL-5 at any timepoint, when cultured in either the presence or absence of OVA (Fig. 5.12B).

A similar pattern of antigen-specific IL-5 production was observed with spleen cells isolated 14 days post-immunisation (Fig. 5.13C), except that in this case a small amount of IL-5 was produced by ISCOMS primed spleen cells after 2 days of culture with OVA. Again, naive spleen cells showed little production of IL-5 *in vitro*, regardless of the culture conditions (Fig. 5.13C).

Taken together, these results confirm that the priming of T_h1 and T_h2 cytokine responses was not restricted to the draining lymph node following s.c. immunisation, but was also disseminated to more distant lymphoid organs.

iii) Cytokine Production by Spleen Cells From Mice Primed Orally with ISCOMS

I next investigated whether oral administration of ISCOMS also primed for antigen-specific cytokine production. Mice were fed six times with 50μg ISCOMS-OVA and, 7 days after the last feed, spleen cells were assayed for OVA-specific cytokine production *in vitro*.

In contrast to naive spleen cells, those taken from mice fed ISCOMS-OVA produced a marked amount of IL-2 when restimulated with OVA *in vitro* (Fig. 5.14A). As in previous experiments on parenterally primed primed mice, the OVA-specific IL-2 was present only after 2 days of culture and not thereafter.

Also consistent with my previous observations, orally primed spleen cells did not produce any IL-4 in response to *in vitro* restimulation with OVA (Table 5.4). However, significant quantities of IL-4 were produced by orally primed and control spleen cells when cultured with Con A and there were no differences between the groups (Table 5.4).

Orally primed spleen cells produced large amounts of both IFN-γ and IL-5 when restimulated *in vitro* with OVA (Fig. 5.14B,C). The kinetics of these responses were similar, with the greatest amounts present in supernatants isolated after 6 days of culture (Fig. 5.14). As in some previous experiments, naive spleen cells also produced significant amounts of IFN-γ after 6 days of culture in the presence of OVA, although these were markedly lower than the IFN-γ levels produced by spleen cells from mice fed ISCOMS-OVA (Fig. 5.14B).

Together, these results indicate that oral immunisation with ISCOMS primes an identical pattern of systemic cytokine production

to that observed following parenteral immunisation, suggesting that ISCOMS are able to prime both T_h1 and T_h2 type responses regardless of their route of administration.

C) Priming of Antigen-Specific IgG1 and IgG2a Production by ISCOMS

To examine whether the pattern of cytokine production I observed *in vitro* was reflected by immune function *in vivo*, I measured the priming of OVA-specific IgG isotypes by parenteral immunisation with ISCOMS-OVA (227, 228). No OVA-specific IgG isotype responses could be detected after primary immunisation with ISCOMS-OVA (data not shown), but one week after challenge with heat-aggregated OVA, ISCOMS-OVA primed mice produced high titres of OVA-specific IgG1 and IgG2a antibodies (Fig. 5.15). In contrast, control mice challenged with OVA alone produced very little OVA-specific IgG1 or IgG2a antibody (Fig. 5.15).

Conclusions

The results of these *in vitro* studies show that ISCOMS prime strong antigen-specific proliferative responses in the draining lymph node after parenteral immunisation. These were entirely dependent on $CD4^+$ T cells as they were ablated by depletion of this subset and I was unable to obtain any evidence that $CD8^+$ T cells played any role in the proliferative responses. Removal of $CD8^+$ T cells had no effect on the responses of primed lymph node cells and there was no proliferative response to the H-$2K^b$-restricted peptide epitope OVA 257-264, even in the presence of exogenous IL-2.

These findings correlate well with my earlier observations on the induction of $CD8^+$ CTL by ISCOMS *in vivo*, which also required the presence of $CD4^+$ T cells, but do raise the question of how these CTL are generated *in vivo*.

In contrast to the PLN responses, antigen-specific proliferative responses could not be detected in ISCOMS primed spleen cell populations after either oral or parenteral immunisation. However, parenteral immunisation with ISCOMS primed for substantial amounts of antigen-specific cytokine production by both PLN and spleen cells following restimulation with antigen *in vitro*. Thus, efficient dissemination of the initially localised response must occur after immunisation with low amounts of protein antigen in ISCOMS (10μg OVA).

The pattern of cytokine production by ISCOMS primed lymphoid cells restimulated with antigen *in vitro* was characterised by a transient IL-2 response, followed by the later accumulation of both IFN-γ and IL-5. This indicates that both T_h1 and T_h2 type $CD4^+$ T cells were activated by immunisation with ISCOMS. This is supported by the wide range of both humoral and cell-mediated immune responses primed by ISCOMS *in vivo*. and was confirmed in the present study by the priming of both IgG1 and IgG2a serum antibody responses.

Priming of both proliferative and cytokine responses also occurred after oral immunisation with ISCOMS. Strong antigen-specific proliferative responses were detected in draining MLN cells isolated from orally primed mice, while spleen cells from these mice produced IL-2, IFN-γ and IL-5 when restimulated with antigen *in vitro*. The proliferative and cytokine responses induced by feeding ISCOMS showed the same characteristics as those

primed by parenteral immunisation, indicating that ISCOMS were able to prime these local and systemic responses in a similar manner, regardless of the route of immunisation.

Together, the findings presented in this chapter emphasise the range, strength and dissemination of the immune responses induced by oral and parenteral immunisation with ISCOMS. In particular, the efficient generation of $CD4^+$ T cell dependent proliferative responses and both T_h1 and T_h2 cytokine responses, indicates the potent adjuvant effect of ISCOMS on this cell population, which is central to the development of effector immune responses. The biological and physico-chemical basis of these adjuvant properties will be explored further in chapters 7 and 8.

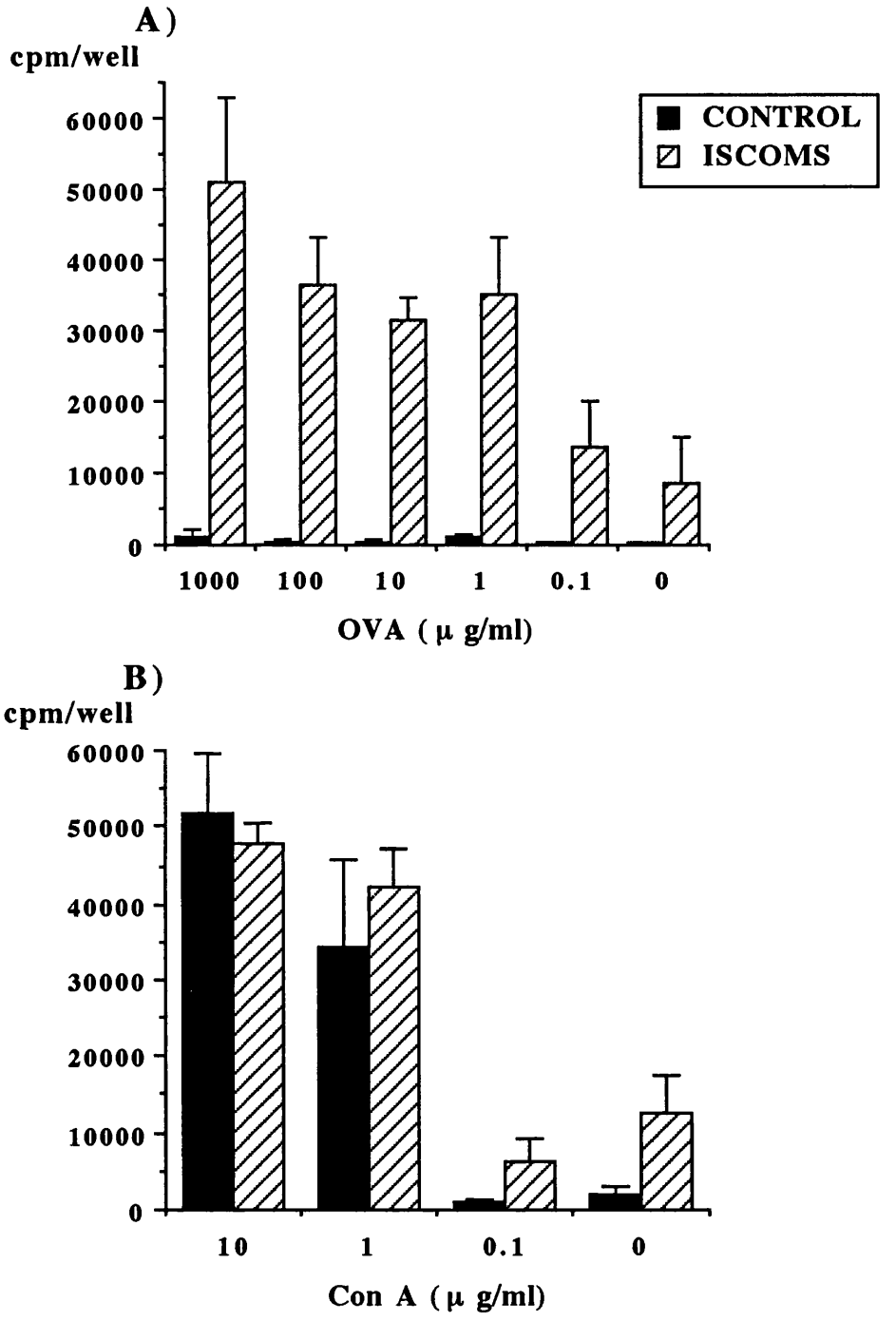

Figure 5.1.

Proliferative responses of popliteal lymph node cells isolated 7 days after priming with 10μg ISCOMS-OVA or saline s.c., after restimulation with OVA (A) or Con A (B) *in vitro*. The data represent mean cpm values ± 1S.D. from quadruplicate cultures harvested after 3 (B) or 6 days (A) of culture.

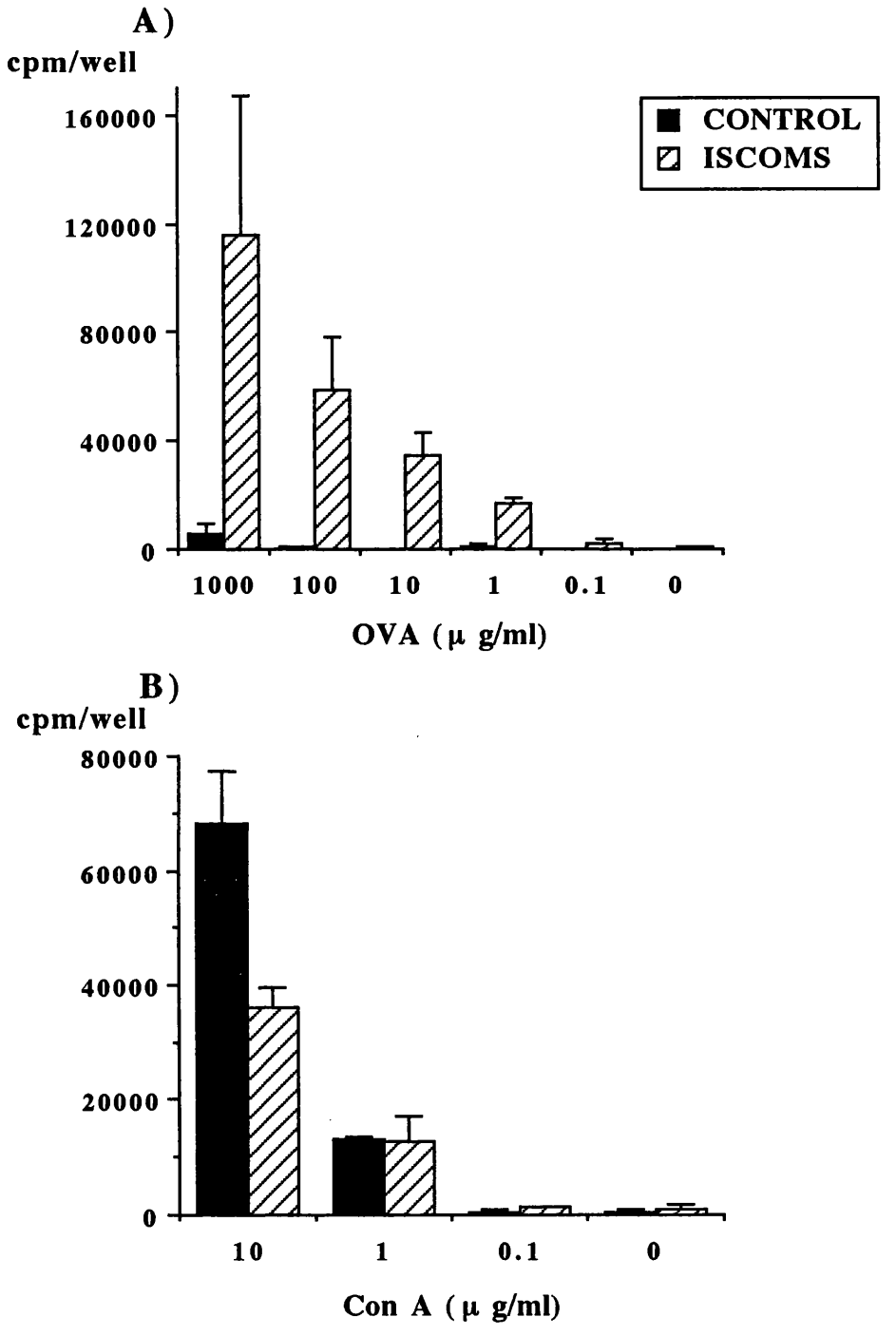

Figure 5.2.

Proliferative responses of popliteal lymph node cells isolated 14 days after priming with 10μg ISCOMS-OVA or saline s.c., after restimulation with OVA (A) or Con A (B) *in vitro*. The data represent mean cpm ± 1S.D. from quadruplicate cultures harvested after 3 (B) or 6 days (A) of culture.

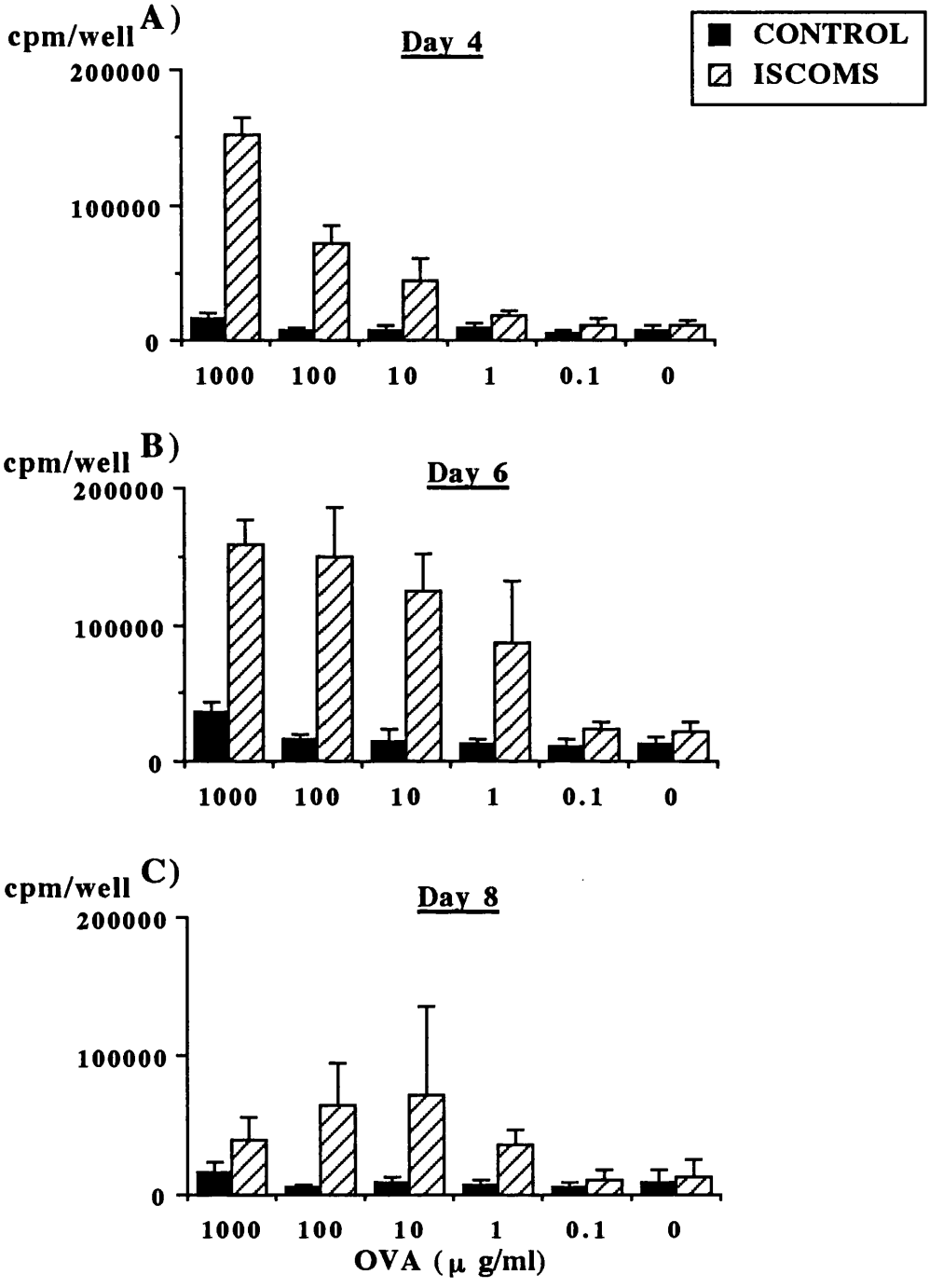

Figure 5.3.

Kinetics of *in vitro* OVA-specific proliferative responses of primed popliteal lymph node cells isolated 14 days after immunisation with 10μg ISCOMS-OVA or saline s.c. The data represent mean cpm ± 1S.D. from quadruplicate cultures harvested after 4 (A), 6 (B) or 8 days (C) of culture.

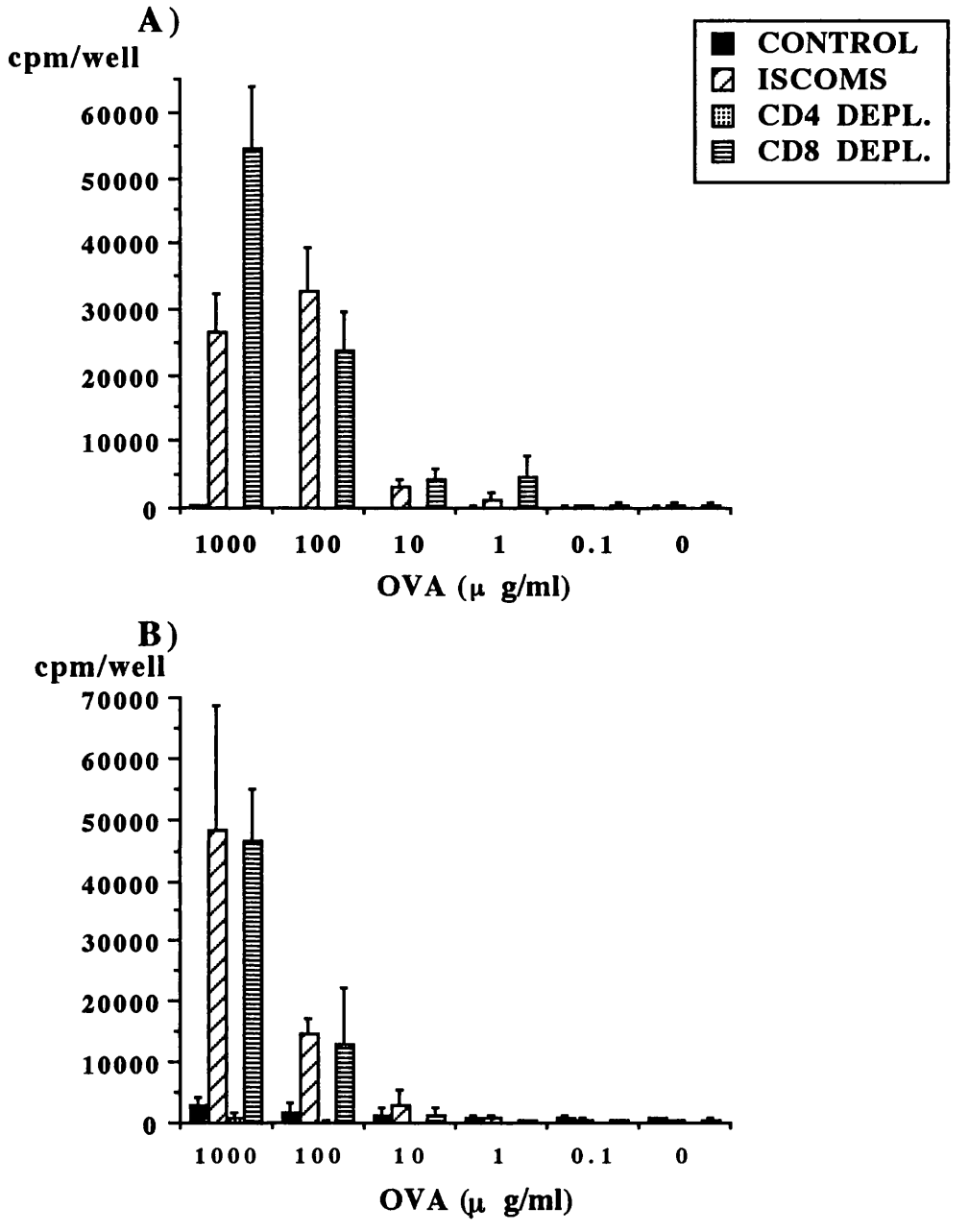

Figure 5.4.

Role of CD4+ and CD8+ T cells in OVA-specific proliferative responses of ISCOMS primed LN cells. Popliteal lymph node cells were isolated 7 (A) or 14 days (B) after priming with 10μg ISCOMS-OVA s.c. and depleted of CD4+ or CD8+ T cells using mAbs plus complement, before restimulation with OVA *in vitro*. The data represent mean cpm ± 1S.D. from quadruplicate cultures of PLN harvested after 6 days of *in vitro* culture.

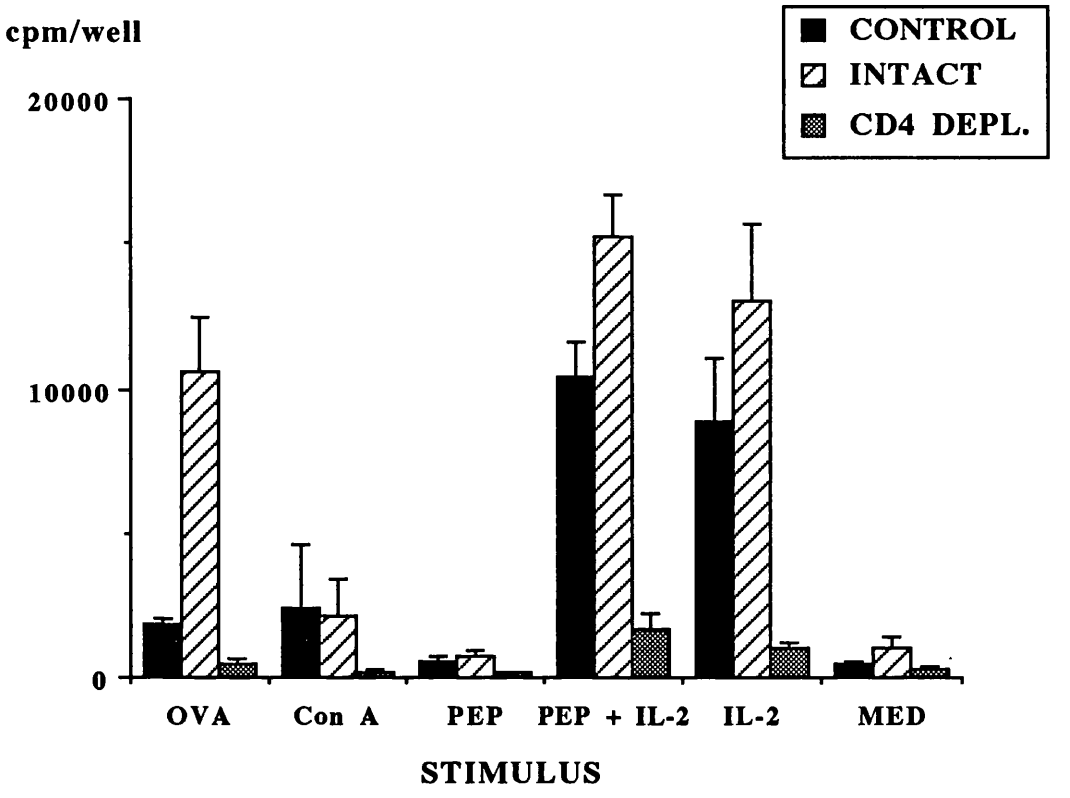

Figure 5.5.

Proliferative responses of ISCOMS-OVA primed cells and control cells to the OVA peptide 257-264. Popliteal lymph node cells were isolated from C57Bl/6 mice 7 days after priming and used intact or after depletion of $CD4^+$ T cells. Cells were cultured with soluble OVA (1mg/ml), Con A (10μg/ml) or OVA 257-264 (200nM) ± rh IL-2 (10U/ml). The data represent mean cpm ± 1S.D. from quadruplicate cultures harvested after 6 days of culture.

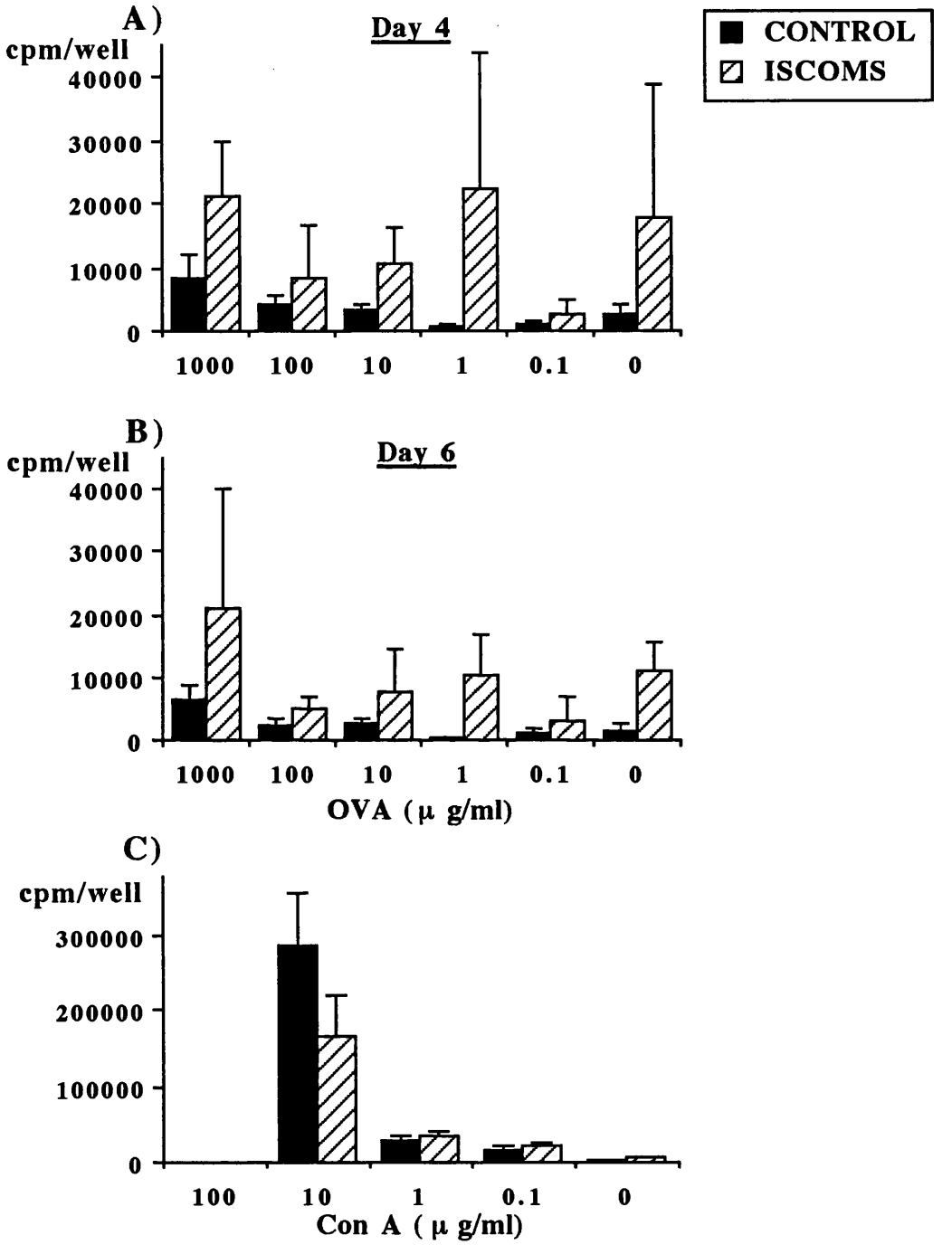

Figure 5.6.

Proliferative responses of spleen cells from mice primed with 5μg ISCOMS-OVA or saline i.p. to stimulation with OVA (A,B) or Con A (C) *in vitro*. The data represent mean cpm ± 1S.D. from quadruplicate cultures harvested after 4 (A and C) or 6 days (B) of culture.

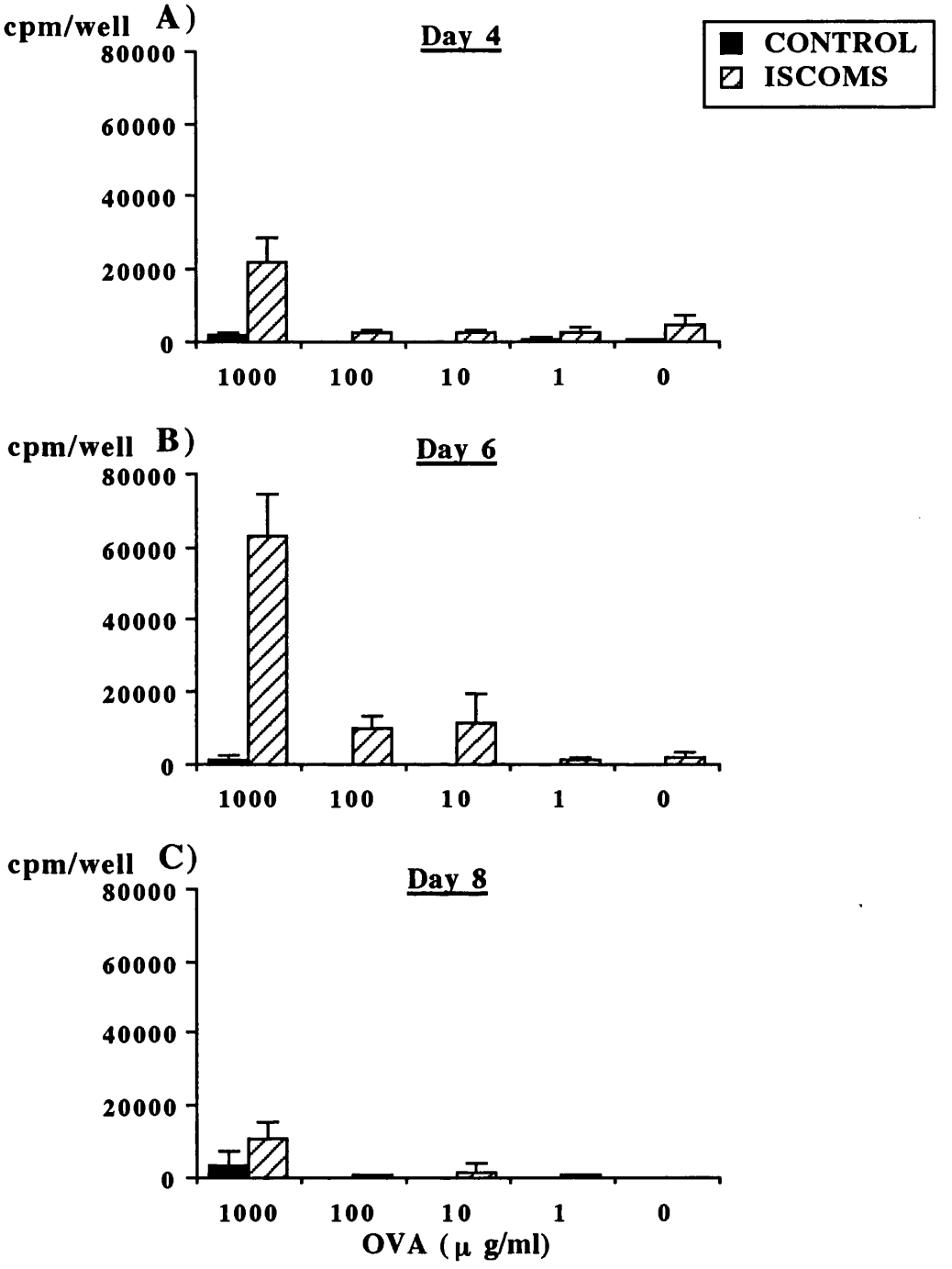

Figure 5.7.

Proliferative responses to OVA of mesenteric lymph node (MLN) cells isolated from mice primed orally with ISCOMS-OVA. Mice were fed three times with 50μg ISCOMS-OVA or saline and MLN cells taken 7 days after the last feed. The data represent mean cpm values ± 1S.D. from quadruplicate cultures harvested after 4 (A), 6 (B) or 8 days (C) of culture.

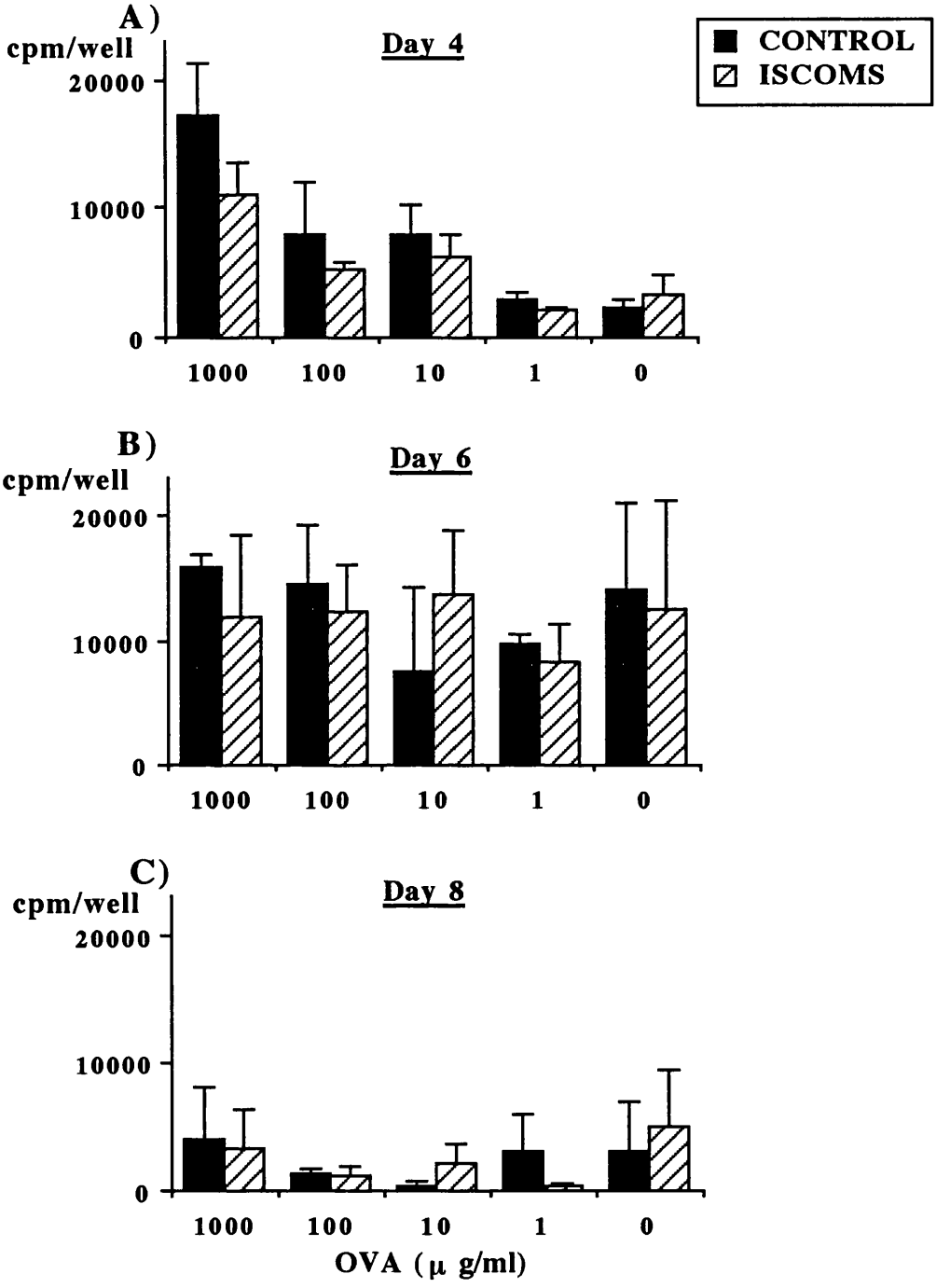

Figure 5.8.

Proliferative responses of spleen cells isolated from mice primed orally with ISCOMS-OVA and stimulated with OVA *in vitro*. Mice were fed three times with 50μg ISCOMS-OVA or saline and spleen cells isolated 7 days after the last feed. The data represent mean cpm ± 1S.D. from quadruplicate cultures harvested after 4 (A), 6 (B) or 8 days (C) of culture.

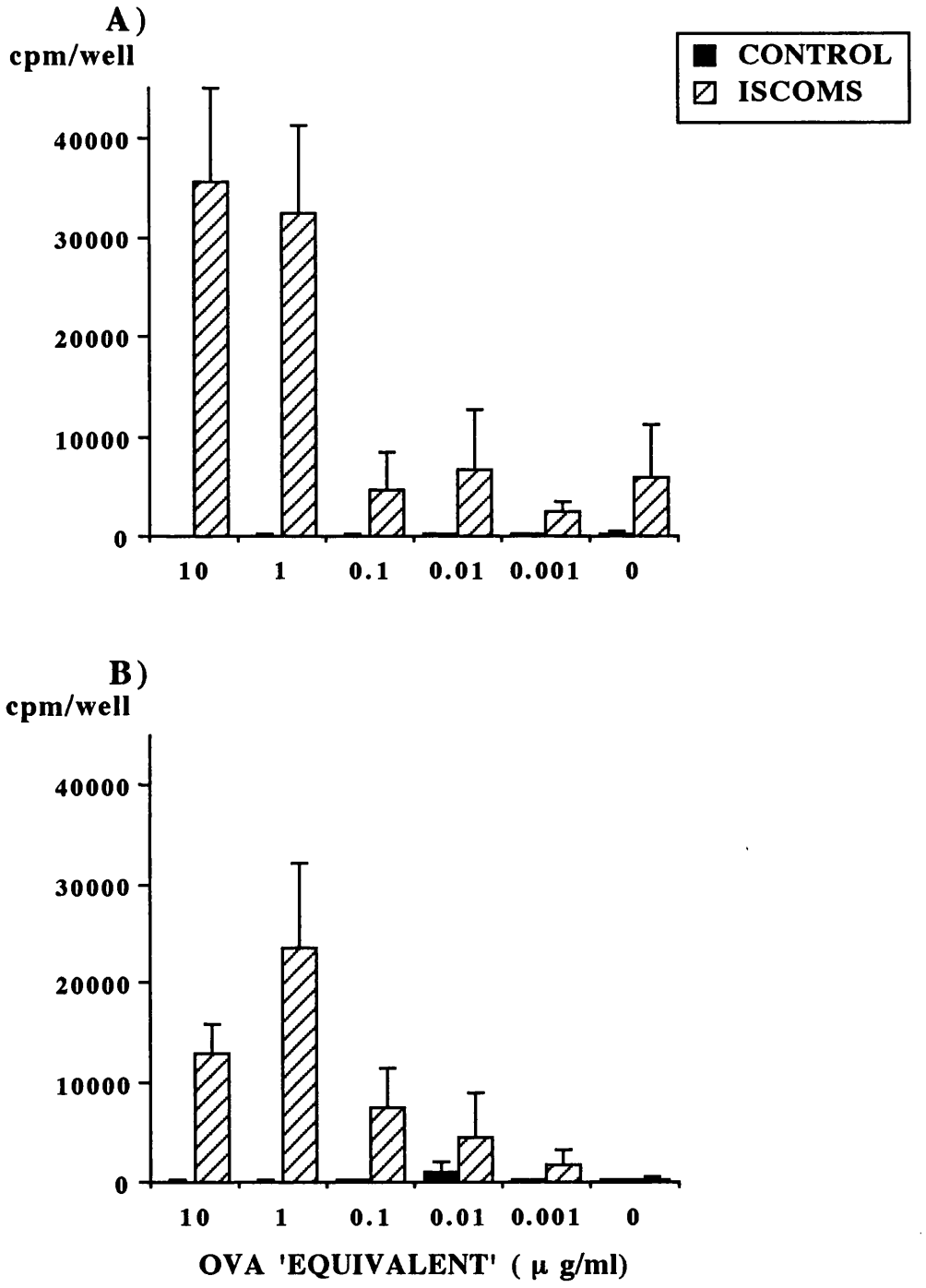

Figure 5.9.

Stimulation of proliferative responses using ISCOMS-OVA as recall antigen *in vitro*. Popliteal lymph node cells were isolated 7 (A) or 14 days (B) after priming with 10μg ISCOMS-OVA or saline s.c. The data represent mean cpm ± 1S.D. from quadruplicate cultures harvested after 6 days of culture.

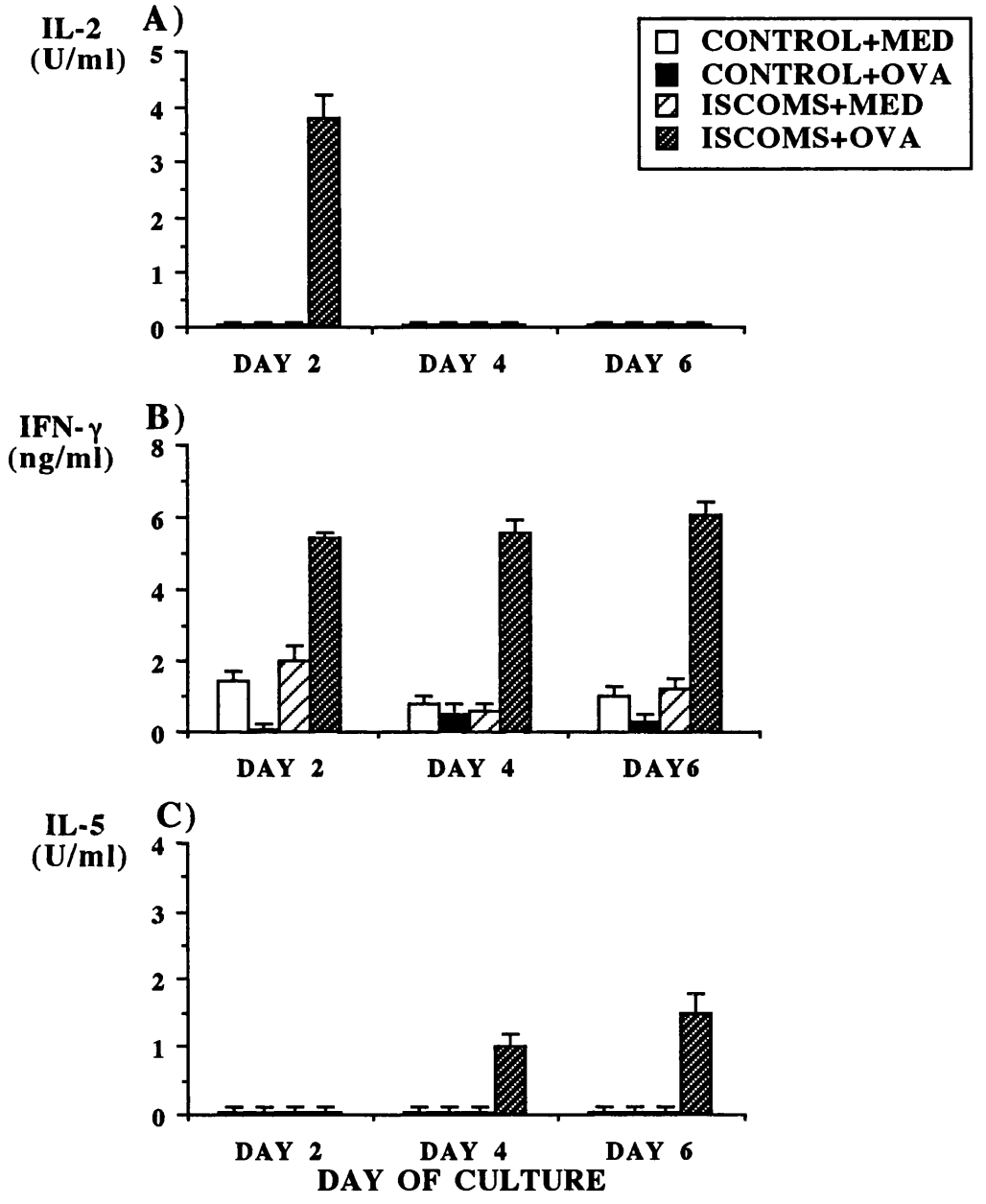

Figure 5.10.

Cytokine production by popliteal lymph node cells isolated 7 days after priming of BALB/c mice with 10μg ISCOMS-OVA or saline s.c. PLN cells were cultured for 2-6 days in the presence (+OVA) or absence (+MED) of 1mg/ml OVA and the supernatants assayed for IL-2 (A), IFN-γ (B) and IL-5 (C) using sandwich ELISAs. The data represent mean cytokine concentrations ± 1S.D. of supernatants assayed in quadruplicate.

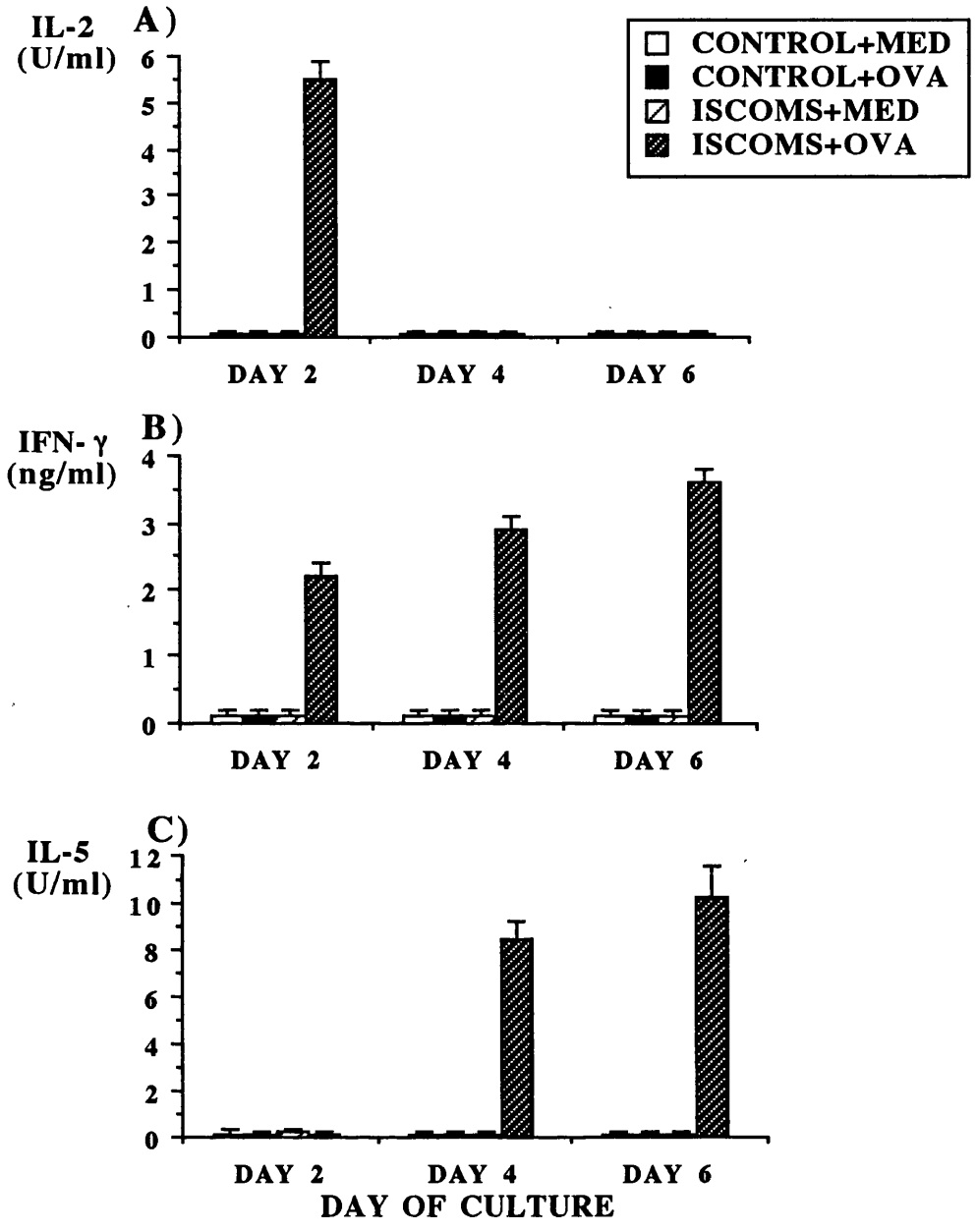

Figure 5.11.

Cytokine production by popliteal lymph node cells isolated 14 days after priming of BALB/c mice with 10μg ISCOMS-OVA or saline s.c. PLN cells were cultured for 2-6 days in the presence (+OVA) or absence (+MED) of 1mg/ml OVA and the supernatants assayed for IL-2 (A), IFN-γ (B) and IL-5 (C) using sandwich ELISAs. The data represent mean cytokine concentrations ± 1S.D. of supernatants assayed in quadruplicate.

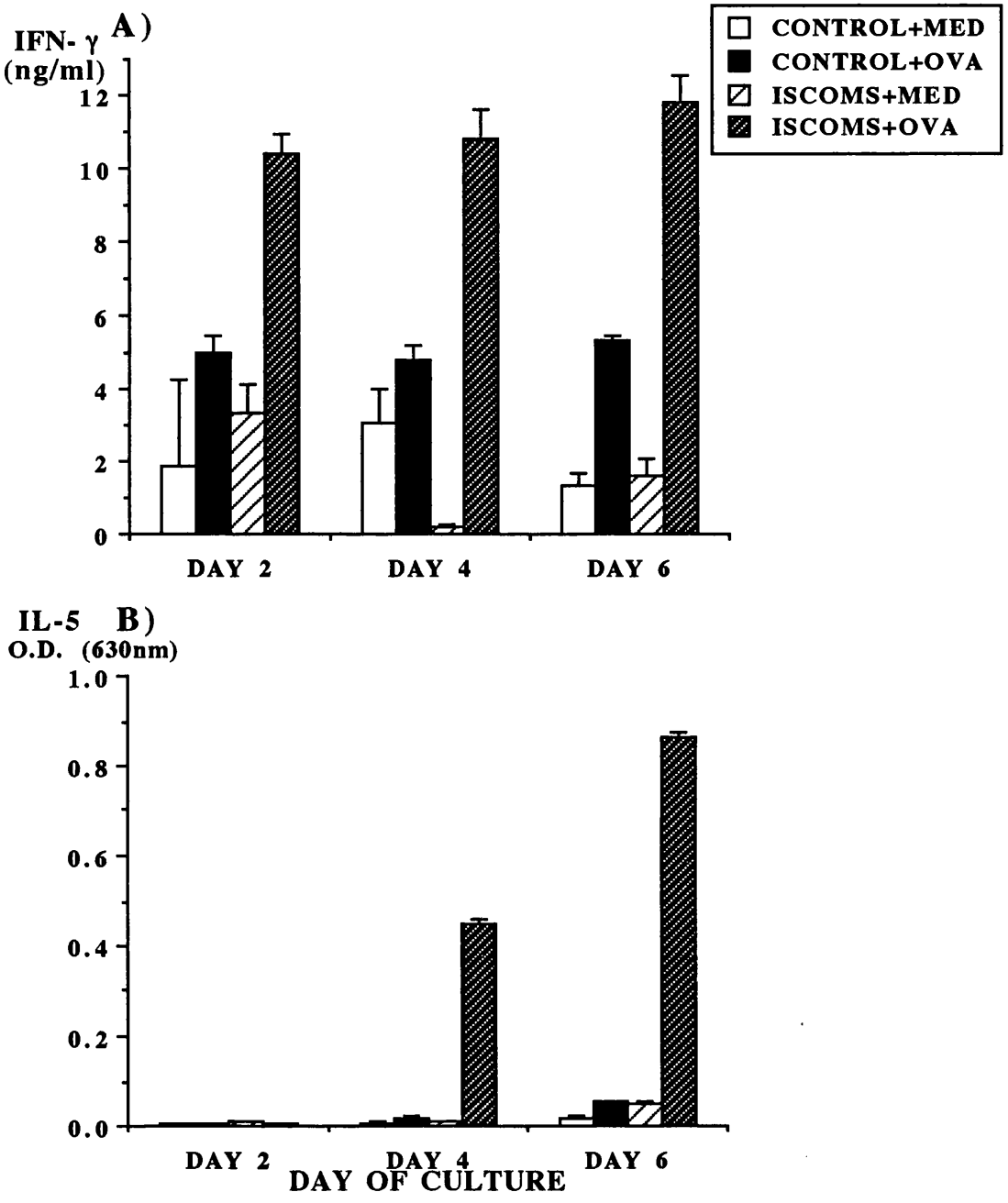

Figure 5.12.

Cytokine production by spleen cells isolated 7 days after priming of BALB/c mice with 10μg ISCOMS-OVA or saline s.c. Spleen cells were cultured for 2-6 days in the presence (+OVA) or absence (+MED) of 1mg/ml OVA and the supernatants assayed for IFN-γ (A) and IL-5 (B) using sandwich ELISAs. The data represent mean cytokine concentrations ± 1S.D. of supernatants assayed in quadruplicate.

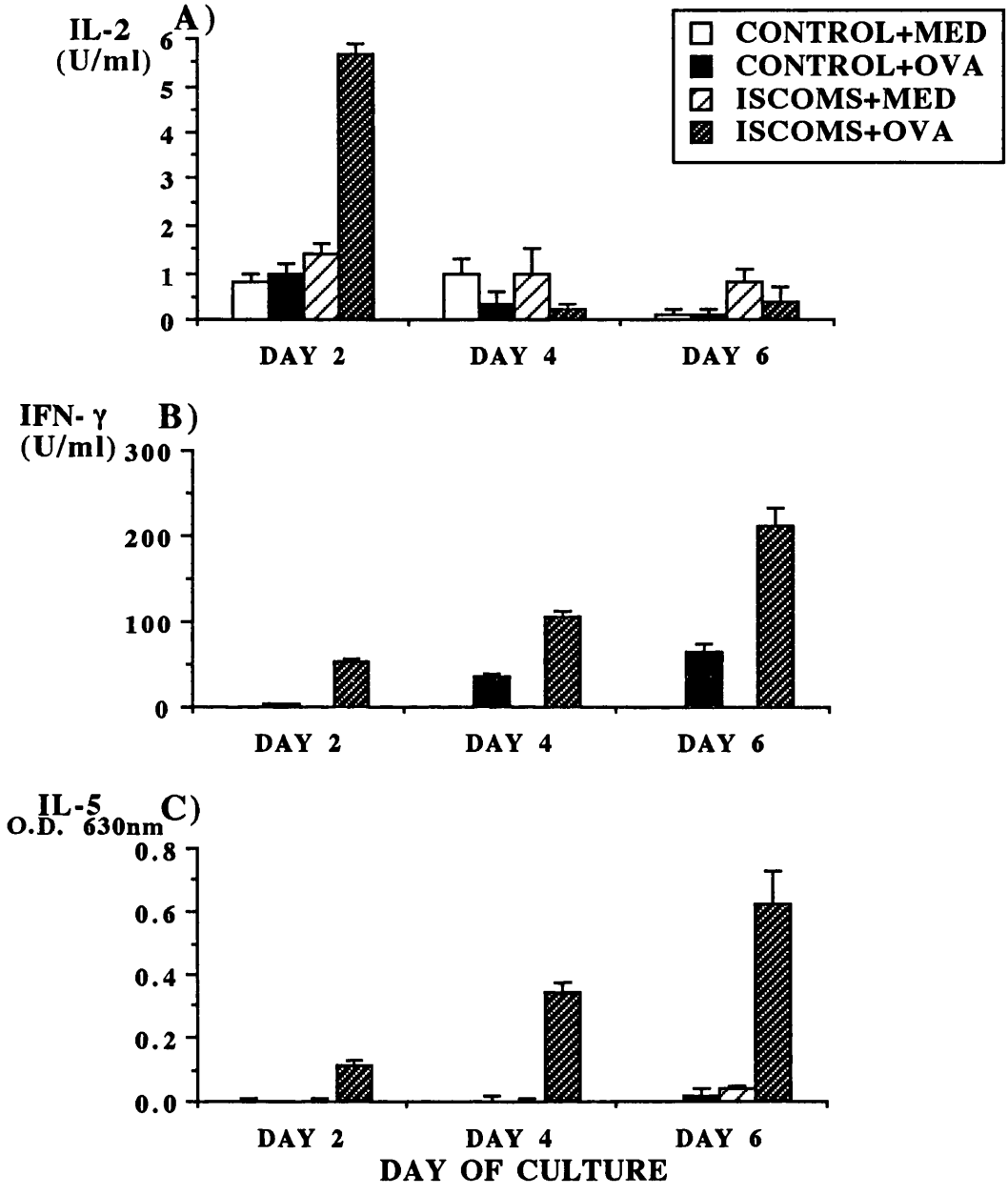

Figure 5.13.

Cytokine production by spleen cells isolated 14 days after priming of BALB/c mice with 10μg ISCOMS-OVA or saline s.c. Spleen cells were cultured for 2-6 days in the presence (+OVA) or absence (+MED) of 1mg/ml OVA and the supernatants assayed for IL-2 (A), IFN-γ (B) and IL-5 (C) using sandwich ELISAs. The data represent mean cytokine concentrations ± 1S.D. of supernatants assayed in quadruplicate.

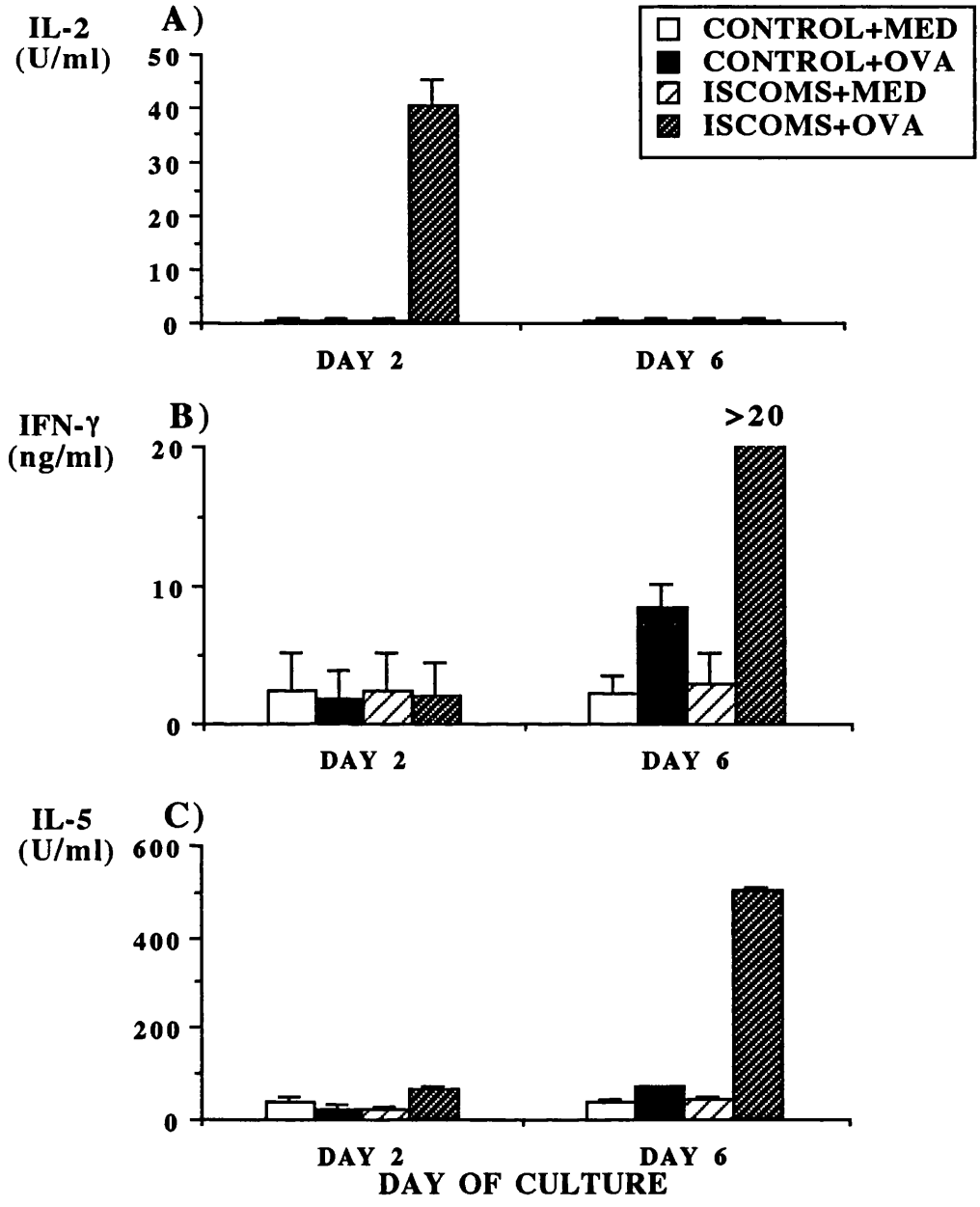

Figure 5.14.

Cytokine production by spleen cells from BALB/c mice fed three times with 50μg ISCOMS-OVA. Spleen cells isolated 7 days after the last feed were cultured for 2-6 days in the presence (+OVA) or absence (+MED) of 1mg/ml OVA and the supernatants assayed for IL-2 (A), IFN-γ (B) and IL-5 (C) using sandwich ELISAs. The data represent mean cytokine concentrations ± 1S.D. of supernatants assayed in quadruplicate.

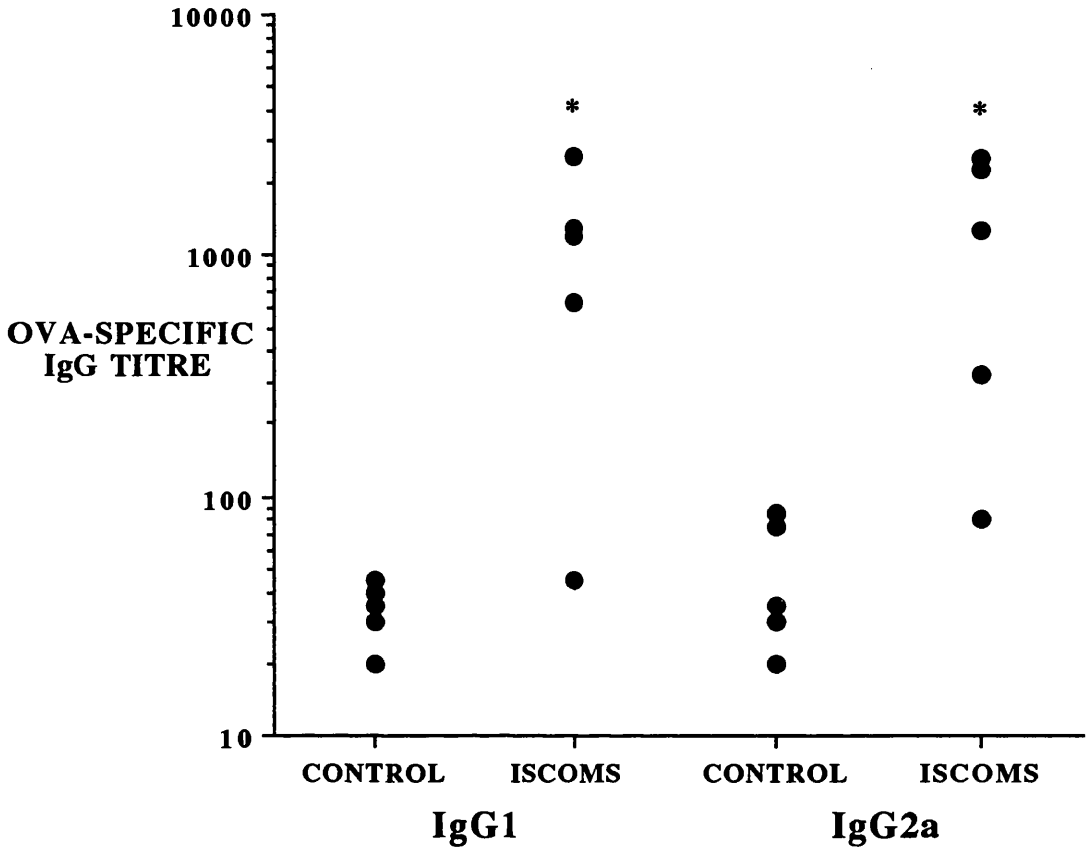

Figure 5.15.

Priming of OVA-specific serum IgG isotypes by parenteral immunisation with ISCOMS-OVA. Secondary antibody levels in BALB/c mice primed 14 days previously with 10μg ISCOMS-OVA or saline only s.c., were assayed one week after footpad challenge with 100μg heat-aggregated OVA. Each symbol represents the titres from an individual mouse.

* $p<0.05$ v. CONTROL.

Table 5.1: Production of IL-4 by Popliteal Lymph Node Cells After Priming with ISCOMS-OVA s.c.

i) Day 7 Post-Immunisation

CELLS	STIMULUS	Day 2	Day 4	Day 6
	Medium	0.7±0.2	0.2±0.1	0.2±0.2
Control:	Con A	2.7±0.3	1.8±0.5	0.5±0.2
	OVA	0.2±0.2	0.3±0.2	0.2±0.2
	Medium	0.1±0.1	0.1±0.1	0.1±0.1
ISCOMS:	Con A	1.8±0.4	1.7±0.5	0.2±0.1
	OVA	0.3±0.3	0.2±0.1	0.2±0.1

ii) Day 14 Post-Immunisation

CELLS	STIMULUS	Day 2	Day 4	Day 6
	Medium	0.1±0.1	0.2±0.1	0.1±0.1
Control:	Con A	1.1±0.2	2.2±0.3	0.4±0.1
	OVA	0.1±01	0.1±0.1	0.1±0.1
	Medium	0.1±0.1	0.2±0.1	0.1±0.1
ISCOMS:	Con A	0.8±0.2	1.5±0.4	0.2±0.1
	OVA	0.1±01	0.1±0.1	0.1±0.1

Popliteal lymph node cells isolated 7 or 14 days after priming of BALB/c mice with 10μg ISCOMS-OVA or saline s.c. were cultured for 2-6 days in the presence of 1mg/ml OVA, 10μg/ml Con A or medium only. The supernatants were assayed for the presence of IL-4 using a sandwich ELISA and the results represent mean IL-4 concentrations (U/ml) ± 1S.D. of supernatants assayed in quadruplicate.

Table 5.2: Production of IL-2 by Spleen Cells After Priming with ISCOMS-OVA s.c.

CELLS	STIMULUS	Day 2	Day 4	Day 6
	Medium	1.9±0.2	12.6±0.5	0.9±0.1
Control:	Con A	>200	>200	51.2±4.4
	OVA	1.1±0.4	5.5±0.2	0.1±0.1
	Medium	2.5±0.2	13.8±0.6	1.6±0.4
ISCOMS:	Con A	>200	>200	62.1±8.4
	OVA	1.1±0.3	0.9±0.5	0.2±0.2

Spleen cells isolated 7 days after priming of BALB/c mice with 10μg ISCOMS-OVA or saline s.c. were cultured for 2-6 days in the presence of 1mg/ml OVA, 10μg/ml Con A or medium only. The supernatants were assayed for the presence of IL-2 using a sandwich ELISA and the results represent mean IL-2 concentrations (U/ml) ± 1S.D. of supernatants assayed in quadruplicate.

Table 5.3: Production of IL-4 by Spleen Cells After Priming with ISCOMS-OVA s.c.

i) Day 7 Post-Immunisation

CELLS	STIMULUS	Day 2	Day 4	Day 6
	Medium	0.1±0.1	0.1±0.1	0.1±0.1
Control:	Con A	8.7±0.6	7.3±0.7	5.4±2.3
	OVA	0.1±0.1	0.1±0.1	0.1±0.1
	Medium	0.1±0.1	0.1±0.1	0.1±0.1
ISCOMS:	Con A	8.5±1.8	6.2±0.7	2.2±1.1
	OVA	0.1±0.1	0.1±0.1	0.1±0.1

ii) Day 14 Post-Immunisation

CELLS	STIMULUS	Day 2	Day 4	Day 6
	Medium	0.1±0.1	0.1±0.1	0.1±0.1
Control:	Con A	14.0±0.9	9.8±0.5	2.3±1.8
	OVA	0.1±01	0.1±0.1	0.1±0.1
	Medium	0.1±0.1	0.1±0.1	0.1±0.1
ISCOMS:	Con A	11.3±0.9	8.4±1.5	4.4±1.2
	OVA	0.1±01	0.1±0.1	0.1±0.1

Spleen cells isolated 7 or 14 days after priming of BALB/c mice with 10μg ISCOMS-OVA or saline s.c. were cultured for 2-6 days in the presence of 1mg/ml OVA, 10μg/ml Con A or medium only. The supernatants were assayed for the presence of IL-4 using a sandwich ELISA and the results represent mean IL-4 concentrations (U/ml) ± 1S.D. of supernatants assayed in quadruplicate.

Table 5.4: Production of IL-4 by Spleen Cells After Oral Immunisation with ISCOMS-OVA

CELLS	STIMULUS	Day 2	Day 6
	Medium	0.2±0.1	4.5±0.5
Control:	Con A	15.7±0.9	37.0±0.8
	OVA	0.2±0.1	0.2±0.1
	Medium	0.2±0.1	3.1±0.3
ISCOMS:	Con A	14.5±0.8	39.3±4.1
	OVA	0.2±0.1	0.2±0.1

BALB/c mice were fed three times with 50μg ISCOMS-OVA or saline and spleen cells isolated 7 days after the last feed were cultured for 2-6 days in the presence of 1mg/ml OVA, 10μg/ml Con A or medium only. The supernatants were assayed for the presence of IL-4 using a sandwich ELISA and the results represent mean IL-4 concentrations (U/ml) ± 1S.D. of supernatants assayed in quadruplicate.

CHAPTER 6: PRIMING OF LONG-TERM MEMORY RESPONSES BY ISCOMS

Introduction

A vital attribute of any potential vaccine vector is the ability to elicit long-lasting specific immunological memory without the need for repeated booster inoculations. As many current vaccines have to be administered as multiple doses, the identification of vectors which promote long-lasting protection by single immunisation would be of great practical and economical benefit.

The experiments I have described thus far have illustrated that ISCOMS are potent stimulators of primary immune responses *in vivo*. Earlier studies in the laboratory had shown that the primary DTH and CTL responses induced by ISCOMS-OVA *in vivo* were relatively short-lived, usually undetectable one month after priming (165, 229). However, this did not exclude the possibility that the immune system was still primed beyond this time, as it might be anticipated that relatively high numbers of antigen-specific T cells would be required to maintain primary immunity *in vivo*, whereas a few specific memory cells may remain for longer periods.

In this chapter, I therefore compared the persistence of primary immune responses for up to one year after a single parenteral immunisation with ISCOMS-OVA and the ability of these animals to generate secondary immunity after challenge *in vivo* with OVA.

Experimental Protocol

BALB/c mice were immunised s.c. with 10μg ISCOMS-OVA into one footpad, while control mice received an equivalent volume of saline. At four-monthly intervals up to one year thereafter, groups of primed or control mice were bled for the measurement of primary IgG responses and were challenged in the opposite footpad with 100μg heat-aggregated OVA to assess the primary DTH response. One week after challenge, the mice were bled again to measure the secondary IgG response.

At the same timepoints after priming, additional groups of primed and control mice were sacrificed without challenge *in vivo* and the proliferative and cytokine responses of their pooled popliteal lymph node cells or spleen cells assayed after restimulation with OVA *in vitro*.

Results

i) Persistence of Primary DTH Responses Primed by ISCOMS

The first parameter I examined was the longevity of the primary DTH responses induced by ISCOMS-OVA. In contrast to earlier experience in the laboratory (165), I found that mice primed with a single dose of 10μg ISCOMS-OVA s.c. exhibited primary OVA-specific DTH when challenged with OVA for up to 8 months after immunisation (Fig. 6.1). At this time, the footpad response of ISCOMS-primed mice remained significantly greater than that in control mice. Four months after immunisation, ISCOMS-primed mice also had footpad increments around twice those observed in the

control group, but this was not statistically significant, perhaps reflecting the generally small magnitude of the DTH responses in all groups in this experiment. One year after immunisation, no detectable OVA-specific DTH response remained in the ISCOMS-primed group (Fig. 6.1).

These results indicate that the DTH responses primed by ISCOMS may be more long-lived than previously reported.

ii) Persistence of Primary OVA-Specific IgG Responses Induced by ISCOMS-OVA

I next examined the longevity of OVA-specific IgG responses primed by ISCOMS-OVA. Four months after immunisation, significant levels of primary OVA-specific IgG antibodies were present in all mice immunised with ISCOMS-OVA (Fig. 6.2A). Similar findings were made 8 months after immunisation and even one year after immunisation, a small primary OVA-specific IgG response persisted in all ISCOMS-OVA primed mice. As anticipated, there was a gradual decrease in the levels of primary OVA-specific IgG antibodies over the period of the experiment.

The challenge protocol used to determine primary DTH responses allowed me to examine the secondary IgG antibody responses in the same mice in which primary IgG responses had been assessed. Unfortunately, the serum samples obtained from mice challenged with heat-aggregated OVA four months after priming were lost and I was unable to determine their secondary responses. However, all mice challenged with heat-aggregated OVA 8 months after immunisation mounted a potent secondary OVA-specfic IgG response to heat-aggregated OVA (Fig. 6.2B). All mice challenged one year after immunisation also made secondary OVA-

specific IgG responses (Fig. 6.2B), although these were of a much lower magnitude than those observed 8 months after priming. Unprimed control mice challenged with heat-aggregated OVA made no detectable primary IgG responses, indicating the poor immunogenicity of the challenge immunisation itself.

These results demonstrate that ISCOMS induced primary antigen-specific systemic IgG responses which persisted for at least 12 months after a single immunisation and that these were accompanied by potent priming of secondary humoral immunity to challenge with sub-optimal dose of antigen.

iii) Persistence of *In Vitro* Proliferative Responses Primed by ISCOMS-OVA

I next examined whether the long-term persistence of primary DTH and antibody responses induced by ISCOMS-OVA was accompanied by other T cell dependent responses, as measured by proliferation and cytokine production *in vitro*.

Popliteal lymph node cells taken four months after priming with ISCOMS-OVA showed no antigen-specific proliferative response when cultured with OVA *in vitro* (Fig. 6.3). However, there was an unusually low background level of ^{3}H-thymidine incorporation in this assay indicating there may have been a general problem with this culture assay, rather than a specific absence of T cell responsiveness.

This was supported by the fact that when popliteal lymph node cells were isolated from the same group of mice 8 months after priming with ISCOMS-OVA, they showed a marked OVA-specific proliferative response when cultured with OVA *in vitro* (Fig. 6.4). As described in earlier chapters, these responses were detectable

after either 4 (Fig. 6.4A) or 6 days (Fig. 6.4B) of culture *in vitro.* Control lymph node cells showed no proliferative response when cultured with OVA *in vitro.*

Popliteal lymph node cells taken one year after immunisation also showed specific proliferative responses to soluble OVA *in vitro* (Fig. 6.5). However, these responses were of a much lower magnitude than those observed 8 months after priming and were significant only after 4 days of culture *in vitro* (Fig. 6.5A).

Together, these results confirm that ISCOMS primed long-lived T cell responses which could be restimulated by *in vitro* challenge for at least 8 months after immunisation and gradually waned after this time.

iv) Persistence of Cytokine Responses Primed by ISCOMS-OVA

It was also of interest to determine whether the pattern of proliferative responses observed over time after immunisation with ISCOMS-OVA was reflected in the production of antigen-specific cytokines. In these experiments, spleen cells were isolated from the mice used to examine the longevity of popliteal lymph node proliferative responses. By using spleen cells in this case, I was able both to obtain sufficient cells to examine the full range of cytokine responses and to explore the ability of local immunisation with ISCOMS to promote long-term disseminated immunity in other lymphoid tissues.

Spleen cells isolated four months after priming with ISCOMS-OVA produced both IL-2 and IL-5 in response to *in vitro* restimulation with OVA (Fig. 6.6). The kinetics of these responses were similar to those observed previously, with IL-2 only present

on days 2 and 4 of culture *in vitro* and IL-5 peaking after 6 days (Fig. 6.6). Control spleen cells made no IL-2 or IL-5 in response to OVA, nor did ISCOMS-OVA primed spleen cells when cultured in the absence of OVA. No OVA-specific IL-4 or IFN-γ was produced by spleen cells taken four months after immunisation, despite the fact that both control and ISCOMS-OVA primed cells produced comparable amounts of these cytokines when stimulated with Con A *in vitro* (Table 6.1).

An almost identical pattern of cytokine production was observed when spleen cells were isolated 8 months after immunisation and restimulated with OVA *in vitro*, with early production of IL-2 and later accumulation of IL-5 (Fig. 6.7). Small amounts of IL-5 were produced by control cells early in culture when restimulated with OVA, but this response was not present at the later timepoints. ISCOMS-OVA primed cells also produced some IL-5 in the absence of OVA after 6 days of culture *in vitro*, but this was much lower than the antigen-specific IL-5 response and its significance unclear. Again, no significant antigen-specific IL-4 or IFN-γ production could be detected from ISCOMS-OVA primed spleen cells or naive control cells cultured with OVA (Table 6.2). Both control and ISCOMS-primed spleen cells produced IL-4 and IFN-γ in response to *in vitro* culture with Con A (Table 6.2).

One year after immunisation, primed spleen cells showed little or no production of any cytokines when restimulated *in vitro* with OVA. The IL-2 responses observed at earlier timepoints were now absent (Fig. 6.8A) and the magnitude of the IL-5 response was greatly diminished, with only very small amounts found after 6 days of culture *in vitro* (Fig. 6.8B). As before, no OVA-specific production of IL-4 or IFN-γ was observed, although mitogenic

stimulation induced production of these cytokines by both control and ISCOMS primed spleen cells (Table 6.3).

These results indicate that the priming of T_h1 and T_h2 dependent cytokine responses by ISCOMS shows similar kinetics to the other T cell dependent responses assayed above, with strong IL-2 and IL-5 responses persisting for at least 8 months after immunisation and a decline thereafter.

Conclusions

The results described in this chapter demonstrate that a single immunisation with 10μg protein antigen in ISCOMS primes long-lasting memory responses, which include all aspects of the immune response *in vivo* and *in vitro*.

Long-term priming of memory T cell responses was demonstrated by the persistence of antigen-specific DTH responses *in vivo*, as well as antigen-specific proliferation and cytokine production *in vitro*, for at least 8 months after immunisation. These T cell responses included both T_h1 and T_h2 type responses, as IL-2 and IL-5 responses were both present.

The kinetics of all these responses were similar, with a marked reduction in magnitude observed 12 months after priming. However this may have been partly attributable to the effects of ageing on immune responsiveness (230), as approximately 25% of both immunised and control mice had died prior to the conclusion of this experiment.

In addition to effector T cell responses, primary antigen-specific systemic IgG responses remained detectable even one year after immunisation, supporting the persistence of priming of T_h2

dependent responses. That memory B cells were also present was suggested by the potent secondary IgG responses found after *in vivo* challenge with OVA. Again, these immune responses decreased gradually over the course of the study.

In summary, these results highlight that ISCOMS are potent inducers of long-lasting immunity, encompassing both humoral and cell-mediated immune responses and activation of both T_h1 and T_h2 memory responses. These responses can be recalled both *in vivo* and *in vitro* even when primary immune responses are no longer evident. These observations on the longevity of ISCOMS-primed immune responses further underline their potential use as future vaccine vectors.

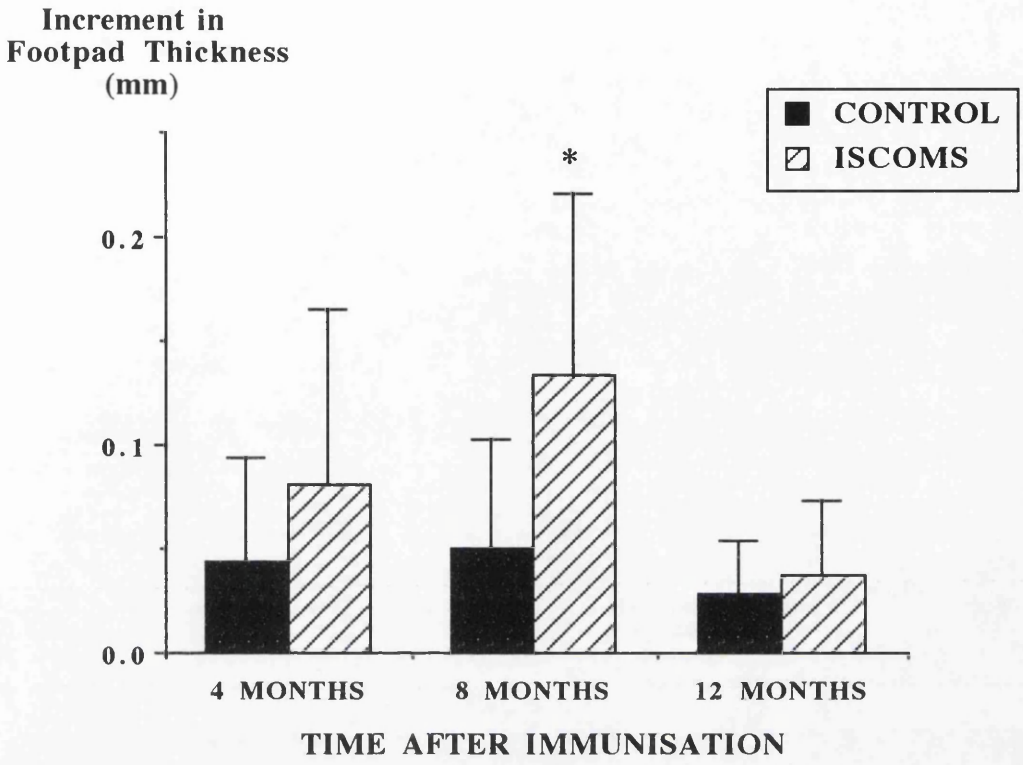

Figure 6.1.

Persistence of primary DTH responses after parenteral immunisation with ISCOMS. DTH responses were measured 24hr after challenge with 100μg heat-aggregated OVA of mice immunised at the timepoints indicated with 10μg ISCOMS-OVA or saline into the opposite rear footpad. The results shown are means ± 1 S.D. for six mice per group.

* $p<0.05$ v. Control.

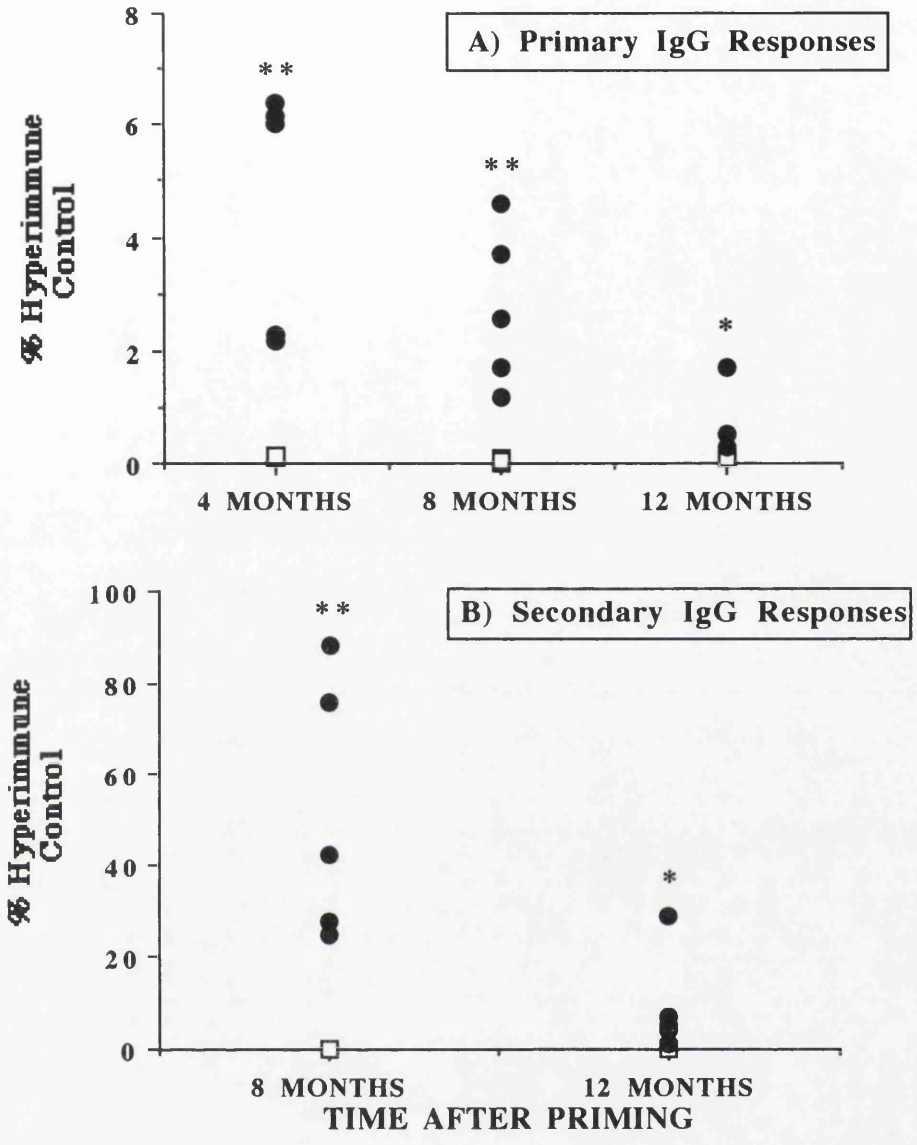

Figure 6.2.

ISCOMS prime long-term primary and secondary OVA-specific serum IgG responses. Primary IgG responses (A) were assayed at the timepoints indicated after immunisation with 10μg ISCOMS-OVA (filled circles) or saline (open squares) into the rear footpad. Secondary IgG responses (B) were assayed in the same mice one week after challenge with 100μg heat-aggregated OVA into the opposite rear footpad. Results from individual mice (six mice per group) are expressed as the % of hyperimmune standard. * $p<0.02$ v. Control.

** $p<0.02$ v. Control, 12 months.

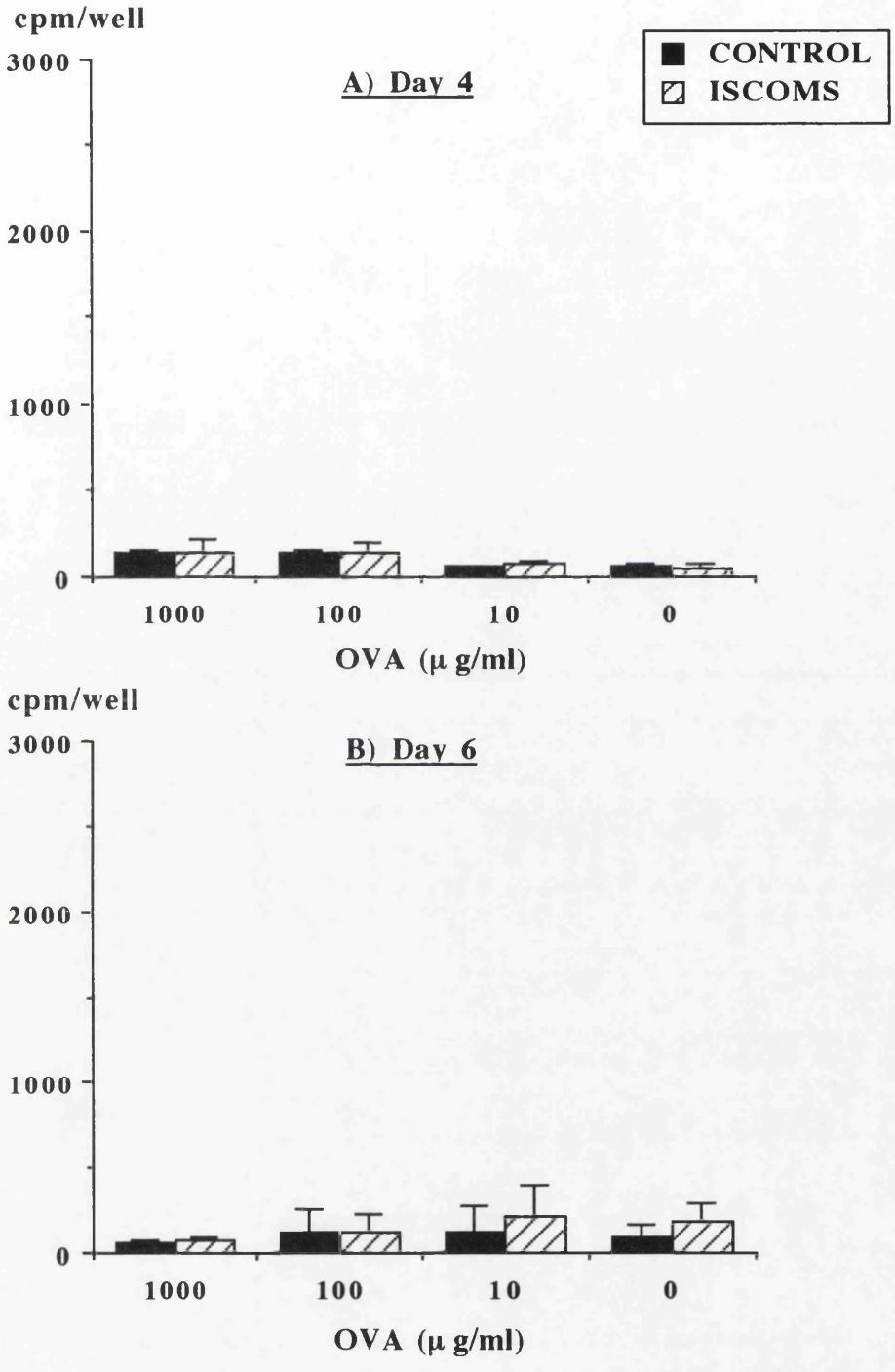

Figure 6.3.

Proliferative responses of popliteal lymph node cells isolated 4 months after immunisation with ISCOMS-OVA. Draining PLN cells were taken from mice primed with 10μg ISCOMS-OVA or saline s.c. and restimulated with OVA *in vitro*. The data represent mean cpm values ± 1S.D. from quadruplicate cultures harvested after 4 (A) or 6 days (B) of culture.

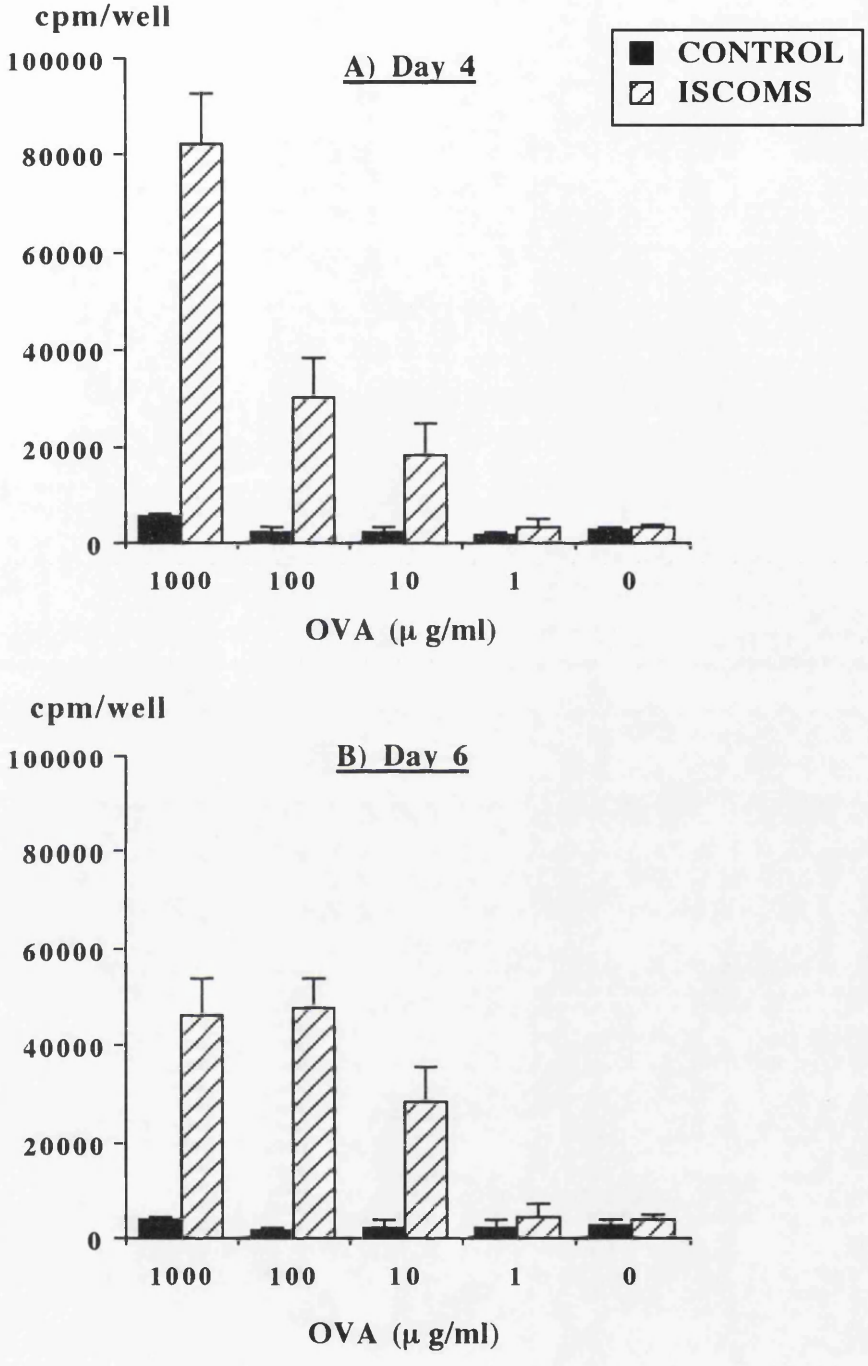

Figure 6.4.

Proliferative responses of popliteal lymph node cells isolated 8 months after immunisation with ISCOMS-OVA. Draining PLN cells were taken from mice primed with 10μg ISCOMS-OVA or saline s.c. and restimulated with OVA *in vitro*. The data represent mean cpm values ± 1S.D. from quadruplicate cultures harvested after 4 (A) or 6 days (B) of culture.

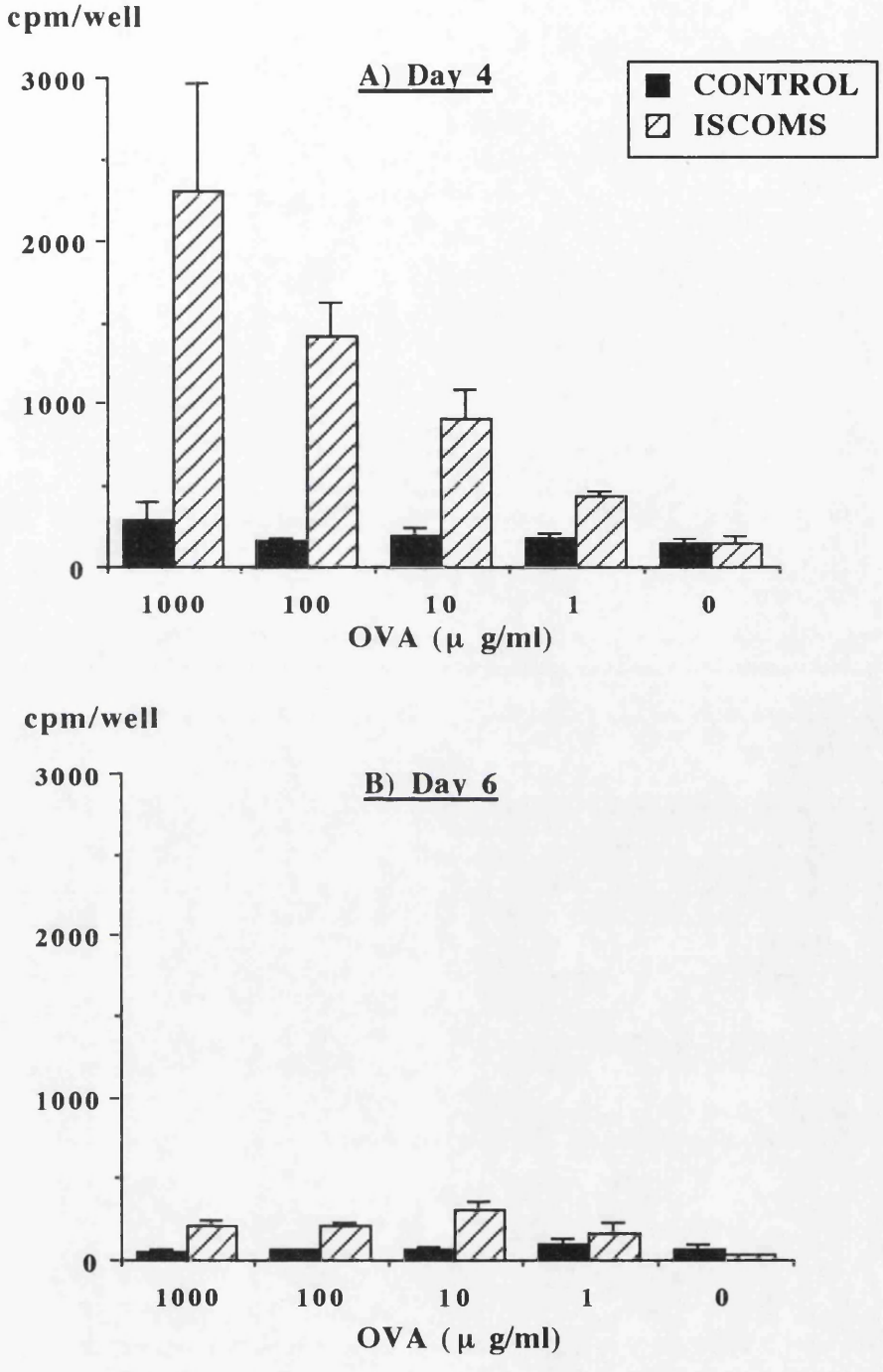

Figure 6.5.

Proliferative responses of popliteal lymph node cells isolated 12 months after immunisation with ISCOMS-OVA. Draining PLN cells were taken from mice primed with 10μg ISCOMS-OVA or saline s.c. and restimulated with OVA *in vitro*. The data represent mean cpm values ± 1S.D. from quadruplicate cultures harvested after 4 (A) or 6 days (B) of culture.

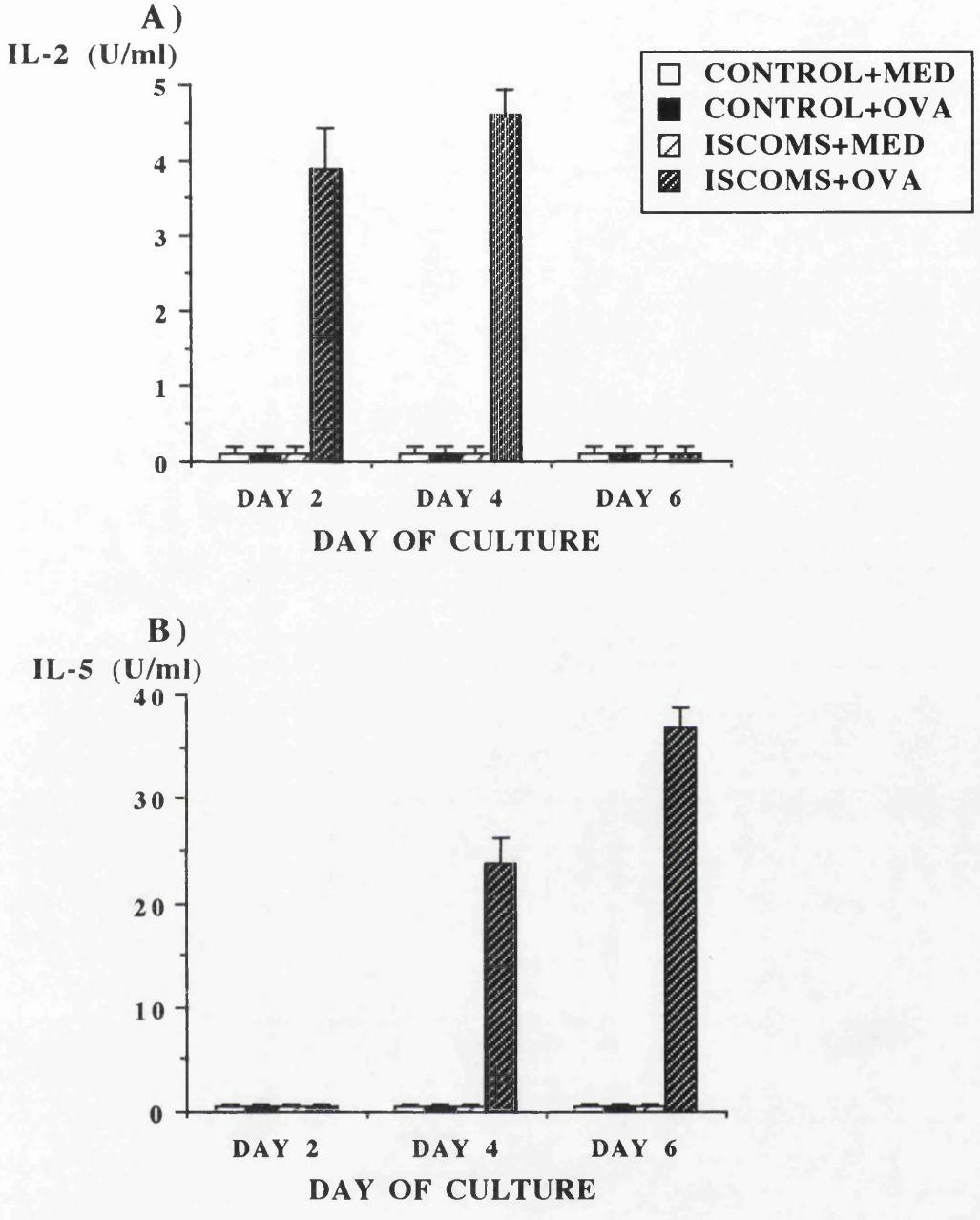

Figure 6.6.

Cytokine production by spleen cells isolated 4 months after immunisation with 10μg ISCOMS-OVA or saline s.c. Spleen cells were cultured for 2-6 days in the presence (+OVA) or absence (+MED) of 1mg/ml OVA and the supernatants assayed for the presence of IL-2 (A) and IL-5 (B) using sandwich ELISAs. The data represent mean cytokine concentrations ± 1S.D. of supernatants assayed in quadruplicate.

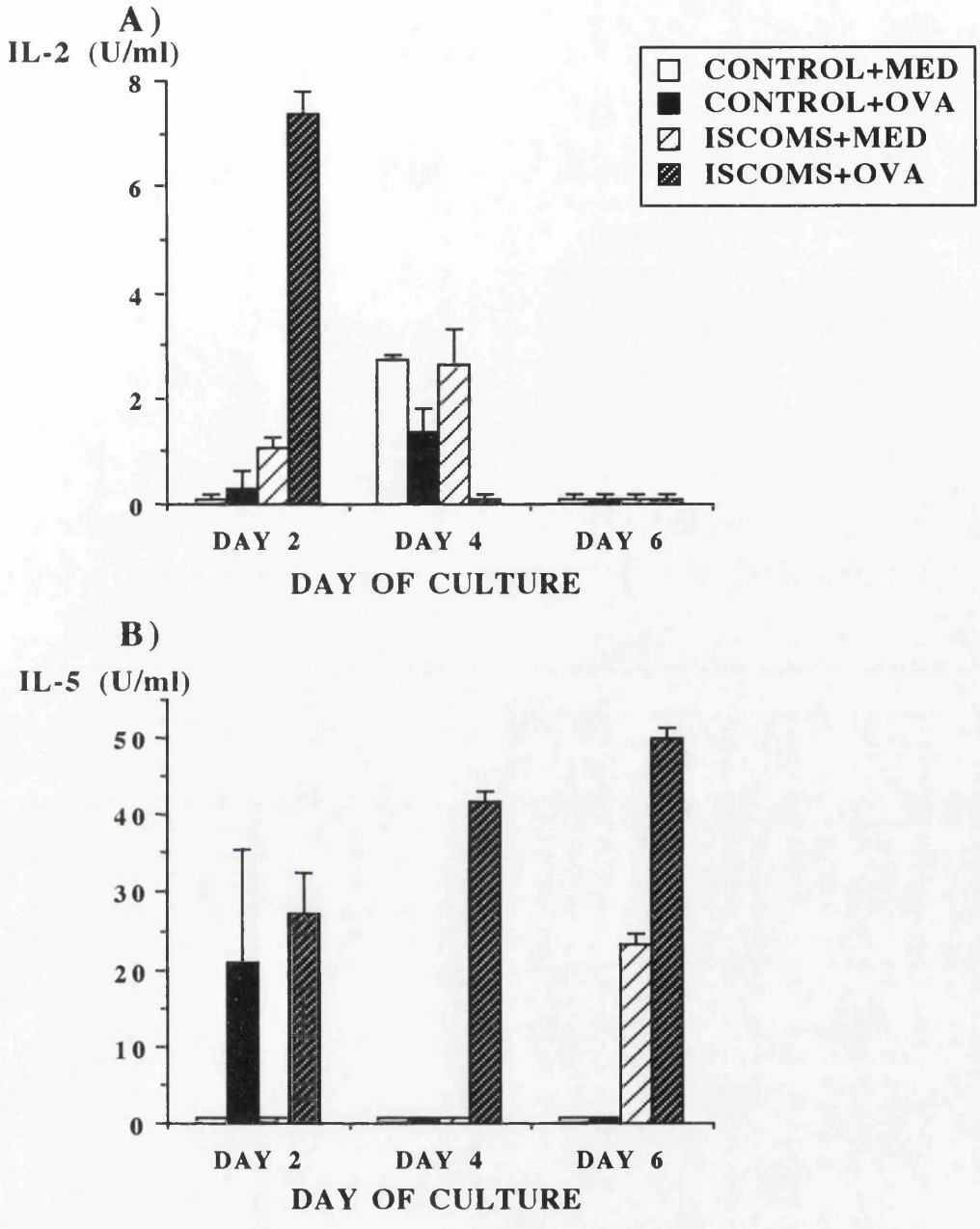

Figure 6.7.

Cytokine production by spleen cells isolated 8 months after immunisation with 10μg ISCOMS-OVA or saline s.c. Spleen cells were cultured for 2-6 days in the presence (+OVA) or absence (+MED) of 1mg/ml OVA and the supernatants assayed for the presence of IL-2 (A) and IL-5 (B) using sandwich ELISAs. The data represent mean cytokine concentrations ± 1S.D. of supernatants assayed in quadruplicate.

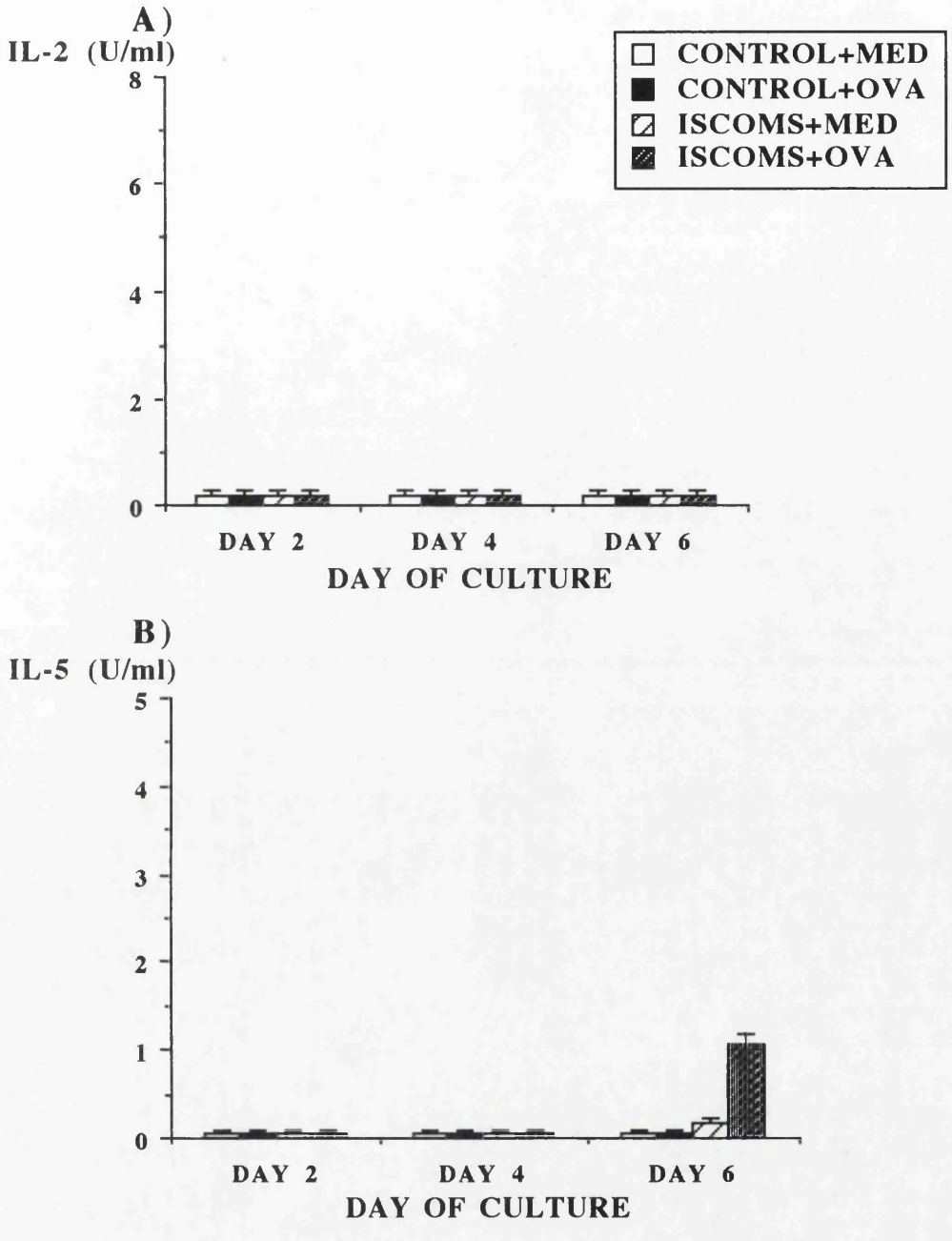

Figure 6.8.

Cytokine production by spleen cells isolated 12 months after immunisation with 10μg ISCOMS-OVA or saline s.c. Spleen cells were cultured for 2-6 days in the presence (+OVA) or absence (+MED) of 1mg/ml OVA and the supernatants assayed for the presence of IL-2 (A) and IL-5 (B) using sandwich ELISAs. The data represent mean cytokine concentrations ± 1S.D. of supernatants assayed in quadruplicate.

Table 6.1: Cytokine production by spleen cells isolated 4 months after parenteral immunisation with ISCOMS-OVA

i) IL-4 Production (U/ml):

CELLS	STIMULUS	Day 2	Day 4	Day 6
	Medium	32.5±11.2	39.9±6.5	36.5±11.0
Control:	Con A	115.2±5.0	85.2±5.2	47.1±8.8
	OVA	20.4±4.1	25.3±5.8	0.4±0.1
	Medium	34.3±9.9	42.3±4.7	21.9±11.4
ISCOMS:	Con A	63.7±17.2	50.2±14.0	47.7±8.1
	OVA	22.5±3.5	15.7±7.8	0.4±0.1

ii) IFN-γ Production (ng/ml):

CELLS	STIMULUS	Day 2	Day 4	Day 6
	Medium	0.1±0.1	0.1±0.1	0.1±0.1
Control:	Con A	>20.0	>20.0	>20.0
	OVA	0.1±01	0.1±0.1	0.1±0.1
	Medium	0.1±0.1	0.1±0.1	0.1±0.1
ISCOMS:	Con A	>20.0	>20.0	>20.0
	OVA	0.1±01	0.1±0.1	0.1±0.1

Spleen cells isolated 4 months after immunisation with 10μg ISCOMS-OVA or saline s.c. were cultured for 2-6 days and the supernatants assayed for the presence of IL-4 (A) and IFN-γ (B) using sandwich ELISAs. The data represent mean cytokine concentrations ± 1S.D. of supernatants assayed in quadruplicate.

Table 6.2: Cytokine production by spleen cells isolated 8 months after parenteral immunisation with ISCOMS-OVA

i) IL-4 Production (U/ml):

CELLS	STIMULUS	Day 2	Day 4	Day 6
	Medium	0.2±0.1	0.2±0.1	0.2±0.1
Control:	Con A	26.7±1.4	41.2±8.2	37.8±0.6
	OVA	0.2±0.1	0.2±0.1	0.2±0.1
	Medium	0.2±0.1	0.2±0.1	0.2±0.1
ISCOMS:	Con A	57.2±1.2	31.7±2.6	75.3±1.6
	OVA	0.2±0.1	0.2±0.1	0.2±0.1

ii) IFN-γ Production (ng/ml):

CELLS	STIMULUS	Day 2	Day 4	Day 6
	Medium	0.1±0.1	0.1±0.1	4.9±0.4
Control:	Con A	>20.0	>20.0	>20.0
	OVA	0.1±01	4.1±0.5	6.8±0.2
	Medium	0.1±0.1	0.1±0.1	4.7±0.3
ISCOMS:	Con A	>20.0	>20.0	>20.0
	OVA	0.1±01	6.2±0.3	6.4±0.3

Spleen cells isolated 8 months after immunisation with 10μg ISCOMS-OVA or saline s.c. were cultured for 2-6 days and the supernatants assayed for the presence of IL-4 (A) and IFN-γ (B) using sandwich ELISAs. The data represent mean cytokine concentrations ± 1S.D. of supernatants assayed in quadruplicate.

Table 6.3: Cytokine production by spleen cells isolated 12 months after parenteral immunisation with ISCOMS-OVA

i) IL-4 Production (U/ml):

CELLS	STIMULUS	Day 2	Day 4	Day 6
	Medium	0.2±0.1	0.2±0.1	0.2±0.1
Control:	Con A	0.2±0.1	19.6±2.0	15.4±1.6
	OVA	0.2±0.1	0.2±0.1	0.2±0.1
	Medium	0.2±0.1	0.2±0.1	0.2±0.1
ISCOMS:	Con A	0.2±0.1	17.4±2.3	20.2±1.3
	OVA	0.2±0.1	0.2±0.1	0.2±0.1

ii) IFN-γ Production (ng/ml):

CELLS	STIMULUS	Day 2	Day 4	Day 6
	Medium	0.1±0.1	0.1±0.1	0.1±0.1
Control:	Con A	17.6±0.6	18.7±0.7	17.3±0.6
	OVA	0.1±01	0.1±0.1	0.1±0.1
	Medium	0.1±0.1	0.1±0.1	0.1±0.1
ISCOMS:	Con A	13.7±0.7	13.5±0.4	11.8±0.5
	OVA	0.1±01	0.1±0.1	0.1±0.1

Spleen cells isolated 12 months after immunisation with 10μg ISCOMS-OVA or saline s.c. were cultured for 2-6 days and the supernatants assayed for the presence of IL-4 (A) and IFN-γ (B) using sandwich ELISAs. The data represent mean cytokine concentrations ± 1S.D. of supernatants assayed in quadruplicate.

CHAPTER 7: THE ROLE OF QUIL A IN THE ADJUVANTICITY OF ISCOMS

Introduction

The results presented in previous chapters have described some of the host factors involved in the unusual adjuvant effects of ISCOMS. I next went on to examine the properties of the ISCOMS themselves which contribute to their adjuvanticity.

The adjuvant properties of saponins have been known for some time (194) and it therefore seemed likely that Quil A played a crucial role in the activity of ISCOMS. Quil A itself enhances both humoral and cell-mediated immune responses when administered either parenterally or orally (194, 199, 200). However, a very narrow range of responses have been measured and there have been no comparative studies of Quil A and intact ISCOMS. Thus, the exact contribution of Quil A to the actions of ISCOMS is unknown.

In this chapter, I therefore compared directly the adjuvant effects of Quil A and ISCOMS on the range of local and systemic OVA-specific immune responses described previously. In addition, I examined the effects of Quil A on aspects of innate immunity, which may be important in the induction of cell-mediated immune responses. These were the ability to activate macrophages (Mϕ) and NK cell activity.

Experimental Protocol

Systemic IgG, DTH, proliferative and cytokine responses were measured in BALB/c mice which had been immunised s.c. with 10μg OVA, either as a soluble protein mixed with free Quil A, or incorporated in ISCOMS. Primary antibody responses were measured 7 days after priming and mice were challenged with 100μg of heat-aggregated OVA on day14 post-priming. Systemic DTH responses were measured 48hr later and secondary IgG responses and *in vitro* proliferation and cytokine production assayed one week later.

Splenic CTL responses were assayed in C57Bl/6 mice immunised i.p. with 10μg OVA mixed with free Quil A or incorporated in ISCOMS.

Intestinal IgA reponses were assayed in BALB/c mice which received 6 feeds of 50μg OVA mixed with Quil A or incorporated in ISCOMS. The ability of oral immunisation to induce systemic CTL activity was measured using spleen cells from C57Bl/6 mice which had been fed 6 times with 50μg OVA plus Quil A or in ISCOMS.

To assess the effects of ISCOMS and Quil A on non-specific immunity, BALB/c mice were injected i.p. with Quil A or ISCOMS, together with 5-10μg OVA. Splenic NK cell activity was measured using a standard ^{51}Cr release assay with the YAC-1 target cell line, while macrophage activation was measured in peritoneal exudate cells by assessing respiratory enzyme burst activity using a chemiluminescence assay (213).

In all experiments control mice received identical priming doses of either soluble OVA, free Quil A or saline only.

Results

A) Parenteral Immunisation With Quil A

i) Parenteral Administration of OVA and Quil A Primes for Antigen-Specific IgG and DTH Responses

My first experiments compared the adjuvant activity of free Quil A and Quil A incorporated in ISCOMS for parenteral immunisation with OVA.

Although virtually no primary IgG antibody response was observed in the serum of BALB/c mice immunised s.c. with either OVA mixed with 1-20μg Quil A or ISCOMS-OVA 7 days after immunisation, both Quil A and ISCOMS primed mice to develop secondary OVA-specific IgG responses when challenged with heat-aggregated OVA (Fig. 7.1). The secondary IgG responses in mice immunised with OVA and free Quil A were dependent on the dose of Quil A given, with significant responses only occurring when 10-20μg Quil A was used. Although these optimal responses were comparable to the secondary IgG responses obtained after immunisation with ISCOMS-OVA, lower doses of Quil A, including that equivalent to the amount given in ISCOMS (1μg Quil A), did not prime mice to develop significant secondary IgG responses (Fig. 7.1).

Both free Quil A and ISCOMS also induced significant primary DTH responses and again the responses elicited by free Quil A were dependent on the dose used (Fig. 7.2). The highest dose of Quil A (20μg) provoked very potent primary DTH responses, which were greater than those elicited by the same amount of OVA in ISCOMS (Fig. 7.2). Mice receiving 5-10μg free Quil A had similar DTH

responses to the ISCOMS primed group, but mice primed with 1μg Quil A, the amount present in the ISCOMS, did not mount any significant DTH responses (Fig. 7.2).

Thus, a single parenteral immunisation of antigen with Quil A primes both humoral and cell-mediated immunity in a dose-dependent manner, but Quil A is more efficient when incorporated within ISCOMS.

ii) Priming of Antigen-Specific Proliferative Responses by Parenteral Immunisation with Quil A

I next examined the ability of free Quil A to prime for antigen-specific proliferative responses *in vitro*.

Popliteal lymph node cells from BALB/c mice primed with either OVA plus 1-20μg Quil A or ISCOMS-OVA exhibited marked proliferative responses when restimulated with 100-1000μg/ml soluble OVA *in vitro* (Fig. 7.3). In contrast to the responses *in vivo*, the level of the proliferative response induced by free Quil A did not show a clear dependence on the dose of Quil A used. In fact the highest dose of Quil A (20μg) induced the poorest proliferative responses (Fig. 7.3). In addition, even the lower doses of Quil A, including the equivalent of that contained in ISCOMS (1μg), elicited proliferative responses similar to those primed by ISCOMS themselves (Fig. 7.3).

Thus, free Quil A is able to prime local antigen-specific proliferative T-cell responses, with equivalent efficacy to those induced by ISCOMS.

iii) Induction of Antigen-Specific Cytokine Production by Parenteral Immunisation with Quil A

As the proliferative responses suggested that Quil A primed antigen-specific T cell responses very efficiently, I examined the pattern of cytokines produced by spleen cells isolated from mice primed with different amounts of free Quil A and OVA.

a) IL-2

Spleen cells isolated from BALB/c mice 14 days after footpad immunisation with OVA and Quil A or in ISCOMS produced marked amounts of IL-2 when restimulated *in vitro* with soluble OVA (Fig. 7.4). Spleen cells from control mice did not produce any IL-2 in the presence of OVA and immunised cells produced no IL-2 when cultured without OVA (Fig. 7.4). Consistent with my previous observations in chapter 5, the IL-2 responses were transient, being detected only after 2 days of culture and not at 6 days (Fig. 7.4). In contrast to the proliferative responses, the IL-2 responses induced by all doses of free Quil A were lower than those of the ISCOMS primed cells. The amount of IL-2 produced was directly dependent on the priming dose of free Quil A, with the spleen cells from mice primed with the dose present in ISCOMS (1μg) producing very little IL-2 in response to OVA *in vitro* (Fig. 7.4).

b) IFN-γ

In addition to IL-2, spleen cells from mice primed with OVA plus free Quil A also produced large amounts of IFN-γ when restimulated with OVA *in vitro* (Fig. 7.5). These responses were first detectable after 2 days of culture, but persisted until day 6. In contrast to the IL-2 response, there did not appear to be any clear

correlation between the amount of IFN-γ produced and the dose of Quil A used for priming. In general however, none of the doses of free Quil A induced equivalent levels of IFN-γ to those produced by ISCOMS primed cells, especially early in culture (Fig. 7.5).

In these studies, Quil A or ISCOMS primed spleen cells also produced significant amounts of IFN-γ when cultured for 6 days in the absence of OVA (Fig. 7.5B), although these responses were markedly lower than those found after restimulation with OVA.

c) IL-4

In agreement with my earlier observations, little or no IL-4 was produced by Quil A or ISCOMS primed spleen cells restimulated with OVA *in vitro* (Table 7.1). However, all groups produced similar amounts of this cytokine after restimulation with Con A (Table 7.1).

d) IL-5

Spleen cells from mice primed with OVA plus free Quil A produced IL-5 in response to restimulation with OVA (Fig. 7.6). ISCOMS primed spleen cells generally produced the highest levels of IL-5 and these showed a similar pattern to the responses described in chapter 5, with increasing amounts of IL-5 detected as the time of culture increased (Fig. 7.6). In the Quil A primed groups, the amount of IL-5 produced was dependent on the dose of Quil A, with cells from mice immunised with 20μg Quil A producing the most IL-5 and those from mice which received 1μg Quil A making very little IL-5 upon restimulation with OVA *in vitro* (Fig 7.6).

These results demonstrate that free Quil A is capable of priming the same range of antigen-specific T_h1 and T_h2 cytokine

responses as intact ISCOMS, but incorporation of Quil A into ISCOMS increases the efficiency of these responses.

iv) Induction of Antigen-Specific CTL Responses by Parenteral Immunisation with Quil A

The above findings suggested that free Quil A was capable of priming CD4+ T cell dependent responses to co-administered protein antigen. I next compared the ability of free Quil A and Quil A in ISCOMS to induce OVA-specific CTL responses, as this is one of the most novel adjuvant properties of ISCOMS and has important implications for the development of vaccine vectors.

Spleen cells from mice primed with OVA either with 1-20μg free Quil A or incorporated within ISCOMS (containing 1μg Quil A) i.p. 7 days before and restimulated with EG7.OVA cells *in vitro* exhibited potent OVA-specific CTL activity (Fig. 7.7). The priming of these responses by free Quil A was dose-dependent, with 20μg Quil A producing the highest levels of OVA-specific CTL activity and 10μg Quil A inducing CTL responses comparable in magnitude to those elicited by ISCOMS-OVA (Fig. 7.7). There was a slight decrease in the OVA-specific CTL activity in mice primed with 5μg free Quil A, but when only 1μg was used, the equivalent dose of Quil A contained in ISCOMS, much less CTL activity was observed (Fig. 7.7).

Unexpectedly, cells from mice given free Quil A and OVA also showed some non-specific cytotoxicity, as evidenced by the lysis of the the non-OVA expressing EL4 cell line (Fig. 7.7B). This non-specific cytotoxicity was present even with the lowest dose of free Quil A (1μg), but was not observed using ISCOMS primed spleen cells, confirming my previous findings in chapter 4. Thus, the OVA-specific cytotoxic response induced by free Quil A is considerably

less than that induced by ISCOMS. This ability of free Quil A to induce non-specific cytotoxicity is discussed further below.

These results show that free Quil A can prime antigen-specific CTL responses when administered parenterally, but this is of lower efficiency and specificity when compared with ISCOMS.

B) Oral Immunisation With Quil A

i) Induction of Intestinal IgA and Systemic IgG Responses by Feeding OVA and Quil A

I next examined whether orally administered Quil A could stimulate the primary intestinal IgA and systemic IgG responses found in mice fed ISCOMS. Mice fed six times with 50μg OVA and free Quil A had significant OVA-specific IgA antibodies in intestinal secretions and again the magnitude of the response was dependent on the dose of Quil A used (Fig. 7.8). High doses of Quil A (20-40μg) induced potent OVA-specific IgA responses, while lower doses of Quil A, including that equivalent to the amount given in ISCOMS (5μg), were unable to prime significant IgA production (Fig. 7.8).

A similar pattern of OVA-specific IgG production was observed in the serum of mice fed Quil A plus OVA (Fig. 7.9). The responses were again dose-dependent, with mice fed with 20-40μg Quil A having equivalent OVA-specific IgG responses to those fed ISCOMS, while lower doses of Quil A (5-10μg) failed to prime significant primary IgG responses (Fig. 7.9).

Thus, Quil A is capable of inducing both local and systemic antigen-specific antibody responses when administered orally, but does so more efficiently when incorporated within ISCOMS.

ii) Induction of Antigen-Specific CTL following Oral Immunisation with Quil A

As Quil A was capable of adjuvant activity when administered orally and ISCOMS can prime CTL responses by this route, I compared the ability of Quil A and ISCOMS to induce antigen-specific CTL responses in mice fed OVA.

As I have described in previous chapters, mice fed 50μg ISCOMS-OVA on 6 occasions over a 10 day period developed high levels of OVA-specific CTL in the spleen (Fig. 7.10). Mice fed the same amount of OVA with free Quil A also generated strong OVA-specific CTL responses (Fig. 7.10A). However, as with systemic immunisation, the CTL responses primed by oral administration of Quil A and OVA were highly dose-dependent, with doses in excess of 20μg Quil A necessary for optimal priming. The 5μg Quil A dose, equivalent to that found in ISCOMS-OVA, elicited only low levels of OVA-specific systemic CTL (Fig. 7.10A).

As found after parenteral immunisation, there was also a significant non-specific cytotoxic component to the responses induced by free Quil A, as evidenced by the lysis of non-OVA expressing EL4 (Fig. 7.10B). This non-specific cytotoxicity was not observed following immunisation with ISCOMS-OVA and again appeared to be associated with the presence of free Quil A (Fig. 7.10B).

Thus, Quil A is able to prime potent CTL responses when administered orally, but again, ISCOMS induce more efficient and specific responses by this route.

C) Activation of Innate Immunity by Quil A

The results described above indicate that both Quil A and ISCOMS are potent adjuvants, especially for T cell dependent immunity. One factor which has been suggested to be involved in the activity of many adjuvants is the stimulation of non-specific inflammatory mechanisms important in the initial stages of activation of antigen-specific T lymphocytes (231, 232). I examined this by investigating the ability of ISCOMS and Quil A to activate macrophages and natural killer (NK) cells.

i) Chemiluminescence of Peritoneal Macrophages Induced by Quil A

To examine macrophage activation, I employed a chemiluminescence assay that can be used to discriminate between macrophage populations which have been elicited by an inflammatory or immunological stimulus, the latter being characterised by their enhanced oxidative metabolic capacity (213).

In this assay, peritoneal exudate cells (PEC) were obtained from BALB/c mice which had been injected i.p. with 10μg OVA plus 1μg Quil A or incorporated in ISCOMS-OVA, 4 days before. Adherent Mϕ were then stimulated with PMA in the presence of luminol and oxidative metabolism measured in a luminometer (213). Heat-killed *Corynebacterium parvum* was used as a stimulus which induces immunologically activated Mϕ, while thioglycollate broth was used as an example of a non-specific inflammatory agent (213).

As shown in Figure 7.11, PEC elicited by *C. parvum* exhibited a rapid oxidative burst after stimulation with PMA, which peaked after 3-4 mins and gradually declined thereafter. In contrast,

thioglycollate induced PEC did not make any significant oxidative response upon stimulation with PMA, confirming the entirely inflammatory nature of these cells (Fig. 7.11). PEC from ISCOMS-OVA primed mice produced a small, but marked, oxidative burst in response to PMA stimulation, although this was of much lower magnitude than the response of the *C. parvum* elicited cells (Fig. 7.11). Similarly, PEC primed by Quil A plus OVA also mounted a small oxidative response after PMA stimulation, which was only slightly lower than that obtained from ISCOMS primed cells (Fig. 7.11).

These results suggest that ISCOMS and free Quil A may activate Mϕ in an immunological manner, albeit to a lesser degree than strong immunological stimuli such as heat-killed *C.parvum.*

ii) Enhancement of Natural Killer Cell Activity by Quil A

As my previous results showed that parenteral immunisation with free Quil A, but not ISCOMS, induced non-specific cytotoxicity in the spleen, I investigated whether natural killer (NK) cell activity was enhanced by Quil A.

Spleen cells isolated one day after injection of 1-20μg free Quil A showed a small but consistent enhancement of NK cell activity against YAC-1 cells, compared with control cells (Fig. 7.12A). This augmentation was dependent on the dose of Quil A adminstered, with 20μg Quil A producing the greatest increase and 1μg Quil A having very little effect on NK activity (Fig 7.12A).

The enhancement of NK activity was more pronounced when spleen cells were isolated 3 days after injection and again directly correlated with the dose of Quil A administered (Fig. 7.12B). The increase in NK activity appeared to be a transient phenomenon

however, as spleen cells isolated 7 days after injection did not exhibit enhanced NK cell activity, compared with controls (Fig. 7.12C).

These results demonstrate that free Quil A induces a dose-dependent but transient increase in splenic NK cell activity.

Conclusions

The results in this chapter demonstrate that Quil A acts as an adjuvant for the same range of humoral and cell-mediated immune responses induced by oral or parenteral immunisation with ISCOMS. However, by directly comparing equivalent doses of free Quil A and Quil A in ISCOMS, I have been able to show that free Quil A is less efficient than the intact vector.

Parenteral immunisation with OVA and Quil A primed systemic antigen-specific DTH , CTL and IgG antibody responses *in vivo*, as well as T cell proliferative activity and T_h1 and T_h2-type cytokine production *in vitro*. In addition, oral immunisation with OVA and Quil A induced local antigen-specific intestinal IgA production, together with systemic IgG and CTL responses. These findings indicate that, like intact ISCOMS, free Quil A allows antigen to gain access to both the exogenous and endogenous pathways of antigen processing and presentation *in vivo*. In addition to its effects on the presentation of exogenous protein antigen to specific lymphocytes, Quil A also had marked effects on innate immune responses including the activation of $M\phi$ and NK cells, as well as the non-specific stimulation of regulatory cytokines such as IFN-γ.

In summary, my results indicate that the oral and parenteral adjuvant properties of ISCOMS are shared by Quil A itself. However,

my findings that equivalent doses of Quil A were generally much more efficient when incorporated into ISCOMS, show that additional factors must be important for the potent adjuvanticity of intact ISCOMS.

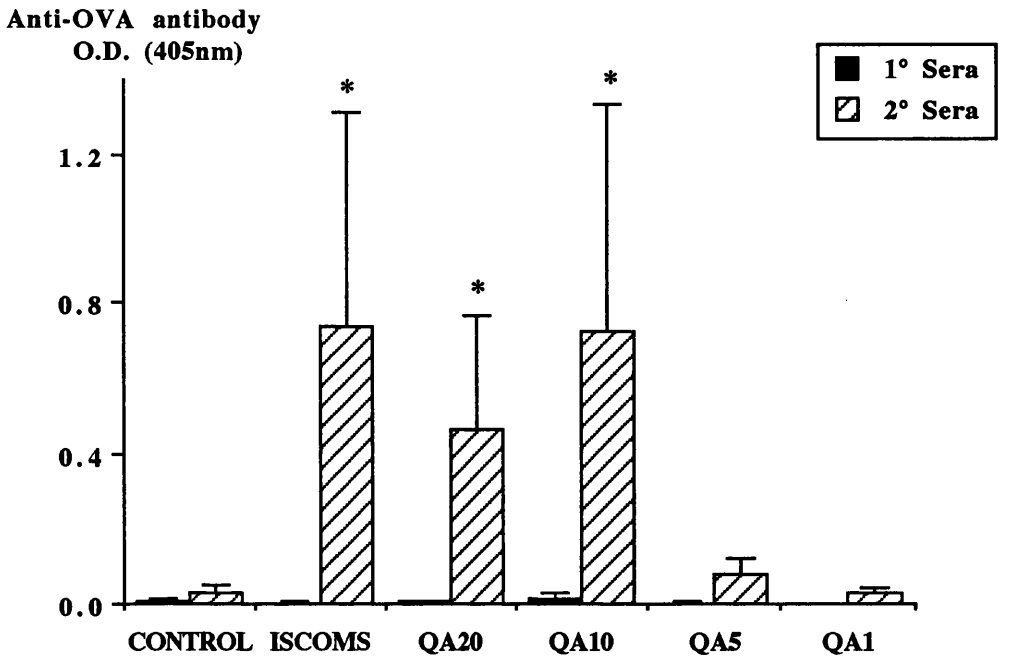

Figure 7.1.

Priming of OVA-specific serum IgG responses by parenteral immunisation with Quil A and OVA. Primary IgG responses were measured 14 days after immunisation with 10μg OVA plus 1-20μg Quil A (QA), 10μg ISCOMS-OVA or saline only s.c., while secondary IgG responses were assayed one week after footpad challenge of primed mice with 100μg heat-aggregated OVA. The data represent mean O.D. (405nm) values ± 1 S.D. from six mice per group.

* $p<0.05$ v. Control.

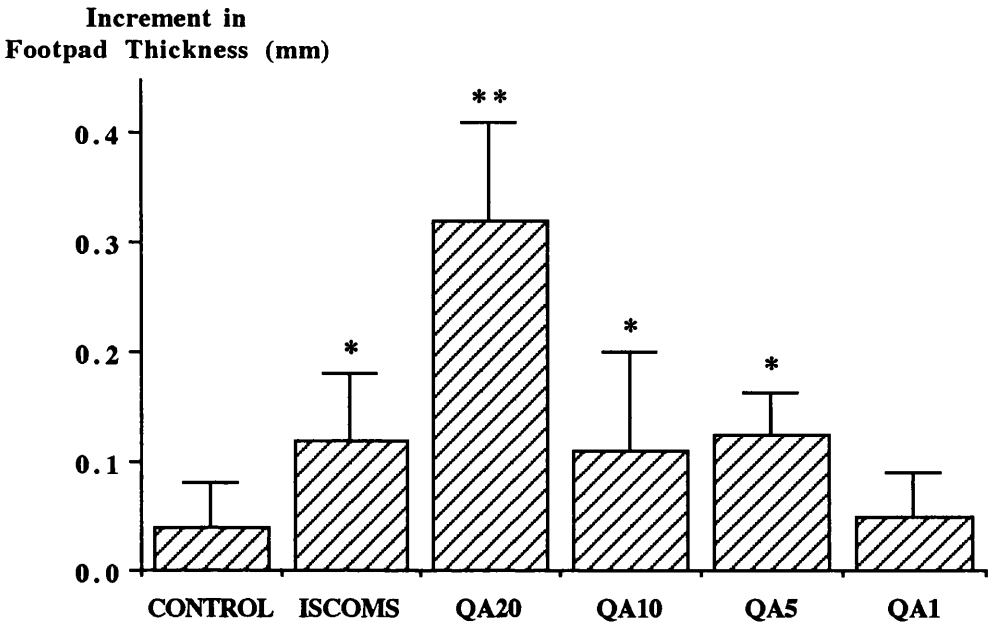

Figure 7.2.

Priming of OVA-specific DTH responses by parenteral immunisation with Quil A and OVA. DTH responses were measured 24hr after challenge with 100μg heat-aggregated OVA in mice immunised 14 days previously with 10μg OVA plus 1-20μg Quil A (QA), 10μg ISCOMS-OVA or saline only s.c. Results shown are means ± 1S.D. for six mice per group.

* $p<0.05$ v. Control.

** $p<0.002$ v. Control and QA1; $p<0.02$ v. all other groups.

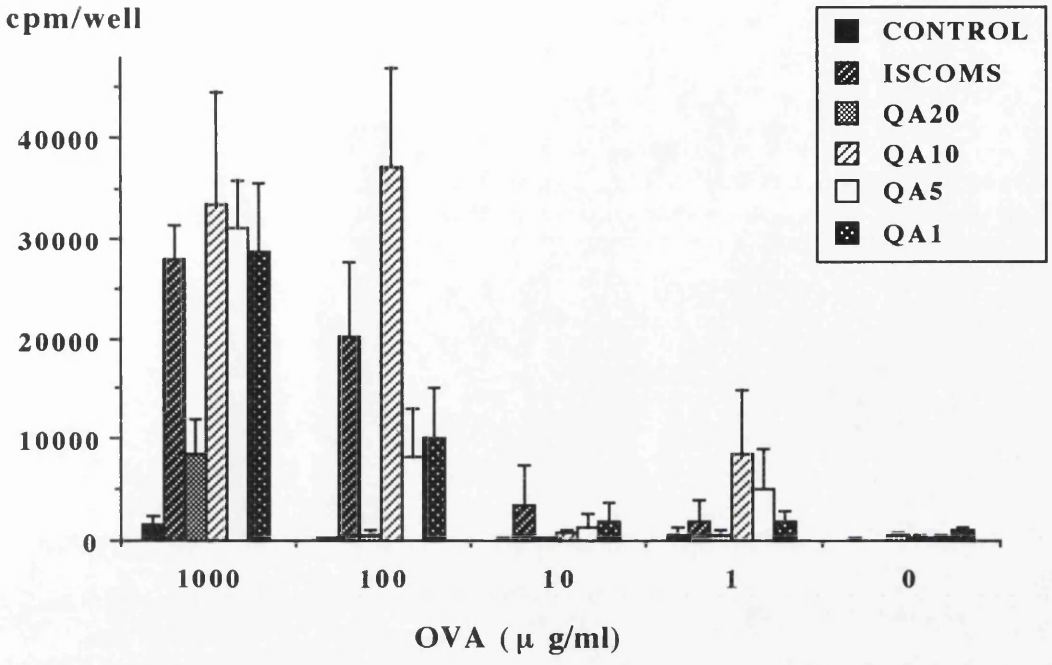

Figure 7.3.

Priming of antigen-specific proliferative responses by parenteral immunisation with Quil A and OVA. Proliferative responses of draining popliteal lymph node cells isolated 14 days after immunisation with 10μg OVA plus 1-20μg Quil A (QA), 10μg ISCOMS-OVA or saline only s.c., were assayed to stimulation with OVA *in vitro*. The data represent mean cpm values ± 1S.D. from quadruplicate cultures harvested after 6 days of culture.

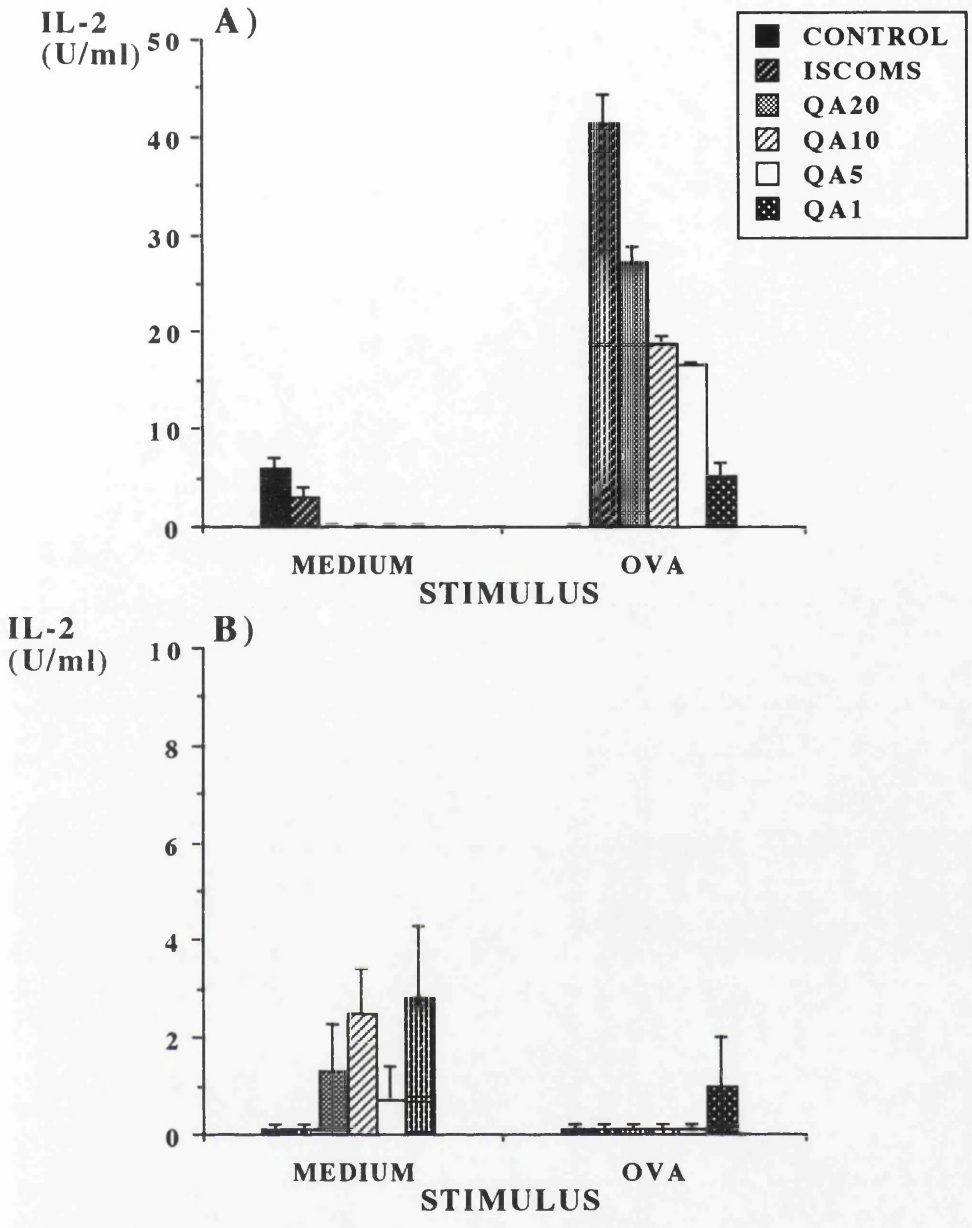

Figure 7.4.

IL-2 production by spleen cells from mice primed with Quil A and OVA. Spleen cells taken 14 days after immunisation with 10μg OVA plus 1-20μg Quil A (QA), 10μg ISCOMS-OVA or saline only s.c., were cultured for 2 days (A) or 6 days (B) in the presence or absence of 1mg/ml OVA and the supernatants assayed for IL-2 by ELISA. The data represent mean cytokine concentrations ± 1S.D. of supernatants assayed in quadruplicate.

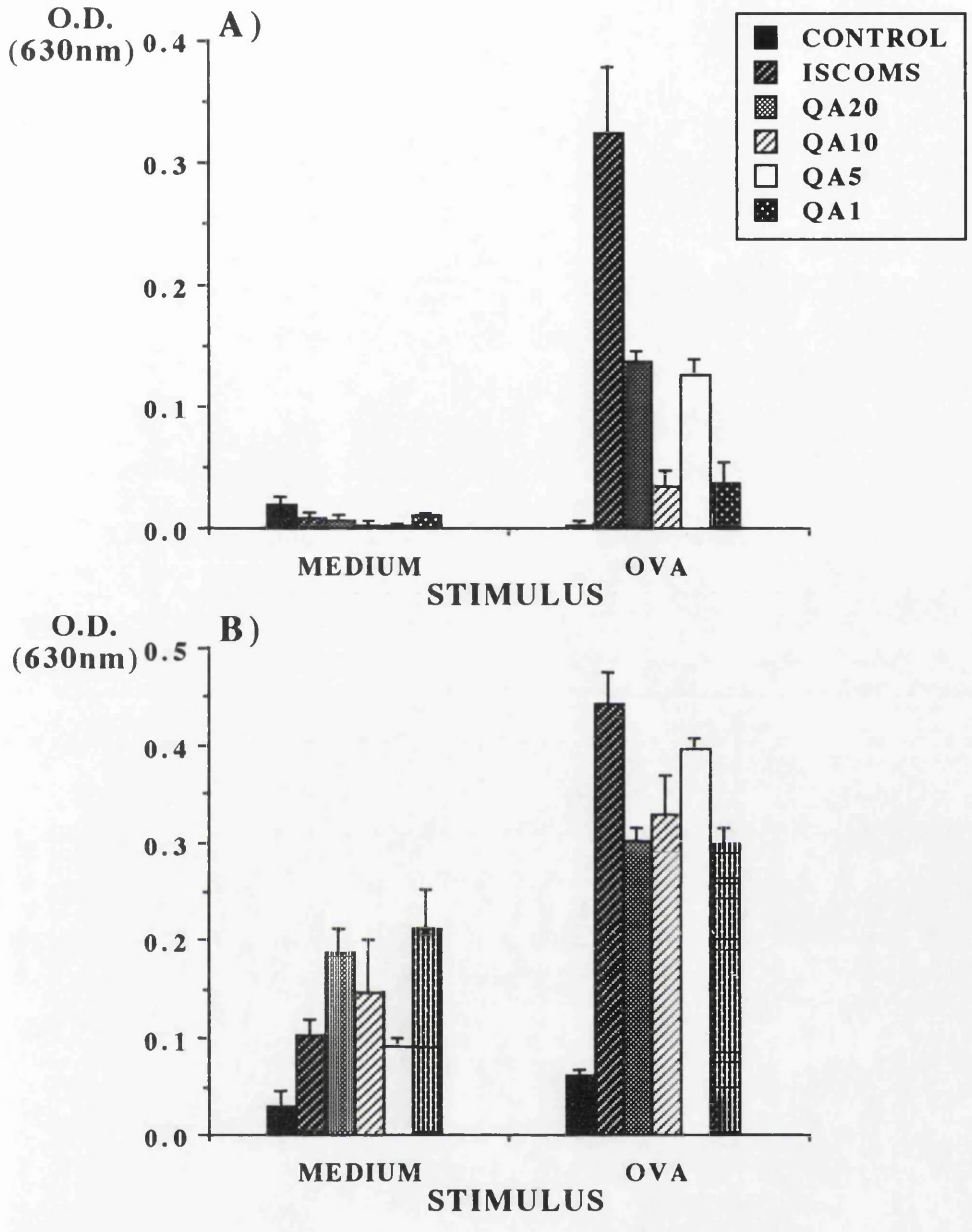

Figure 7.5.

IFN-γ production by spleen cells from mice primed with Quil A and OVA. Spleen cells taken 14 days after immunisation with 10μg OVA plus 1-20μg Quil A (QA), 10μg ISCOMS-OVA or saline only s.c., were cultured for 2 days (A) or 6 days (B) in the presence or absence of 1mg/ml OVA and the supernatants assayed for IFN-γ by ELISA. The data represent mean O.D. 630nm values ± 1S.D. of supernatants assayed in quadruplicate.

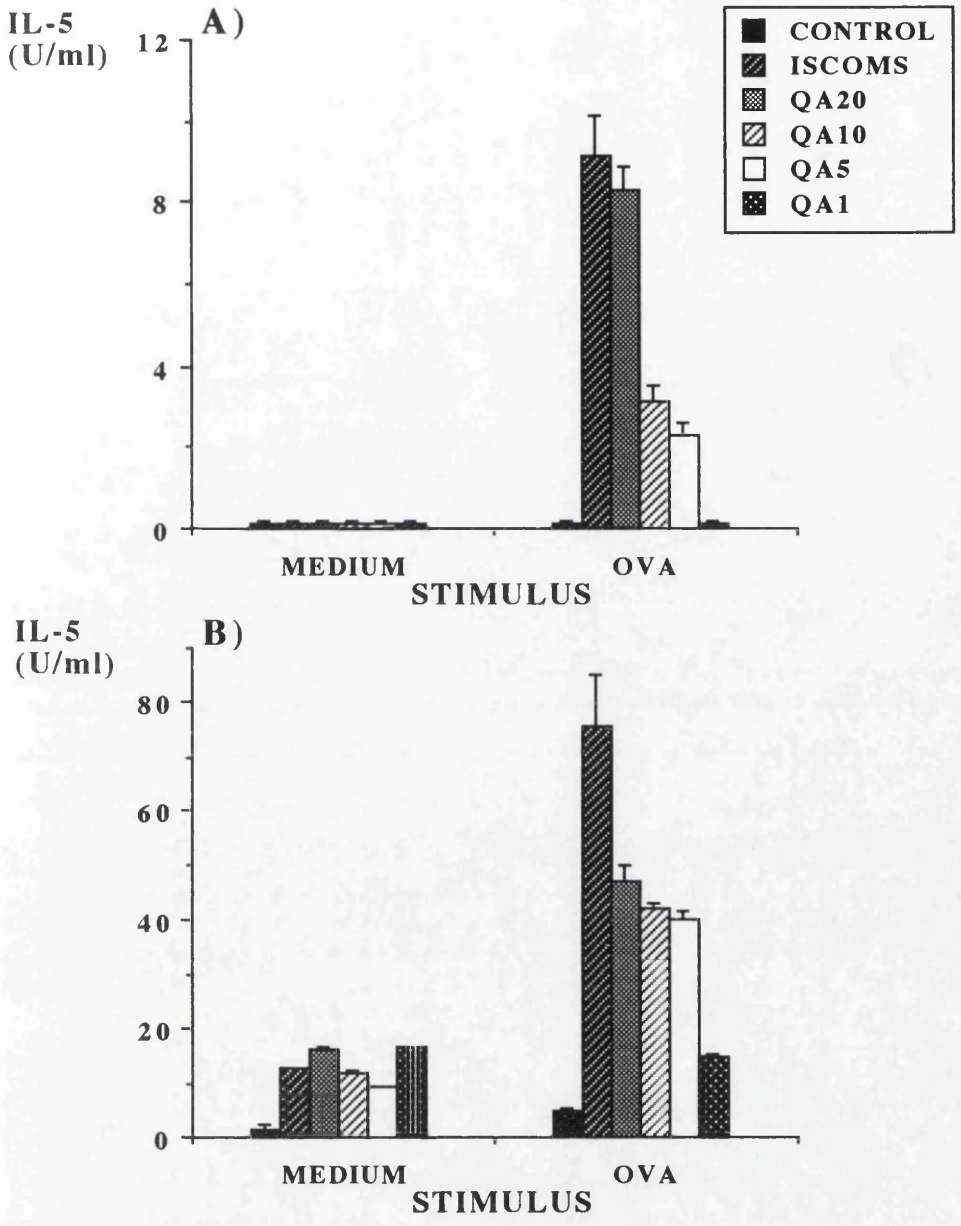

Figure 7.6.

IL-5 production by spleen cells from mice primed with Quil A and OVA. Spleen cells taken 14 days after immunisation with 10μg OVA plus 1-20μg Quil A (QA), 10μg ISCOMS-OVA or saline only s.c., were cultured for 2 days (A) or 6 days (B) in the presence or absence of 1mg/ml OVA and the supernatants assayed for IL-5 by ELISA. The data represent mean cytokine concentrations ± 1S.D. of supernatants assayed in quadruplicate.

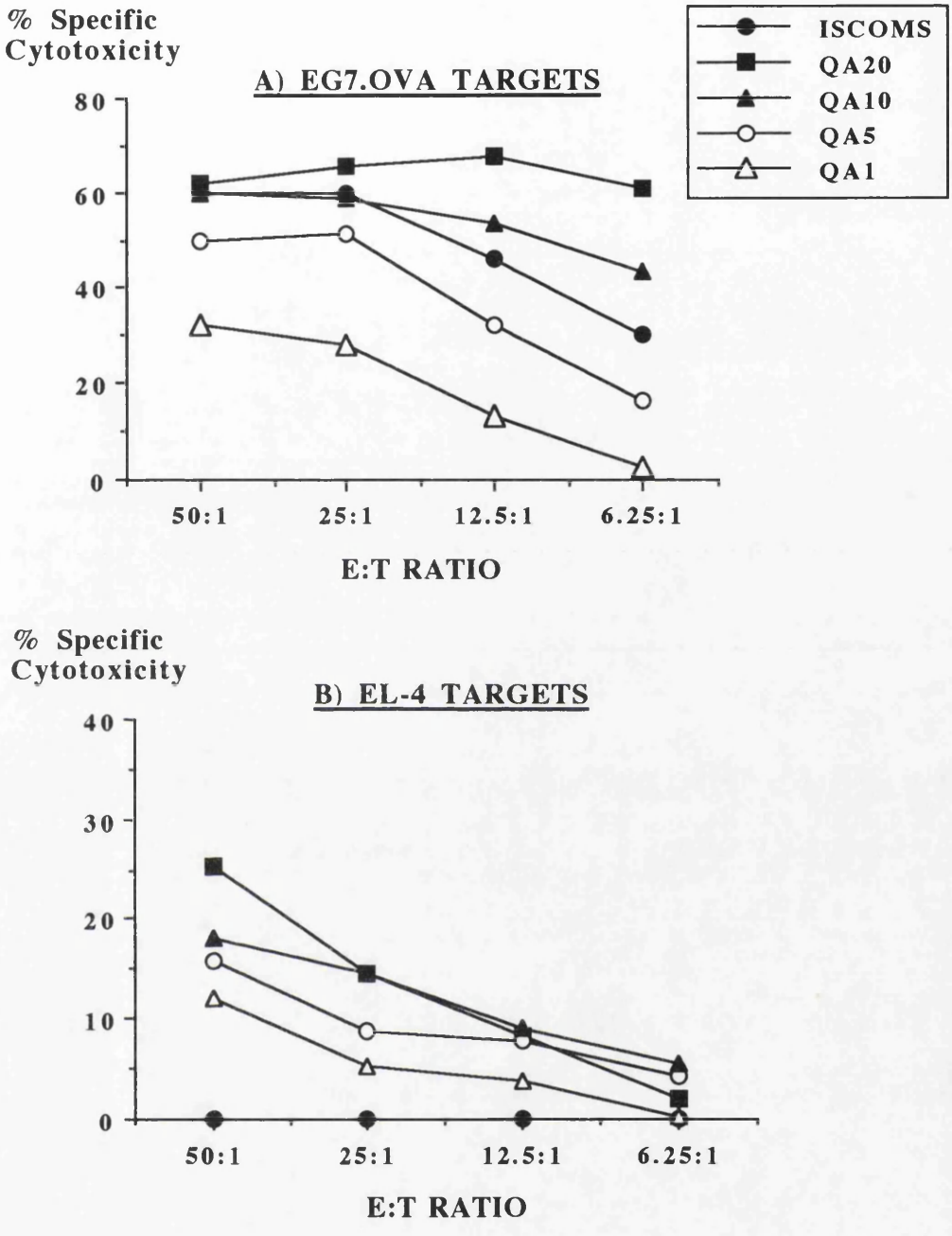

Figure 7.7.

Induction of OVA-specific CTL responses by parenteral immunisation with Quil A and OVA. Splenic CTL activity was assayed in C57Bl/6 mice immunised 7 days before with 10μg OVA plus 1-20μg Quil A (QA), 10μg ISCOMS-OVA or saline only i.p., after restimulation *in vitro* for 5 days with EG7.OVA cells. Results shown are percent specific cytotoxicity using spleen cells pooled from four mice per group.

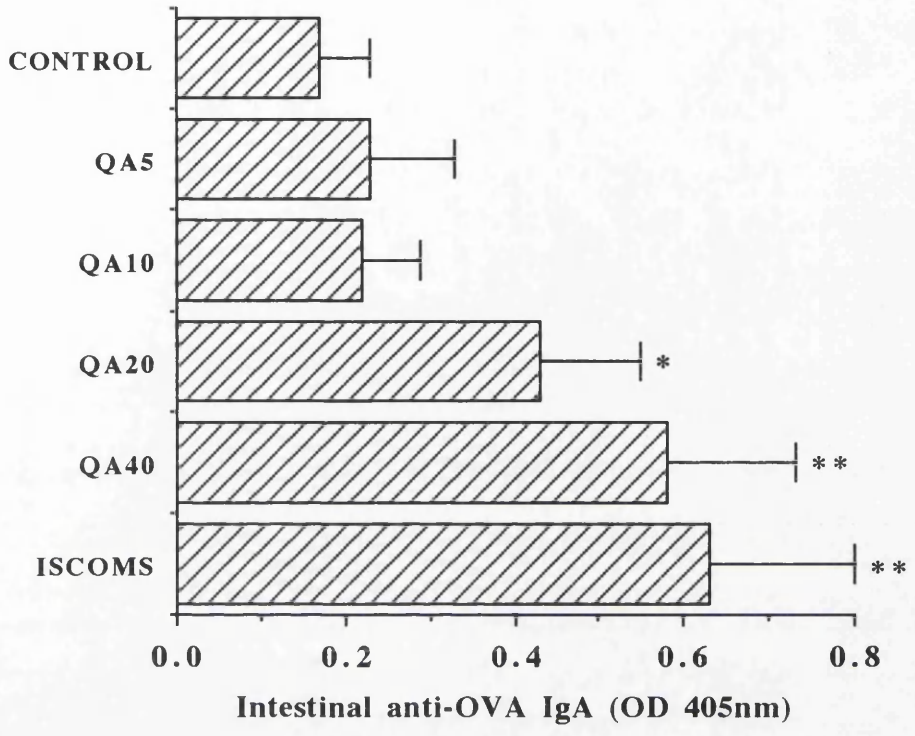

Figure 7.8.

Induction of OVA-specific intestinal IgA responses by oral immunisation with Quil A and OVA. Intestinal washes were obtained from BALB/c mice 7 days after the last of six feeds of 50μg OVA plus 5-40μg Quil A (QA), 50μg ISCOMS-OVA or saline only. Results shown represent mean O.D. 405nm values ± 1S.D. from six mice per group.

*$p<0.05$ v. CONTROL.

**$p<0.02$ v. CONTROL.

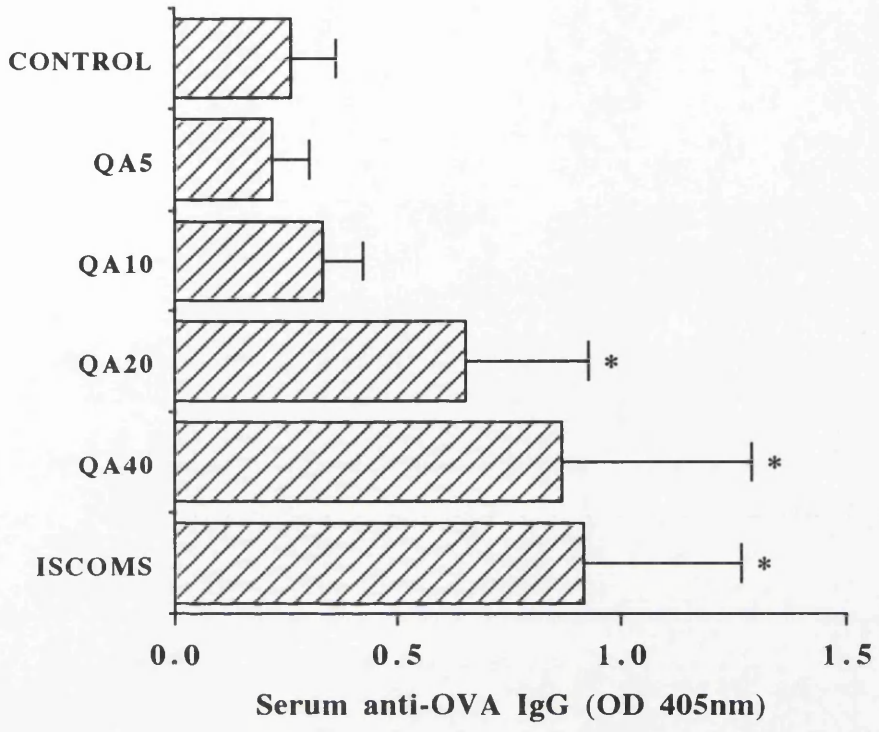

Figure 7.9.

Induction of OVA-specific serum IgG responses by oral immunisation with Quil A and OVA. Sera were obtained from BALB/c mice 7 days after the last of six feeds of 50μg OVA plus 5-40μg Quil A (QA), 50μg ISCOMS-OVA or saline only. Results shown represent mean O.D. 405nm values ± 1S.D. from six mice per group. *$p<0.05$ v. CONTROL.

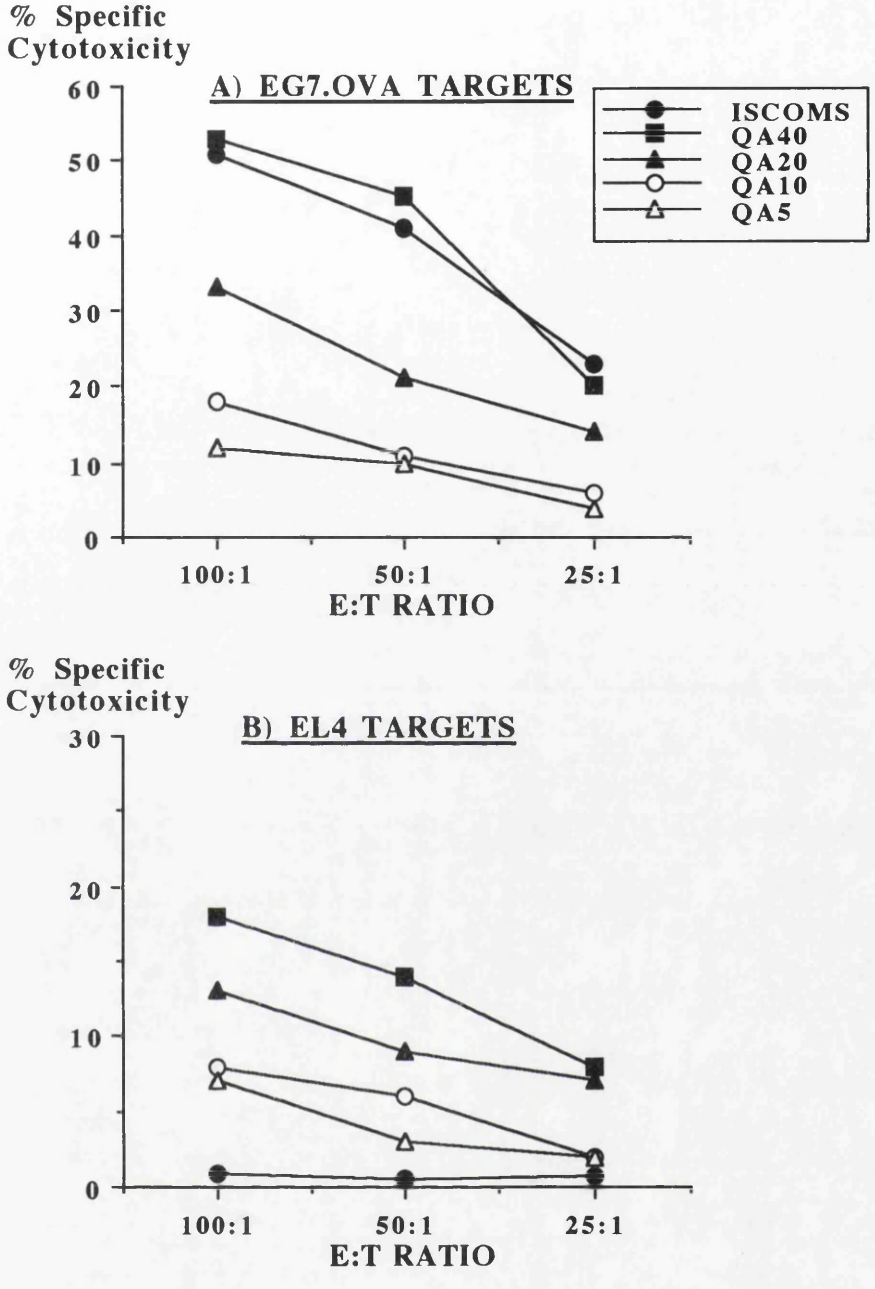

Figure 7.10.

Induction of OVA-specific CTL responses by oral immunisation with Quil A and OVA. Splenic CTL activity was assayed in C57Bl/6 mice 7 days after the last of six feeds of 50μg OVA plus 5-40μg Quil A (QA), 50μg ISCOMS-OVA or saline only, after restimulation *in vitro* for 5 days with EG7.OVA cells. Results shown are percent specific cytotoxicity using spleen cells pooled from four mice per group.

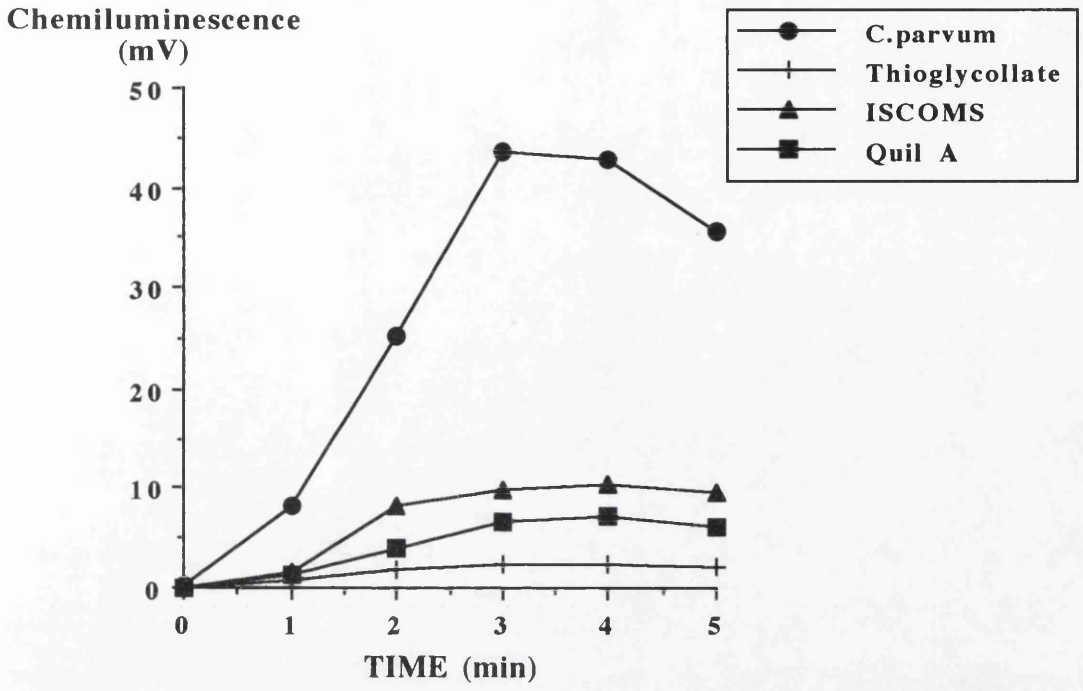

Figure 7.11.

Respiratory burst activity of peritoneal exudate cells (PEC) after administration of Quil A or ISCOMS. Adherent PEC were isolated from mice 4 days after i.p. administration of 1μg free Quil A, ISCOMS-OVA containing 1μg Quil A, 1mg heat-killed *Corynebacterium parvum* or 2mls thioglycollate broth and their oxidative metabolic capacity assayed by chemiluminescence following stimulation with 0.5μg/ml PMA *in vitro*.

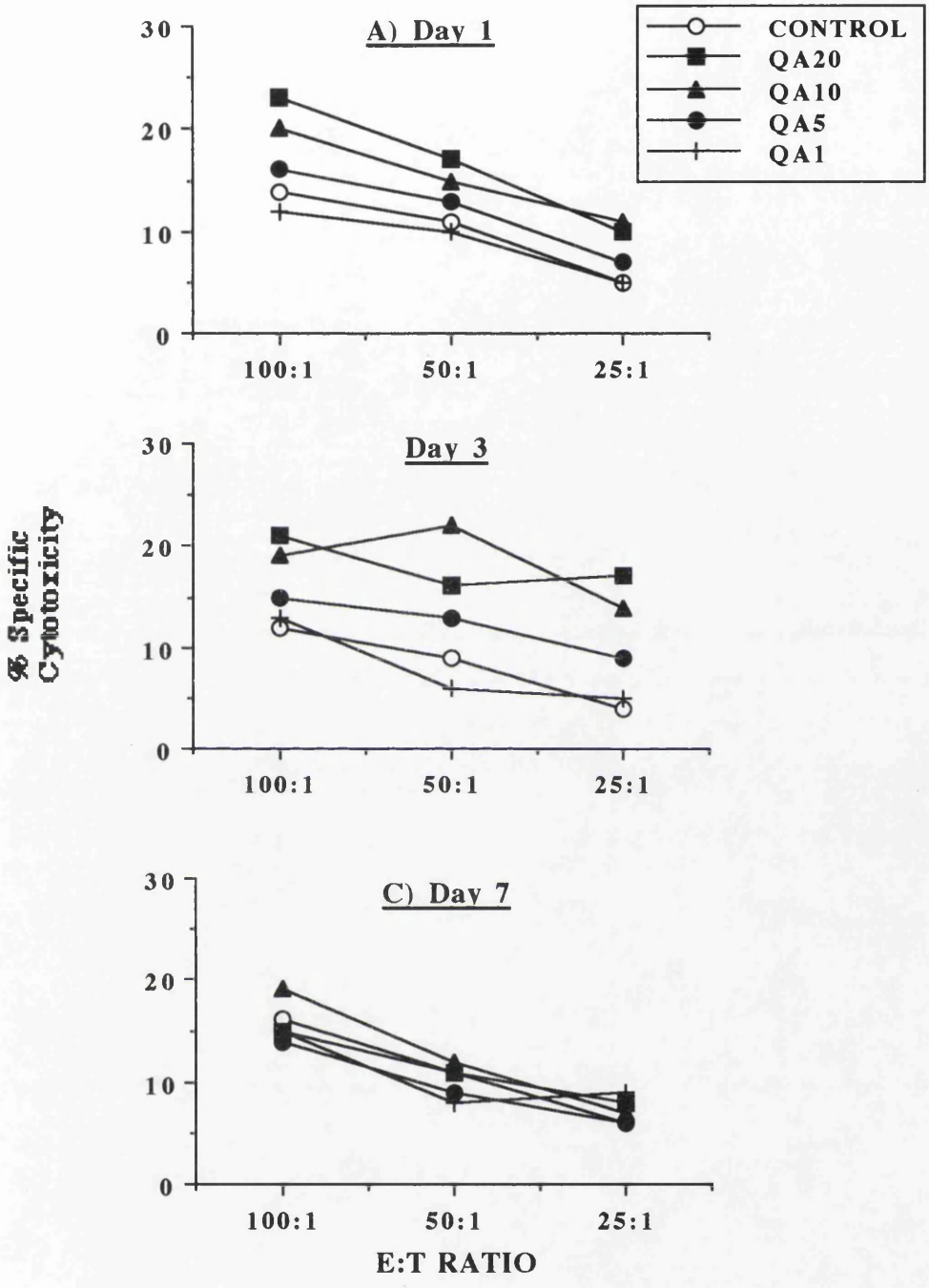

Figure 7.12.

Enhancement of splenic natural killer (NK) cell activity by Quil A. Splenic NK cell activity was assayed in BALB/c mice 1 day (A), 3 days (B) or 7 days (C) after injection of 1-20μg Quil A (QA) or saline only i.p. Results shown are percent specific cytotoxicity against YAC-1 target cells using spleen cells pooled from two mice per group.

Table 7.1: IL-4 production by spleen cells from mice primed with Quil A and OVA

CELLS	STIMULUS	Day 2	Day 6
Control:	Medium	0.1±0.1	0.6±0.3
	Con A	14.5±4.9	6.7±3.5
	OVA	0.1±0.1	0.3±0.2
ISCOMS:	Medium	0.1±0.1	0.1±0.1
	Con A	28.7±1.3	31.2±2.6
	OVA	1.8±1.0	0.1±0.1
QA20:	Medium	0.1±0.1	1.7±0.6
	Con A	34.9±2.3	21.4±7.1
	OVA	0.6±0.6	0.3±0.3
QA10:	Medium	0.1±0.1	2.1±0.8
	Con A	23.4±1.0	19.7±2.7
	OVA	0.3±0.2	0.7±0.4
QA5:	Medium	0.1±0.1	1.3±1.0
	Con A	33.9±2.1	21.3±2.1
	OVA	0.1±0.1	0.7±0.6
QA1:	Medium	0.1±0.1	2.7±2.0
	Con A	30.2±2.2	39.3±7.6
	OVA	0.1±0.1	0.8±0.6

Spleen cells taken 14 days after immunisation with 10μg OVA plus 1-20μg Quil A (QA), 10μg ISCOMS-OVA or saline only s.c., were cultured for 2 or 6 days in the presence or absence of 1mg/ml OVA and the supernatants assayed for IL-4 by ELISA. The data represent mean IL-4 concentrations (U/ml) ± 1S.D. of supernatants assayed in quadruplicate.

CHAPTER 8: INDUCTION OF MUCOSAL AND SYSTEMIC IMMUNE RESPONSES BY IMMUNISATION WITH OVA ENTRAPPED IN POLY(LACTIDE-CO-GLYCOLIDE) MICROPARTICLES

Introduction

The results described in previous chapters have shown that ISCOMS are potent adjuvants when administered either orally or parenterally and a substantial part of this activity is due to the saponin component Quil A. However, Quil A itself was not as potent as when incorporated into ISCOMS and in this chapter, I have attempted to define how the particulate nature of the ISCOMS contributes to their adjuvant effects by comparing them with another particulate vaccine vector which lacked Quil A.

Biodegradable microparticles composed of poly(lactide-co-glycolides) (PLG) release antigen over a long period after *in vivo* administration (233). Parenteral or oral immunisation with antigen entrapped in microparticles induces systemic and mucosal antibody responses, indicating the potential usefulness of this approach for the induction of mucosal and systemic immune responses (108, 234). The availability of PLG microparticles containing OVA thus allowed me to compare the mucosal adjuvanticity of ISCOMS directly with another particulate vector.

Experimental Protocol

OVA-containing PLG microparticles, prepared as described in the Materials and Methods, were provided by Dr. D.T. O'Hagan (University of Nottingham).

Systemic IgG and DTH responses were measured in BALB/c mice immunised s.c. into the rear footpad with either 100μg OVA entrapped in PLG microparticles or with 10μg ISCOMS-OVA. For the induction of antigen-specific proliferative responses, BALB/c mice were immunised s.c. into the rear footpad with either 50μg OVA in microparticles or 5μg ISCOMS-OVA and the draining popliteal lymph node cells were isolated 14 days later.

Splenic CTL responses were assayed in C57Bl/6 mice which had been immunised 7 days before with 5μg or100μg or OVA in microparticles or 5μg ISCOMS-OVA i.p. To induce popliteal lymph node CTL responses, C57Bl/6 mice were primed s.c. with 50μg OVA in microparticles or 5μg ISCOMS-OVA and the draining popliteal lymph node cells were isolated 4 days later.

To assess mucosal immunogenicity, intestinal IgA responses were assayed in BALB/c mice one week after the last of 6 feeds of 100μg OVA in PLG microparticles. Splenic CTL responses were measured in C57Bl/6 mice one week after the last of 3 or 6 feeds of either 100μg OVA in PLG microparticles or 50μg ISCOMS-OVA.

In all experiments, control mice received identical priming doses of saline only.

Results

i) OVA Entrapped in PLG Microparticles or in ISCOMS Primes for OVA-Specific IgG and DTH Responses

My first experiments set out to confirm the adjuvant activity of PLG microparticles for parenteral immunisation and to compare this with the responses induced with OVA in ISCOMS. Virtually no primary IgG antibody response was observed in mice immunised into the rear footpad with either OVA in PLG microparticles or ISCOMS-OVA 7 days after immunisation (Fig. 8.1). However, both PLG-OVA and ISCOMS-OVA primed mice to develop highly significant OVA-specific IgG responses when challenged with heat-aggregated OVA (Fig. 8.1). The responses of the ISCOMS-OVA primed group were significantly higher than those of the microparticle primed group, despite the fact that the ISCOMS contained 10-fold less antigen.

Both adjuvants induced significant primary DTH responses and there was no significant difference between the ISCOMS and PLG primed groups (Fig. 8.2).

These results confirm previous findings that a single parenteral immunisation of antigen in PLG microparticles or ISCOMS can prime systemic humoral and cell mediated responses and indicate that they are of generally comparable magnitude.

ii) Priming of Antigen-Specific Proliferative Responses by OVA in PLG Microparticles or ISCOMS

As there had been little previous examination of the priming of T cells by PLG microparticles, I next compared the abilities of PLG entrapped OVA and ISCOMS-OVA to prime for antigen-specific

proliferative responses *in vitro*. Popliteal lymph node cells isolated two weeks after s.c. immunisation with either ISCOMS-OVA or OVA in PLG microparticles exhibited marked proliferative responses to restimulation with exogenous soluble OVA (Fig. 8.3A). However, these responses were only evident at the highest concentrations of OVA when microparticle primed cells were used. In contrast, as I have shown before, ISCOMS primed cells responded at concentrations of as little as 1-10μg OVA/ml. Unprimed cells showed no response to OVA *in vitro* and both responded strongly to stimulation with Con A (Fig. 8.3B)

Thus, while these findings clearly show that PLG microparticles can prime local proliferative T cell responses, ISCOMS appear to be more potent for this purpose.

iii) Induction of OVA-Specific CTL by Parenteral Immunisation with OVA Entrapped in Microparticles or in ISCOMS

As PLG microparticles shared the ability of ISCOMS to induce peripheral T cell responses, I went on to examine whether PLG microparticles were also capable of generating OVA-specific CTL responses.

As expected, spleen cells from mice primed with 5μg ISCOMS-OVA i.p. exhibited very high levels of OVA-specific CTL activity, even at E:T ratios as low as 6.25:1 (Fig. 8.4A). In contrast, mice immunised with the same amount of OVA in PLG microparticles had no OVA-specific CTL activity in the spleen (Fig. 8.4A). Mice immunised with a much larger dose of OVA (100μg) in PLG microparticles did generate OVA-specific CTL responses, but these were much reduced at lower E:T ratios when compared with those

found using ISCOMS-OVA primed cells (Fig. 8.4A). These results show for the first time that PLG microparticles can prime CTL *in vivo*, but they are less efficient than ISCOMS in this respect.

To confirm this finding, I examined for the presence of CTL precursors in the draining lymph node following footpad immunisation with 50μg OVA in microparticles or 5μg ISCOMS-OVA, without further restimulation with antigen. As shown in Fig. 8.4B, ISCOMS-OVA primed cells showed substantial OVA-specific CTL activity in this assay, whereas no CTL activity could be detected in the PLG microparticle primed group.

These results confirm that PLG microparticles are less efficient at priming antigen-specific CTL responses than ISCOMS, when administered parenterally.

iv) Induction of OVA-Specific CTL by Oral Immunisation with OVA in PLG Microparticles or ISCOMS

To explore further the ability of PLG microparticles to induce CTL activity, I next compared the ability of PLG and ISCOMS to induce systemic CTL responses when administered orally. As in previous experiments, mice fed 100μg OVA in ISCOMS six times over a 10 day period developed high levels of OVA-specific CTL in the spleen (Fig. 8.5A). Comparable levels of OVA-specific CTL were found in mice fed the same amount of OVA in PLG microparticles (Fig. 8.5A). However, mice fed ISCOMS-OVA on only 3 occasions still exhibited strong splenic CTL responses, whereas those receiving 3 oral immunisations with OVA in PLG microparticles had only very low levels of OVA-specific CTL activity (Fig. 8.5B).

Thus, PLG microparticles are also able to prime CTL responses when administered orally, but again, ISCOMS are more efficient by this route.

v) Induction of Intestinal IgA Responses by Feeding OVA in PLG Microparticles

As previous studies had indicated that feeding antigen entrapped in PLG microparticles could induce systemic and salivary antibody responses, I also examined the induction of OVA-specifc intestinal IgA responses by oral immunisation with OVA in PLG microparticles. Mice fed 6 times with 100μg OVA in PLG microparticles had significant OVA-specific IgA in intestinal secretions (Fig. 8.6), confirming that PLG microparticles are potent inducers of local and systemic antibody responses when administered orally.

Conclusions

These studies show that oral or parenteral immunisation with antigen entrapped in PLG microparticles primes a wide range of humoral and cell-mediated immune responses, both locally and systemically. This extends previous reports of the adjuvant activity of PLG microparticles, by demonstrating the stimulation of T cell responses, particularly antigen-specific CTL activity after either parenteral or oral administration. However, the CTL responses induced by ISCOMS were usually higher than those induced by PLG microparticles, requiring fewer or lower doses of antigen. It was also impossible to detect CTL activity in PLG-OVA immunised mice without *in vitro* restimulation with antigen.

Interestingly, PLG seemed relatively more efficient when given by the oral route a finding supported by the fact that oral immunisation with OVA in PLG induced antigen-specific IgA responses in the small intestine itself. This extends previous studies which showed serum IgG and salivary IgA antibody responses in mice fed PLG-OVA (108, 234).

These preliminary findings indicate that only part of the adjuvant effect of ISCOMS can be ascribed to their particulate nature, supporting evidence from the previous chapter on the adjuvant properties of free Quil A.

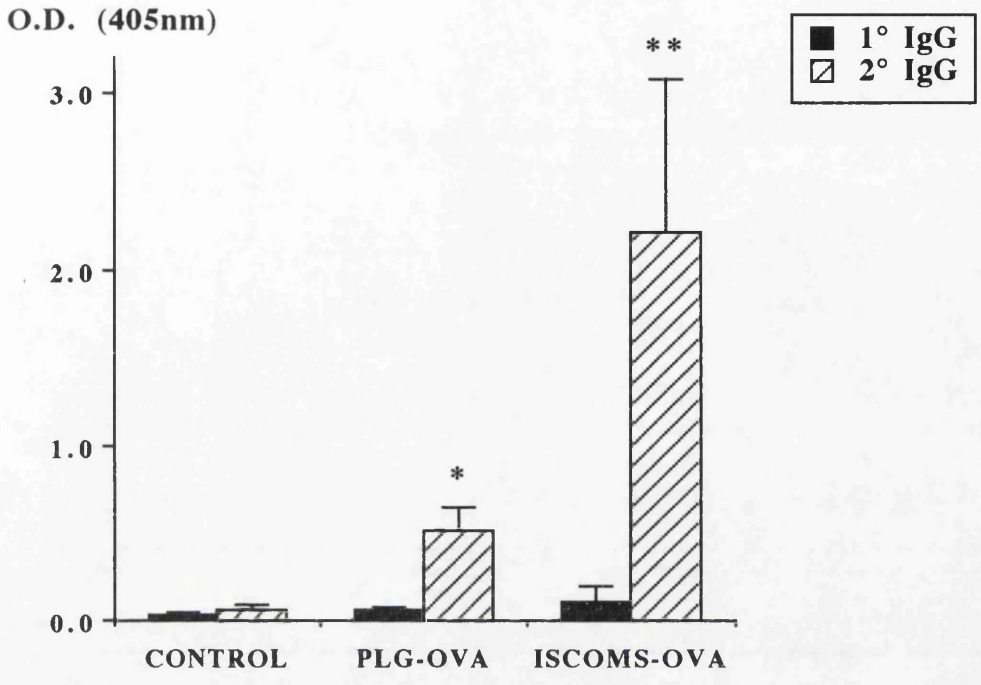

Figure 8.1.

Priming of OVA-specific serum IgG responses by parenteral immunisation with OVA in PLG microparticles. Primary sera were obtained one week after priming of BALB/c mice with either 100μg OVA in PLG microparticles, 10μg ISCOMS-OVA or saline only s.c., while secondary IgG responses were assayed one week after footpad challenge of primed mice with 100μg heat-aggregated OVA. Mean O.D. values ± 1S.D. are shown for 6 mice per group.

* $p<0.001$ v. Control.

** $p<0.001$ v. Control ; $p<0.005$ v. PLG-OVA.

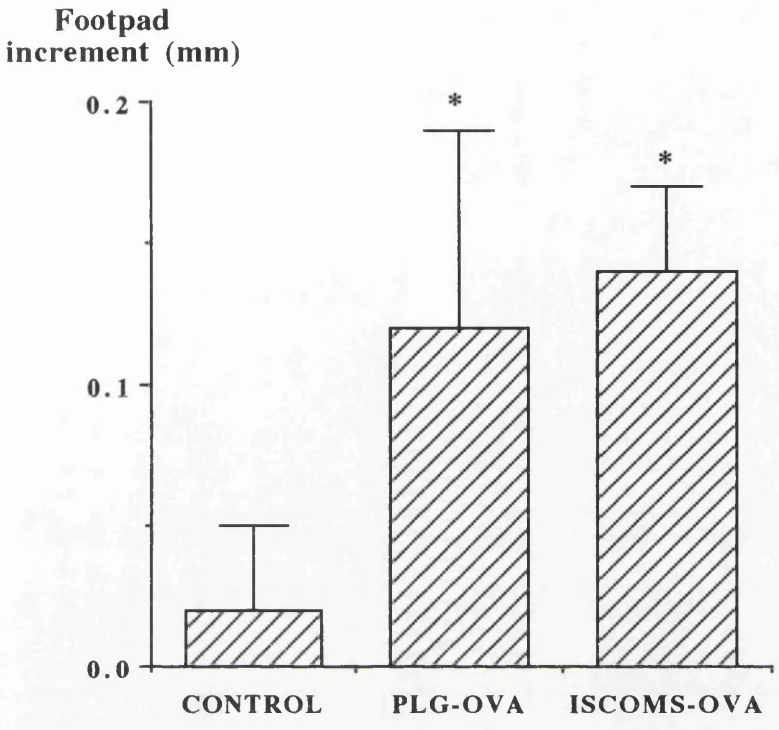

Figure 8.2.

Priming of OVA-specific systemic DTH responses by parenteral immunisation with OVA in PLG microparticles. DTH responses were measured 24hr after footpad challenge with 100μg heat-aggregated OVA in BALB/c mice which had been primed 14 days previously with either 100μg OVA in PLG microparticles, 10μg in ISCOMS-OVA or saline only s.c. Results shown are means ± 1S.D. for 6 mice per group.

* $p<0.05$ v. Control.

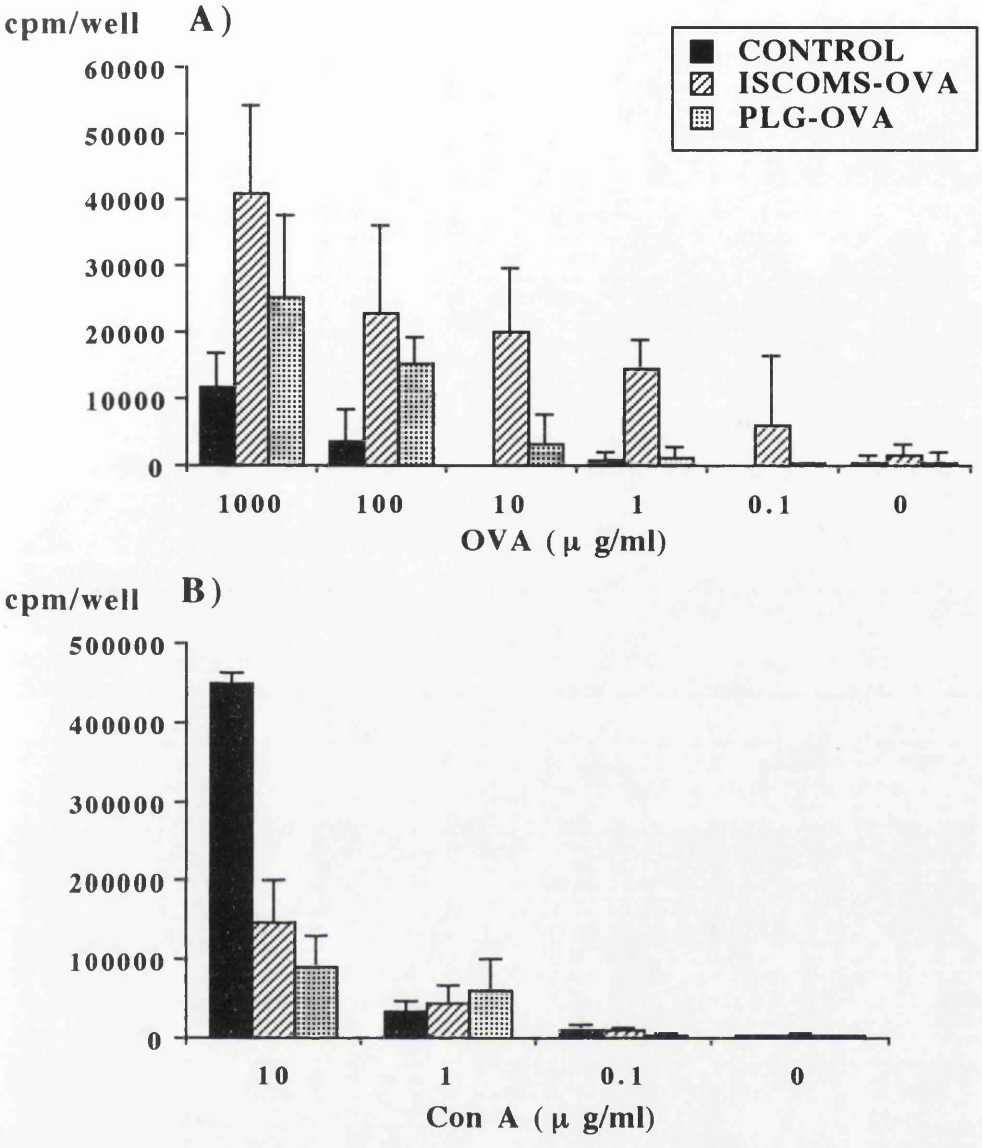

Figure 8.3.

Priming of antigen-specific proliferative responses by OVA in PLG microparticles. Proliferative responses of draining popliteal lymph node cells were assayed 14 days after priming with either 50μg OVA in PLG microparticles, 5μg ISCOMS-OVA or saline only s.c., to *in vitro* restimulation with (A) OVA or (B) Con A. The data represent mean cpm values ± 1S.D. from quadruplicate cultures harvested after 4 days (B) or 6 days (A) of culture.

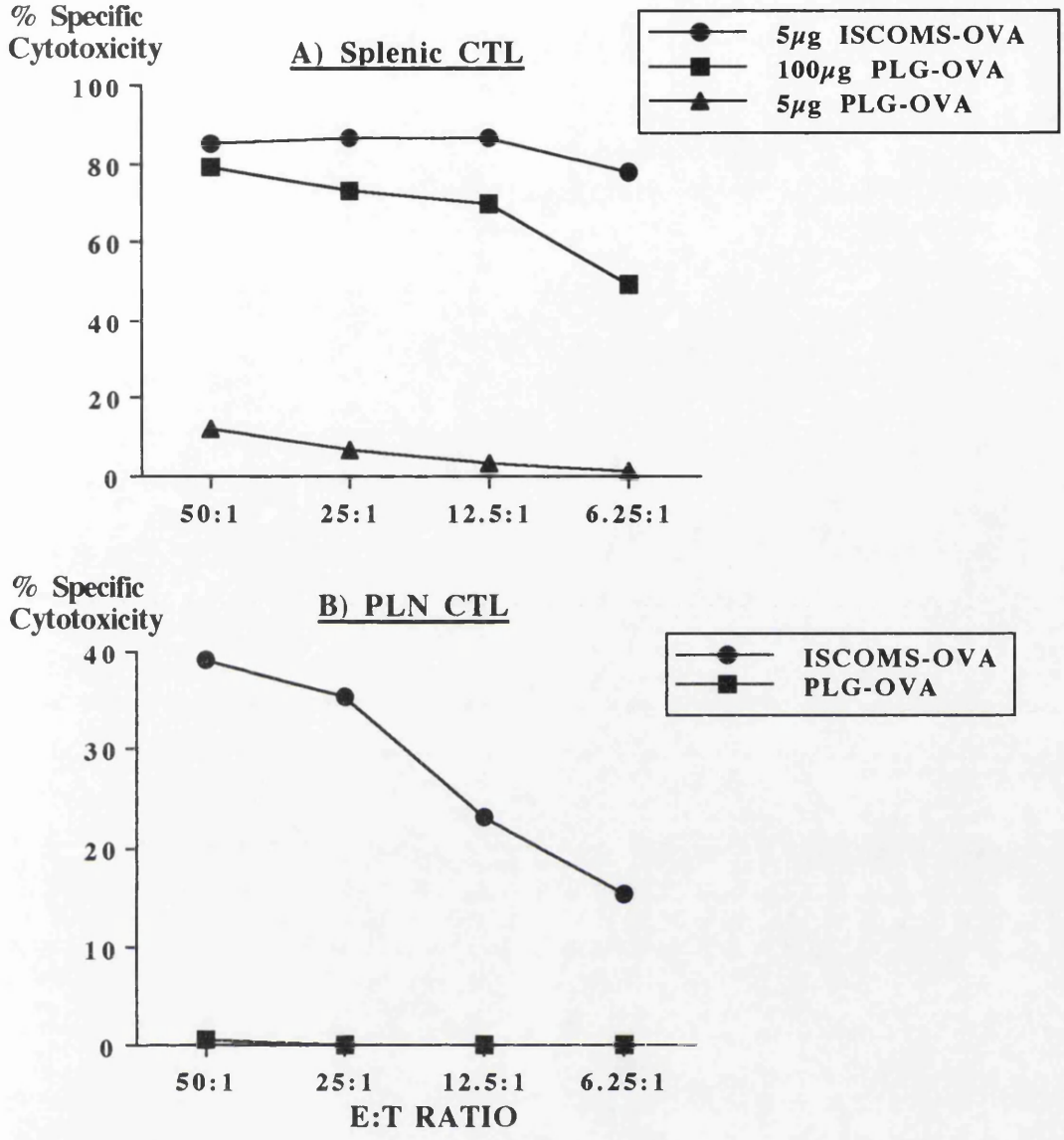

Figure 8.4.

Induction of OVA-specific CTL responses by parenteral immunisation with OVA in PLG microparticles. (A) Splenic CTL responses were assayed one week after priming with either 5μg ISCOMS-OVA or 5μg or 100μg OVA in PLG microparticles i.p., after restimulation *in vitro* with EG7.OVA cells. (B) PLN CTL responses were assayed 4 days after priming with either 50μg OVA in PLG microparticles or 5μg ISCOMS-OVA s.c., after *in vitro* restimulation with rhIL-2. Results shown are specific cytotoxicity against EG7.OVA cells using cells pooled from 4 mice per group. There was no lysis of EL4 cells by any of the effector populations

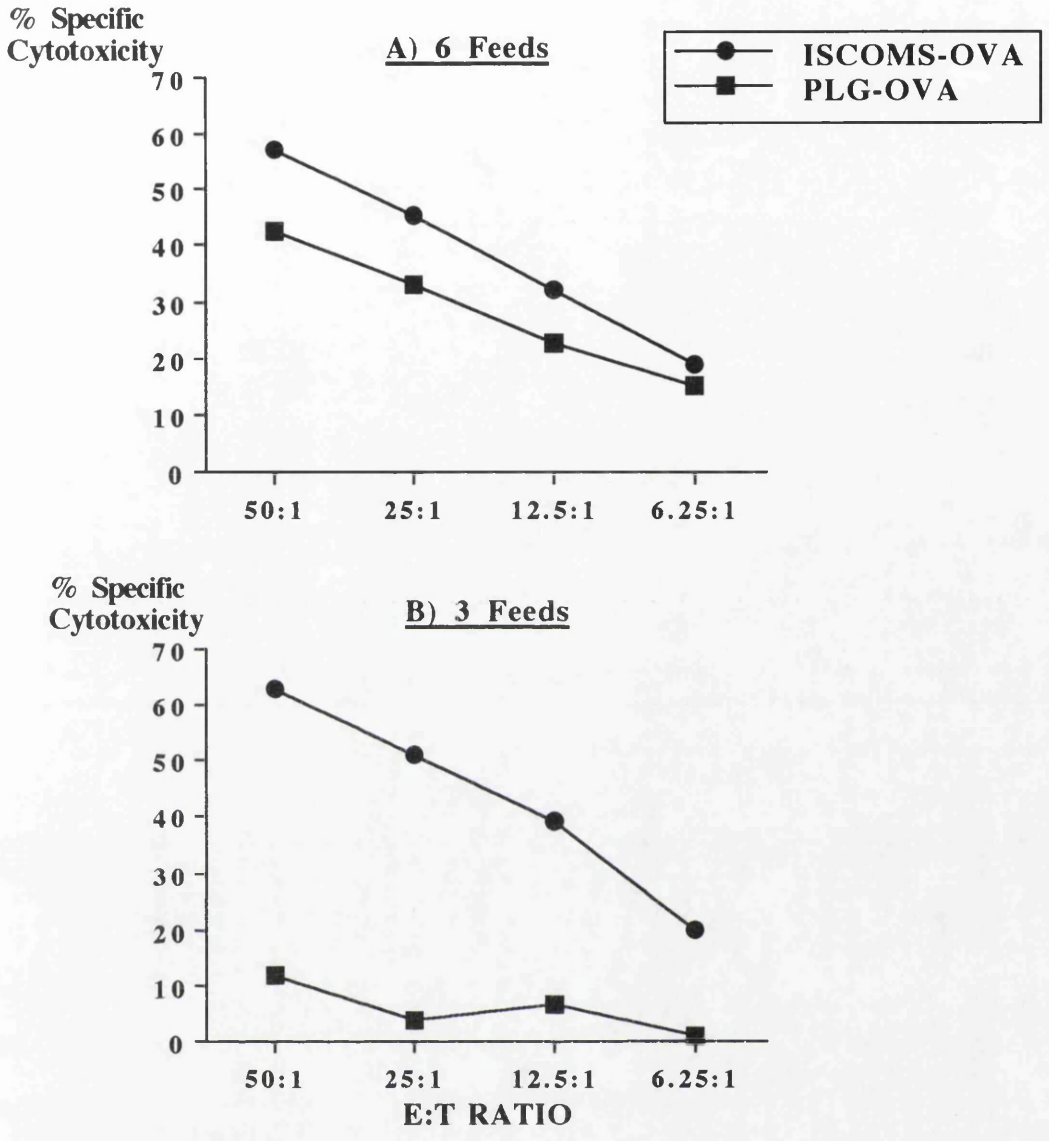

Figure 8.5.

Induction of OVA-specific systemic CTL responses by oral immunisation with OVA in PLG microparticles. Splenic CTL responses were assayed one week after the last of 6 feeds (A) or 3 feeds (B) of either 100μg OVA in PLG microparticles or 50μg ISCOMS-OVA, after restimulation *in vitro* with EG7.OVA cells. Results shown are specific cytotoxicity against EG7.OVA cells using cells pooled from 4 mice per group. There was no lysis of EL4 cells by any of the effector populations.

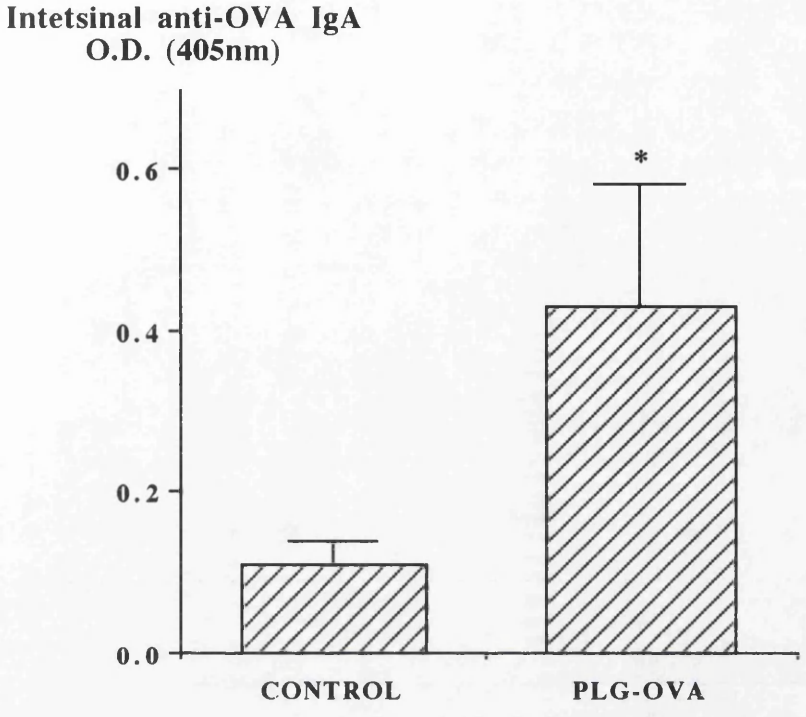

Figure 8.6.

Oral immunisation with OVA in PLG microparticles induces OVA-specific intestinal IgA. BALB/c mice were fed six times over a ten day period with 100μg OVA in PLG microparticles or saline only and intestinal washes obtained one week later. Results shown are mean O.D. (405nm) values ±1S.D. from six mice per group.

* $p<0.05$ v. Control.

CHAPTER 9: DISCUSSION

Introduction

The results presented in this thesis have confirmed and extended previous findings on the induction of immune responses by ISCOMS. In addition, they have highlighted several important features of the host immune response, and of ISCOMS themselves, which contribute to their adjuvant properties. These results have implications not only for oral vaccine development, but also for understanding the regulation of immune responses to protein antigens *in vivo*.

Nature of the Immune Responses Induced by Oral and Parenteral Administration of ISCOMS

The results in chapter 3 showed that ISCOMS primed a wide range of humoral and cell-mediated immune responses when administered either orally or parenterally. These included systemic IgG and secretory IgA antibodies, systemic DTH responses, and antigen-specific CTL responses. Although the ability of ISCOMS to induce both humoral and cell-mediated immunity has been demonstrated with a wide variety of protein antigens, this study was the first to assess the parallel induction of such a wide range of immune responses *in vivo*.

The first important finding was that a single parenteral immunisation with as little as 5-10μg of a soluble protein antigen in ISCOMS primed antigen-specific IgG responses, confirming what we and others had shown previously (159, 165, 169, 170, 172, 173,

179, 181). I also confirmed that this immunisation regime elicited potent antigen-specific DTH responses (165), indicating efficient priming of both B and T cells by antigen incorporated in ISCOMS.

Parenteral immunisation with ISCOMS-OVA also elicited high levels of antigen-specific CTL activity in the spleen, which was entirely mediated by $CD8^+$ T cells. These results suggest that, in addition to processing and presentation of antigen by the exogenous route to class II MHC-restricted $CD4^+$ T cells, incorporation within ISCOMS allowed soluble protein antigen access to the endogenous processing pathway for recognition by $CD8^+$ T cells. This unusual ability of ISCOMS to induce potent CTL responses has been confirmed in a number of other systems (184, 212, 235, 236) and may be of vital importance for protecting against many viral infections.

The most impressive property of ISCOMS to be demonstrated in chapter 3 was their ability to elicit a wide range of local and systemic immune responses when administered orally. This required repeated oral immunisation, with optimal immune responses occurring when ISCOMS were fed six times over a 10 day period. The responses induced included the induction of secretory IgA in the intestine, as well as systemic IgG, DTH and CTL responses. These observations are in contrast to the systemic immunological tolerance normally induced by feeding soluble protein antigen (54), but are consistent with the findings that local and systemic immunity can be induced by intranasal administration of ISCOMS (173, 183, 235). This suggests that presentation in ISCOMS allows protein antigens to avoid the regulatory mechanisms which normally operate at mucosal surfaces. This may be due in part to their ability to withstand the

harsh environments of the stomach and small intestine (166) and may also reflect the particulate nature of antigen in ISCOMS, which may alter both uptake and immunogenicity of orally administered antigen (128). Evidence for this hypothesis has come from recent studies showing that prior administration ISCOMS enhances the uptake of orally administered protein into the systemic circulation (237). In parallel, serum from these mice does not contain the tolerogenic material normally found in animals fed soluble proteins (Furrie E, Mowat AMcI; manuscript in preparation). The exact reasons for this are unclear, but cholera toxin, one of the most well studied oral adjuvants, has many effects on the intestinal environment, including increasing intestinal permeability and antigen uptake (238), altering epithelial cell function (152, 239) and activation of local and systemic APC (148, 151), a process known to interfere with oral tolerance (240). Further studies on the effects of ISCOMS on these parameters are necessary in order to elucidate the mechanisms responsible for their oral adjuvant properties.

The fact that multiple oral immunisations of 10-20 fold more antigen than that used for systemic immunisation were required to efficiently prime via the oral route, suggests that some degradation of either the ISCOMS or of the incorporated antigen did occur after feeding. Nevertheless, effective immunisation with other oral adjuvants such as cholera toxin also requires repeated feeding (143), indicating the general difficulty in stimulating solid immunity at mucosal sufaces. The fact that the most important factor governing the induction of immune responses to orally administered ISCOMS and CT is the frequency of antigen administration, rather than the total dose of antigen given, may

reflect the need for repeated stimulation of a relatively small number of antigen specific precursors in the gut-associated lymphoid tissues (GALT). The site of priming of the immune system by orally administered ISCOMS is unknown, but the presence of secretory IgA responses and CTL responses in the MLN suggests that priming occurs locally in the GALT. In addition, it must be emphasised that feeding ISCOMS simultaneously primed systemic immune responses, although whether this is due to recirculation of primed lymphocytes from the GALT or to systemic dissemination of antigen is not yet clear. Studies using fluorescently labelled ISCOMS have reported that they are preferentially taken up into the Peyer's patches after oral administration (241), but their subsequent fate is not known. Time-course studies looking for the appearance of antigen-specific B and T cells in mucosal and systemic lymphoid tissues following oral administration of ISCOMS could help clarify this issue.

Having illustrated the nature and range of immune responses induced by parenteral or oral immunisation with ISCOMS, I went on to characterise the host immunological mechanisms involved in the generation of immunity against ISCOMS.

Induction of Class I MHC-Restricted CTL Responses by ISCOMS

The novel ability of ISCOMS to stimulate potent antigen-specific CTL responses against exogenous protein antigens make them ideal candidates for vaccines against many viral infections. However, to confirm that these CTL responses were relevant to those induced by endogenous processing of naturally occurring

viral antigens, it was important for me to fully characterise the CTL elicited by ISCOMS and to explore the mechanisms involved in their generation.

i) Phenotype and Specificity of CTL Induced by ISCOMS

My results showed that the CTL primed by ISCOMS-OVA were $CD8^+$ T cells, which efficiently lysed OVA-transfected EG7.OVA cells but not parental non-transfected EL4 cells. As the EG7.OVA cells do not express class II MHC molecules (202) and there was no lysis of OVA-pulsed P815 cells, the ISCOMS primed CTL were conventional class I MHC-restricted T cells. The CTL recognised an endogenously processed form of OVA, as osmotic loading of EL4 cells with OVA sensitised them for lysis, while exogenously added native OVA did not. There was also no component of NK cell activity, as ISCOMS-OVA primed CTL did not lyse the NK-sensitive YAC-1 cell line. I also showed that oral administration of ISCOMS-OVA elicited CTL with an identical phenotype and specificity to those induced after parenteral immunisation, indicating that ISCOMS retained the ability to elicit conventional $CD8^+$ class I MHC-restricted T cells when administered orally.

The ISCOMS-OVA primed CTL recognised an epitope within the OVA 258-276 peptide, as EL4 cells were sensitised for lysis when pulsed with this peptide. Others have shown that this peptide contains an octamer (OVA 257-264) which binds the K^b class I MHC molecule and is the dominant epitope recognised by OVA-specific CTL in H-2^b mice following normal endogenous processing of OVA (33, 221, 242, 243). Notably, the ISCOMS-OVA primed CTL did not recognise EL4 cells pulsed with the peptide OVA 111-122, which contains a H-$2D^b$-restricted epitope that can be recognised by OVA-

specific CTL generated by *in vitro* stimulation with enzymatically cleaved OVA, but is not generated by endogenous processing of OVA *in vitro* or *in vivo* (220). Taken together, these findings further support the hypothesis that ISCOMS faciltate the entry of incorporated protein antigen into the natural endogenous antigen processing and presentation pathway for recognition by $CD8^+$ class I MHC-restricted T cells *in vivo*. My findings are supported by other reports that $CD8^+$ class I MHC-restricted CTL are primed by ISCOMS containing HIV gp160 or influenza haemagglutinin (184) or OVA (212), in which the class I MHC-restriction of OVA-specific CTL was shown formally using RMA-S target cells (212).

The lysis of OVA 258-276 pulsed EL4 cells by ISCOMS-OVA primed CTL was always lower than the lysis of EG7.OVA cells. This could reflect a higher epitope density of the naturally occurring octamer on the surface of the EG7.OVA cells, compared with fragments of exogenous OVA 258-276 which will need processed. Alternatively, the bulk CTL population may contain T cells which recognise class I MHC binding peptides derived from OVA other than the 257-264 epitope. Others have reported similar observations on the recognition of peptide pulsed EL4 cells by ISCOMS-OVA primed CTL (212) and it has recently been shown that these CTL may recognise at least two K^b-restricted OVA peptides, OVA 257-264 and OVA 176-183 (244). These CTL were heterogeneous, as some clones were specific for the individual epitopes, while others recognised both epitopes (244). Thus, ISCOMS-OVA primes a diverse population of CTL *in vivo*, capable of recognising a range of endogenously processed OVA peptides.

In confirmation of other observations (212), ISCOMS-OVA primed CTL did not require *in vitro* restimulation with antigen, as

pCTL in the PLN differentiated when cultured with IL-2 *in vitro*. This indicates that ISCOMS prime a high frequency of precursor CTL *in vivo* and this assay emphasises the potency of this property of ISCOMS.

The induction of class I MHC-restricted CTL responses by ISCOMS-OVA is of particular interest, as exogenous soluble protein generally does not prime $CD8^+$ CTL responses (202, 245). However, recent studies have indicated that soluble protein antigens can induce CTL responses when injected in association with detergents (246, 247) adjuvants such as CFA (248), or cellular debris (215, 245, 249). Furthermore, as will be discussed more fully below, there is increasing evidence that particulate antigens may effectively stimulate class I MHC-restricted CTL responses against associated protein antigens (224, 250-252).

ii) The Uptake, Processing and Presentation of ISCOMS to Class I MHC-Restricted T Cells

The mechanisms underlying the ability of exogenous antigen to prime CTL responses are controversial and I therefore examined some of the factors involved in the induction of these responses by ISCOMS-OVA. I firstly examined the role of macrophages in the induction of CTL, using silica or liposomes containing the toxic compound Cl_2MDP, to paralyse or deplete $M\phi$ *in vivo*. These procedures resulted in a marked reduction in the magnitude of the CTL responses primed by ISCOMS, to around one third of the level of cytotoxicity achieved in the ISCOMS primed group. Thus, efficient priming of CTL by ISCOMS is dependent on the presence of functional phagocytic accessory cells. These observations are consistent with a number of reports documenting a role for

phagocytic cells in the induction of CTL responses *in vivo* (215, 251, 253-255). In addition, they concur with findings that exogenous particulate forms of antigen, such as antigen conjugated to beads (252) or encapsulated within liposomes (224, 250, 251), can efficiently prime class I MHC-restricted T cells *in vivo* and that phagocytic cells are of central importance in the uptake, processing and presentation of such particulate antigens to T cells (131, 132, 251, 256-258).

More recently, direct evidence for the involvement of Mϕ in the uptake and processing of ISCOMS has been provided by the finding that rabies virus antigen presented in ISCOMS was taken up mainly by the marginal metallophilic macrophages in the spleen, a subset of macrophages distinct from those that take up free inactivated rabies virus antigen (241). Depletion of Mϕ with liposomes containing Cl_2MDP also inhibits the development of CTL responses against OVA encapsulated in liposomes, but only for a peroid of about 30 days after injection of Cl_2MDP-containing liposomes (251). The return of CTL priming coincides exactly with the repopulation of marginal metallophilic macrophages in the spleen, while marginal zone macrophages are not repleted until more than 2 months later (209). Taken together, these results suggest that marginal metallophilic macrophages in the spleen play a key role in the induction of class I MHC-restricted CTL responses against exogenous protein antigens presented in ISCOMS or in other particulate vectors. Interestingly, other studies have also highlighted the ability of a subset of splenic macrophages to process and present exogenous protein antigen in association with class I MHC molecules, although the anatomical location of these cells was not identified (259).

It should be noted that some CTL activity was retained in ISCOMS primed mice, even after depletion or paralysis of Mϕ, suggesting that ISCOMS utilise more than one pathway of CTL induction *in vivo*. One candidate APC for this activity are DC, which are also potent inducers of antigen-specific CTL responses (218, 260, 261). Although doubts have been raised over the ability of DC to take up and process particulate antigens (16), recent studies show that they are able to induce antigen-specific CTL responses when pulsed with liposome-encapsulated OVA *in vitro* or *in vivo* (216). EBV-transformed B lymphoblastoid cell lines (B-LCL) can also process and present ISCOMS containing the transmembrane fusion protein of measles virus (MV-F-ISCOMS), or the envelope glycoprotein gp340 of EBV (gp340 ISCOMS), for recogntion by both class I MHC-restricted and class II MHC-restricted CTL clones *in vitro* (236, 262). Whether this presentation pathway operates *in vivo* and occurs for all types of protein antigens incorporated in ISCOMS is not clear, but together with my own observations and the evidence discussed above, they emphasise the possibility that protein antigens in ISCOMS may be processed and presented by a range of different APC types *in vivo*.

The precise intracellular processing route by which protein antigens incorporated in ISCOMS gain entry into the endogenous pathway of antigen processing for presentation in association with class I MHC molecules is also unclear. Recent evidence that presentation of OVA-coated particles by Mϕ for recognition by class I MHC-restricted T cells requires functional TAP proteins and involves processing by proteasomes (263), suggests that the associated antigen gains access to the cytosol. Similarly, the presentation of MV-F-ISCOMS to $CD8^+$ T cells by B-LCL is also TAP-

dependent, indicating that ISCOMS do allow protein antigens to enter the cytosolic pathway of antigen processing (236). This could occur because the lipophilic nature of ISCOMS may allow them to fuse with cellular or endosomal membranes and thus release incorporated antigen directly into the cytosol. This hypothesis is consistent with the ability of other hydrophilic complexes such as lipopeptides (253, 264) and liposomes (224, 250, 251) to induce CTL responses. However, as I shall discuss below, the detergent properties of Quil A may also play a role in allowing antigens access to the endogenous pathway of antigen processing and presentation. Therefore, at present it remains uncertain as to how ISCOMS enter the endogenous pathway of antigen processing and presentation.

iii) The Role of CD4+ T Cells in the Priming of CTL by ISCOMS

I next investigated whether the concomitant activation of CD4+ T cells was involved in the priming of such potent CTL responses by ISCOMS *in vivo*. Mice depleted of CD4+ T cells failed to exhibit any OVA-specific CTL activity after priming with ISCOMS-OVA, indicating that CD4+ T cells play a crucial role in the priming of CTL by ISCOMS. Although these findings are in agreement with a number of other studies showing that CD4+ T cells are necessary for the priming of CD8+ CTL responses (49-51, 265-269), there is conflicting evidence on this subject, as other workers have reported that CTL responses can be efficiently primed in the absence of CD4+ T cells (217, 219, 260, 270).

It is likely that the requirement for CD4+ T cells in the induction of CTL responses is influenced both by the nature of the immunological stimulus and by the strength of non-specific

inflammatory signals present at the time of antigen recognition. Thus, viruses which replicate at high levels in host cells which express class I MHC molecules, but not class II MHC molecules, are likely to elicit CTL responses in the absence of $CD4^+$ T cells (217, 270). Similarly, allospecific CTL responses can be induced in the absence of $CD4^+$ T cells (260, 271), because the frequency of responding $CD8^+$ T cells is unusually high, allowing enough IL-2 to be produced to provide autocrine help (271, 272).

The position with the induction of $CD8^+$ CTL responses by soluble protein antigens is less clear, but most reports support the view that concomitant activation of $CD4^+$ T cells may be required (215, 245-248). Similar findings have been reported with purified peptides, where direct induction of $CD8^+$ T cell responses *in vivo* with free peptide appears difficult and usually requires conjugation to lipid moieities (253, 264), activation of $CD4^+$ T cells (267, 273), or co-administration with inflammatory stimuli such as IFA (219, 267, 274). The induction of CTL responses by influenza-infected or peptide-pulsed DC *in vitro* also requires the presence of $CD4^+$ T cells or their lymphokine products (218). Thus, the ability to present antigen to both $CD4^+$ T cells and CTL is usually essential for the effective induction of CTL responses by DC. Similarly, although $CD8^+$ CTL primed *in vivo* by OVA in liposomes can be restimulated *in vitro* in the absence of $CD4^+$ T cells, this is enhanced by the presence of $CD4^+$ T cells (251). Furthermore, studies in IL-2 deficient mice have indicated that although these mice can make anti-viral CTL responses, their magnitude is reduced by 70-90%, confirming the important role of this cytokine in the expansion of CTL (275, 276).

Taken together with the evidence discussed above, my results suggest that after *in vivo* administration, ISCOMS are taken up by professional APC populations, which process and present antigen to both class I MHC- and class II MHC-restricted T cells. This could create a three-cell cluster providing the antigenic signal and local IL-2 production required for the efficient induction of $CD8^+$ CTL, as has been suggested by other *in vitro* studies of helper-dependent CTL responses (269).

Priming of $CD4^+$ T Cell Responses by ISCOMS

The induction of potent local and systemic antibody responses and systemic DTH responses by ISCOMS, together with requirement for $CD4^+$ T cells in the priming of CTL responses, suggested that the priming of $CD4^+$ T cells was the key event in the induction of immune responses by ISCOMS. To assess this issue more closely, I examined the antigen-specific T cell proliferative responses and the pattern of cytokines produced by ISCOMS-OVA primed cells restimulated *in vitro* with soluble OVA.

I found that parenteral immunisation with ISCOMS primed strong antigen-specific proliferative responses in the draining lymph node, which were entirely mediated by $CD4^+$ T cells. I could find no evidence of $CD8^+$ T cell proliferation *in vitro*, even when primed cells were cultured with the immunodominant CTL epitope in the presence of exogenous IL-2. Although these findings support my earlier observations on the CD4-dependence of CTL responses induced by ISCOMS, they are somewhat paradoxical in view of the potent ability of ISCOMS-OVA to prime class I MHC-restricted CTL which can be restimulated by endogenously processed OVA

epitopes or IL-2 *in vitro* (212). This discrepancy may be explained by the fact that as peptide-specific CTL are present only in a low frequency, it is difficult to detect their proliferation *in vitro*. Alternatively, the kinetics of CTL proliferation *in vitro* could differ from those of CD4+ T cells and by this late timepoint in culture the pCTL in the primed PLN cells may have already fully differentiated into effector CTL. In support of the latter hypothesis, others have noted that CD8+ T cells undergo blastogenesis *in vivo* after priming with ISCOMS-OVA (212). Thus, the *in vitro* proliferative assay I used may not be sensitive enough to detect the proliferation of small numbers of CD8+ T cells. Nevertheless, the results emphasise that relatively more CD4+ T cells must be primed by *in vivo* immunisation with ISCOMS.

My experiments showed for the first time that oral administration of ISCOMS-OVA also primed antigen-specific proliferative responses in the draining lymph nodes (MLN), but these required higher concentrations of antigen for restimulation (1mg/ml vs. 1-10μg/ml for PLN cells). This difference may reflect differences in the proliferative capacity of the different cell populations and/or differences in the proportion of antigen-reactive cells. However, I believe it is more likely that ISCOMS are simply less efficient at priming a high frequency of T cells by the oral route. This would be consistent with my earlier findings on the induction of local and systemic immunity by feeding ISCOMS, where much higher amounts of antigen were required to prime equivalent responses by the oral rather than parenteral route. It would be helpful to investigate this hypothesis using ISCOMS containing radio- or fluorescently-labelled antigen to examine the

concentration of antigen achieved in local lymphoid tissues after oral or parenteral immunisation.

One feature of my studies was the consistent inability to prime detectable antigen-specific proliferative responses in the spleen after either oral or parenteral immunisation with ISCOMS. Although this finding could reflect the lower proportion of T cells in spleen compared with lymph node, ISCOMS primed spleen cells produced cytokines when restimulated with antigen *in vitro* (see below), suggesting that T cell priming may have occurred in the spleen and again indicating the limited sensitivity of the proliferative assay. In this respect, it is notable that others have reported a degree of non-specific proliferation in the spleen for up to 2 weeks after parenteral administration of ISCOMS (182), which could mask the smaller antigen-specific component. In support of this hypothesis, I also observed elevated spontaneous proliferative responses of spleen cells from mice primed parenterally with ISCOMS.

ISCOMS-OVA primed PLN cells also proliferated in response to restimulation with ISCOMS-OVA, showing that ISCOMS themselves can be processed and presented to T cells *in vitro*. Similar findings have been reported using ISCOMS containing influenza virus glycoproteins (182) and these observations are consistent with studies showing that EBV-transformed B cells can process and present ISCOMS associated antigen for recognition by both $CD4^+$ and $CD8^+$ clones *in vitro* (236, 262). Importantly, in agreement with the other reports (182, 236, 262), I found that very low concentrations of antigen in ISCOMS (1μg/ml) could stimulate T cell proliferation *in vitro*, suggesting that when incorporated in ISCOMS antigen is processed and presented very efficiently for recognition by T cells.

This property may prove useful in terms of understanding the mechanisms which are involved in the processing and presentation of ISCOMS to $CD4^+$ and $CD8^+$ T cells *in vivo*, but unfortunately I did not have time to explore this issue during this study.

The strong priming of $CD4^+$ T cells by ISCOMS was emphasised by the substantial amounts of antigen-specific cytokine production by ISCOMS primed lymphoid cells. This included production of the T_h1 cytokines, IL-2 and IFN-γ, as well as production of the T_h2 cytokine, IL-5. In addition, these cytokines were found after both oral and parenteral priming with ISCOMS. The cytokines were produced in a distinctive pattern during *in vitro* culture with antigen, characterised by an early, transient IL-2 response, which peaked on day 2 of culture, whereas IL-5 and IFN-γ accumulated later, with the highest levels of these cytokines being found on day 6 of culture. This pattern may reflect the initial activation of antigen-specific cells *in vitro*, followed by their subsequent proliferation and maturation to express effector functions.

The simultaneous production of IL-2, IL-5 and IFN-γ indicated that both T_h1 and T_h2 subsets of $CD4^+$ T cells were activated by immunisation with ISCOMS, supporting the wide range of humoral and cell-mediated immune responses primed by ISCOMS *in vivo*. Furthermore, ISCOMS-OVA primed mice to produce high titres of antigen-specific IgG1 and IgG2a antibodies, indicative of T_h2 and T_h1 cell activation respectively (227, 228, 277). Others have also reported the secretion of both these immunoglobulin isotypes after immunisation with ISCOMS containing influenza virus proteins (179, 278) and although one of these studies reported the preferential production of IL-2 and IFN-γ (278), subsequent work

showed that IL-4 was also produced (279). Together, these findings confirm that ISCOMS stimulate both subsets of $CD4^+$ T cells *in vivo.*

Surprisingly in view of the marked IgG1 antibody and IL-5 production I observed, I could detect no antigen-specific IL-4 production in any of the tissues after either oral or parenteral immunisation, even when this cytokine was produced when the same cells were stimulated with mitogen. Although it remains possible that the levels of IL-4 produced in responses to antigen were below the sensitivity of the ELISA, or that any IL-4 produced was being utilised rapidly during culture, my findings could represent a true dichotomy in the regulation of IL-4 and IL-5 production during the stimulation of $CD4^+$ T cells by ISCOMS-associated antigen *in vivo.* This idea needs confirmation by direct examination of IL-4 production *in vivo,* using ELISPOT or PCR assays.

In addition to the range of cytokines primed by ISCOMS, oral and parenteral immunisation induced disseminated systemic immunity. Local immunisation in the footpad induced identical profiles of cytokine production in both the draining lymph node and more distantly in the spleen, consistent with the induction of systemic IgG and DTH responses which I observed after s.c. priming with ISCOMS. A similar pattern of cytokine production was obtained with spleen cells from mice primed orally with ISCOMS-OVA. Thus, ISCOMS promote a full range of local and systemic antigen-specific immune responses when administered by mucosal or parenteral routes. This contrasts with the restricted nature of T_h1 or T_h2 responses induced by conventional parenteral adjuvants such as CFA and alum which predominantly stimulate T_h1 and T_h2 responses respectively (53). In addition, existing mucosal adjuvants

such as Salmonella (115) or cholera toxin (154, 155) are believed to stimulate similarly restricted patterns of $CD4^+$ T cell responses (see below). These results emphasise the potential of ISCOMS as vaccine vectors and show that the mucosal immune system is perfectly capable of inducing a variety of different effector responses against a single protein antigen if presented in an appropriate manner.

Priming of Long-Term Memory Responses by ISCOMS

The next part of my thesis addressed some aspects of ISCOMS which were not directly concerned with the host mechanisms involved in the induction of immune responses by ISCOMS, but which were important for evaluating their potential as vaccine vectors. The first area that I examined was whether immunisation with ISCOMS stimulated long-lasting immune priming.

A single parenteral immunisation with 10μg OVA in ISCOMS primed T and B cell memory responses for several months after immunisation. Systemic DTH responses could be recalled for up to 8 months after priming, a much longer period than was suggested by previous studies (165). The presence of memory T cells was confirmed by the fact that proliferative responses of ISCOMS primed PLN cells could be restimulated *in vitro* for at least 8 months after immunisation and gradually waned thereafter. Similar findings were made with cytokine production by spleen cells from these mice, with strong IL-2 and IL-5 responses persisting for at least 8 months after priming and subsequently declining. This suggests that memory T cells of both T_h1 and T_h2 phenotypes persisted after priming with ISCOMS. However, it was noteworthy that there was no antigen-specific production of IFN-γ by ISCOMS

primed cells at any of the timepoints examined, a finding that was surprising in view of the long-term DTH response I observed. This was not due to any inherent defect in the cells, as both primed and control cells produced high amounts of IFN-γ when stimulated with mitogen. Unfortunately, I was not able to measure the isotypes of the persisting primary or memory IgG responses (see below), as these would have provided further evidence on whether both memory T_h1 and T_h2 cells persisted *in vivo* after priming.

Immunisation with ISCOMS induced a small but significant primary IgG response which persisted for one year after priming. Although this primary IgG response was not found in my earlier experiments described in chapter 3, these responses were measured 7 days after priming, which may be too early for the development of an IgG response. Indeed previous work in this laboratory showed that mice immunised with 10μg ISCOMS-OVA did not develop primary IgG antibodies until 21 days after priming (165). Other studies using influenza virus antigens in ISCOMS have also demonstrated the persistence of antigen-specific IgG responses for at least 5 months after a single parenteral immunisation (280, 281). The potent secondary IgG responses I found after challenge with antigen *in vivo* are consistent with the presence of long-lived memory B cells, as well as the memory T cells I described above. As with the T cell responses, both the primary and secondary IgG responses decreased in magnitude over time, but persisted for a full year after immunisation. I did not examine the IgG isotype of the persisting IgG responses, but it has been reported that all IgG subclasses, particularly IgG2a, remained elevated for at least 5 months after priming with ISCOMS (281). Furthermore, multiple parenteral immunisation of macaque monkeys with purified HIV-2

proteins in ISCOMS induced protective immunity which persisted for up to 18 months after the final immunisation (282). Thus, ISCOMS appear capable of inducing long-term immunity in both rodents and primates.

The secondary T and B cell responses declined markedly between 8 and 12 months post-immunisation. This could reflect loss of antigen reactive cells, but may also have been partly due to the effects of ageing. This is known to influence immunological responsiveness (230) and, as around 25% of both primed and control mice had died by the end of my experiments, this clearly could have been an important factor.

I did not directly examine the persistence of the OVA-specific CTL responses primed by ISCOMS-OVA, as previous experiments in our laboratory had shown that these were relatively short lived, being virtually undetectable one month after priming (165, 229). However, other workers have reported the presence of antigen-specific CTL responses for up to 3-4 months after parenteral immunisation with viral proteins in ISCOMS (184, 235) and recent studies have shown that CTL responses primed by live virus persist for extremely long periods *in vivo*, even after adoptive transfer of primed cells into class I MHC-deficient recipients (283, 284). Thus, it seems surprising that ISCOMS-OVA did not induce CTL responses of longer duration in our earlier work and a more detailed re-examination of this issue would be useful. In addition, the use of alternative assays, such as precursor frequency analysis, may give a more accurate estimation of the longevity of the CTL responses primed by ISCOMS.

An important area which I did not have time to investigate was the longevity of immune responses primed by oral

administration of ISCOMS. With the exception of our earlier findings that the secretory IgA and systemic CTL responses were both transient (229), there is no information on the persistence of memory after mucosal immunisation with ISCOMS. Long-lasting priming of local and systemic KLH-specific B and T lymphocytes persisted for at least 8 months after oral immunisation with CT administered with KLH, indicating that solid memory can be achieved by this route (146, 285). Therefore, it would be important to study this directly after oral immunisation with ISCOMS.

While my results showed that ISCOMS primed for long-term memory responses, an investigation of the mechanisms responsible for the maintenance of immunological memory was beyond the scope of this study. However, it would be interesting to examine the phenotype of T cells primed by antigen in ISCOMS to assess their expression of molecules such as MEL-14 (L-selectin) and CD44 which are differentially expressed on naive and memory T cells (286, 287). It would also be interesting to employ the bromodeoxyuridine (BrdU) labelling technique, which allows cells which proliferate at the time of priming to be tracked *in vivo* (288), to explore the longevity of the different lymphocyte subpopulations primed by ISCOMS. Such studies would be assisted by the use of mice transgenic for an OVA-specific TcR, which provide a population of antigen-specific cells that can be adoptively transferred and monitored before and after priming *in vivo* (289). Experiments of this kind are now being established in the laboratory.

In summary, my results clearly demonstrated that a single parenteral immunisation with a small amount (10μg) of protein antigen in ISCOMS primed long-term B cell and T cell memory

responses, which persisted for a full year after priming. Although further work will be required to confirm and extend these findings, they enhance the potential of ISCOMS as future vaccine vectors.

Quil A Plays a Key Role in the Adjuvanticity of ISCOMS

In the latter part of this study, I investigated some of the aspects of the ISCOMS themselves which I thought might be important for their adjuvant activity. The class of compounds extracted from plant sources, termed collectively as saponins because of the detergent properties associated with them, have long been known to possess adjuvant activity (194). Quil A is a saponin fraction prepared from an aqueous extract of the South American soaptree, *Quillaja saponaria molina* (290). It is the adjuvant component of ISCOMS (159, 161) and therefore I thought it important to examine how much of the adjuvant properties of ISCOMS were attributable to the presence of Quil A, or whether the particulate structure of the ISCOM was also required for adjuvanticity.

i) Adjuvant Effects of Free Quil A

I first compared the adjuvant properties of free Quil A with that of Quil A incorporated in ISCOMS. Free Quil A induced the same range of immune responses as ISCOMS, but it was generally less efficient in this form than in intact ISCOMS. These responses included the priming of systemic IgG, DTH and class I MHC-restricted CTL responses, demonstrating that free Quil A has the ability to facilitate the entry of exogenous protein antigen into both

the endogenous and exogenous pathways of antigen processing *in vivo*. Free Quil A also primed for proliferative responses and the production of both T_h1 and T_h2 cytokines *in vitro*, indicating that it is a potent activator of both subsets of $CD4^+$ T cells *in vivo*. Free Quil A exhibited a similar range of adjuvant properties when administered orally, with the induction of local and systemic antigen-specific antibody responses and the priming of class I MHC-restricted CTL.

Others have reported similar findings on the adjuvanticity of parenterally administered Quil A, although direct comparison with ISCOMS in such a wide range of responses has not been performed previously. Many studies have demonstrated that Quil A augments humoral immune responses to both T-dependent and T-independent antigens (199, 200, 291-293). Although I did not examine the isotype distribution after immunisation with OVA plus free Quil A, it has been shown that this effect encompasses both IgG1 and IgG2a isotypes (294, 295). This is consistent with my observations that Quil A primed both T_h1 and T_h2 cytokine production *in vitro*.

The oral adjuvant properties of saponins have also been reported in a number of other studies and this route has the advantage that much higher amounts of saponins can be tolerated when administered orally (194). Co-administration of saponin and inactivated rabies vaccine led to 10-fold higher serum neutralizing antibody responses than elicited when the vaccine was fed alone (296) and led to enhanced lymphocyte proliferation in both the spleen and MLN *in vivo* (192), suggesting that saponins elicit local and systemic immunity. Unfortunately, I was not able to examine the priming of DTH, proliferative and cytokine responses induced

by feeding Quil A. However, subsequent work in the laboratory has shown that Quil A does indeed stimulate both subsets of $CD4^+$ T cells when administered orally.

My findings that Quil A allows priming of CMI responses, including proliferative responses and CTL responses, has been confirmed by other workers using both intact Quil A (192, 292) and an HPLC-purified fraction designated QS-21 (201, 297, 298). Furthermore, recent studies using liposomes containing the immunodominant K^b-restricted CTL epitope, OVA 257-264, showed that this peptide was unable to prime CTL when given in liposomes, unless the liposomes also contained Quil A (299). The antigen-specific CTL primed by co-administration of QS-21 and OVA exhibit an identical phenotype and specificity to those primed by ISCOMS-OVA, suggesting that the saponin alone is able to allow protein antigen to enter into the endogenous pathway of antigen processing and presentation (297). This may be related to the ability of saponins to bind cholesterol and create pores in cellular membranes (162, 196), which could facilitate the delivery of protein antigen into the cytosol. These findings suggest that the induction of antigen-specific CTL responses by ISCOMS may be largely due to the detergent properties of Quil A and are consistent with recent reports that detergents can promote the priming of CTL by soluble protein antigens (246, 247). An alternative explanation for the effects of saponin on CTL is that it may create a local inflammatory reaction, leading to uptake of cellular debris together with exogenous protein antigen by APC, which can result in CTL priming (215). This would be consistent with the findings that the priming of CTL responses by QS-21 requires the presence of phagocytic cells *in vivo* (300). Although similar to ISCOMS, the QS-21 induced

responses were not dependent on $CD4^+$ T cells (300) and therefore the precise pathway of CTL induction requires further study.

In my studies, incorporation within ISCOMS particles greatly augmented the adjuvant properties of Quil A. When compared with parenteral immunisation with ISCOMS containing 1μg of Quil A, around 5-10 fold more free Quil A was required to achieve the same levels of priming of antibody, DTH and CTL responses *in vivo*, as well as cytokine production *in vitro*. In addition, other workers have shown that multiple immunisations with QS-21 were required for the efficient induction of CTL (297), confirming my findings that, although not essential, incorporation within ISCOMS particles greatly increased the efficacy of CTL induction when compared with free saponin preparations. Similarly, oral immunisation with 25-50μg doses of free Quil A was required to provoke antibody and CTL responses equivalent to those obtained after immunisation with ISCOMS containing 5μg Quil A, confirming that the particulate nature of ISCOMS enhances the induction of immune responses at mucosal surfaces.

One exception to the general dose dependency of the effects of free Quil A was that *in vitro* proliferative responses were effectively primed by as little as 1μg of free Quil A and the highest dose (20μg) actually inhibited the priming of this response. The reasons for this paradox are not clear, but they may reflect differences in the kinetics of the *in vitro* proliferative responses and/or toxicity associated with higher doses of saponins (194). Alternatively, the high dose of Quil A may have induced unusually high levels of an inhibitory mediator such as IFN-γ. This idea is supported by the very large DTH responses seen in these mice and by the fact that mice immunised with free Quil A produced higher

levels of spontaneous IFN-γ than mice which received ISCOMS. As high concentrations of IFN-γ are known to be cytostatic *in vitro* (301, 302), this could have contributed to the low proliferative responses in mice given high doses of free Quil A.

ii) Activation of Innate Immune Responses by Quil A

Stimulation of non-specific inflammatory mechanisms has been suggested to be an important property of many adjuvants (231, 303) and this prompted me to examine the ability of free Quil A to activate macrophages and NK cells.

I found that Mϕ populations obtained from PEC elicited with either ISCOMS or free Quil A produced a small, but marked oxidative burst when stimulated with PMA *in vitro*. Although the magnitude of this response did not compare with that obtained using Mϕ elicited with a strong immunological stimulus (*C.parvum*), my results suggested that ISCOMS and free Quil A may activate at least some Mϕ in an immunological manner. In addition, others have demonstrated that ISCOMS and free saponin can enhance the expression of class II MHC molecules in an IFN-γ dependent manner (304, 305) and also that ISCOMS themselves induce the production of inflammatory cytokines such as IL-1 and IL-6 by macrophages (279, 306). It would be interesting to extend these studies by examining the expression of co-stimulatory molecules by ISCOMS and Quil A primed macrophages and also their production of cytokines, such as IL-12, which can influence the nature of the subsequent immune response (307, 308).

One difference between ISCOMS and free Quil A that emerged from my studies, was the ability of orally or parenterally administered free Quil A to stimulate non-specific cytotoxicity, as

evidenced by the marked lysis of EL4 cells. This effect was dependent on the dose of free Quil A administered and was associated with enhanced NK cell activity, as I also found that parenteral administration of free Quil A produced a small, transient increase in NK cell activity, which peaked around 3 days after injection. Importantly, neither enhanced NK cell activity or non-specific cytotoxicity were observed after oral or parenteral immunisation with protein antigen in ISCOMS. As with the non-specific cytotoxic responses, the NK cell activity was dependent on the dose of Quil A administered and was most marked when 10-20μg Quil A was injected. Similar findings on the induction of NK cells and non-specific cytotoxicity by saponin have been reported by others (192) and this could again be partly explained by the stimulation of IFN-γ release.

In this respect, it is notable that mice fed saponin alone showed a significant level of protection against intracerebral challenge with rabies (191, 309) and feeding saponin greatly potentiated the efficiency of parenterally administered rabies vaccine (309). Thus, oral administration of saponin appears to activate non-specific systemic immune mechanisms. Others have also noted the similarities in lymphocyte traffic and enhanced class II MHC expression after parenteral injection of saponin or IFN-γ, suggesting that the adjuvant activities of saponin are partly due to the induction of IFN-γ release (305).

Although they clearly have adjuvant effects on specific and non-specific immune responses, the precise mode of action and cellular targets of saponins remains unclear. As Quil A augments antibody responses to T-independent antigens and is effective in athymic mice, it seems likely that some of the adjuvant effects of

Quil A may be mediated directly through B cells (200). There is also evidence that saponins can increase the permeability of mucosal cells and infleunce macromolecular transport *in vitro* (310), an attribute that is also possessed by CT (238). Studies using radiolabelled antigens co-administered with Quil A could help to define the effects of saponins on the uptake and processing of soluble protein antigens at mucosal surfaces after oral immunisation and by systemic professional APC after parenteral administration.

When taken together, my studies indicate that although free Quil A shares many of their adjuvant properties, ISCOMS have a number of potential advantages for use as vaccine vectors. Incorporation within ISCOMS decreases the amount of Quil A required for effective priming, thus limiting any problems with toxicity. In addition, as antigen and adjuvant are physically linked in the ISCOM particle, the induction of potentially harmful non-specific immune responses is avoided. In contrast, the non-specific responses elicited by free Quil A suggest that caution must be used to avoid the possiblity that priming may occur to soluble self or dietary proteins which may be present at the time of administration of free Quil A.

Induction of Immune Responses with PLG Microparticles

The results described above showed that although Quil A was important for the adjuvant effects of ISCOMS, the structure of the ISCOM itself also played a role in enhancing the efficiency and specificity of immune priming. To investigate if this related to their particulate nature, I compared the oral and parenteral adjuvant

effects of ISCOMS with those of an inert particulate vaccine vector which did not contain a built-in adjuvant. These were PLG microparticles composed of the biodegradable polymer poly(lactide-co-glycolide).

I found that OVA entrapped in PLG microparticles could induce the same range of local and systemic immune responses as OVA incorporated in ISCOMS, but that higher amounts of antigen were required when the PLG microparticle vector was used. A single s.c immunisation with OVA in PLG microparticles primed for strong antigen-specific DTH responses and serum IgG responses *in vivo*, as well as for excellent OVA-specific proliferative responses *in vitro*, indicating that PLG microparticles can prime antigen-specific T and B cells *in vivo*. Although others had reported similar findings on the priming of humoral immune responses by PLG microparticles (108, 136, 311, 312), there had been little prior investigation of their effects on T cell priming. The proliferative responses indicated that PLG microparticles could prime $CD4^+$ T cells and the induction of IgG and DTH responses *in vivo* suggested that they primed both T_h1 and T_h2 subsets. However, an assessment of the IgG isotypes present and cytokines produced by PLG primed mice would be necessary in order to confirm this hypothesis.

I also observed that that PLG microparticles primed both humoral and cell-mediated immune responses when administered orally. My findings are consistent with other reports on the induction of antigen-specific salivary IgA and serum IgG antibodies after oral administration of protein antigens entrapped in PLG microparticles (137, 234) and underline the potential of PLG

microparticles for stimulating local and systemic immunity by the oral route.

A novel finding of my experiments was that PLG-OVA primed antigen-specific class I MHC-restricted CTL responses after either parenteral or oral administration. This had never been demonstrated previously using PLG microparticles and although I did not fully characterise the CTL in this study, they appear very similar to those found after immunisation with ISCOMS. These results confirm that the particulate structure of ISCOMS contributes to the potent induction of CTL responses and are consistent with the evidence noted earlier that particulate antigens may effectively stimulate class I MHC-restricted CTL (224, 250). In addition, it is known that PLG particles are phagocytosed rapidly *in vivo* after oral or parenteral administration (136, 137) and it would be interesting to look at the effects of macrophage depletion on the induction of CTL by PLG microparticles. The CTL responses induced by oral or parenteral immunisation with ISCOMS were higher than those induced by PLG, requiring fewer or lower doses of antigen. It was also impossible to detect CTL activity in PLG-OVA immunised mice without *in vitro* restimulation with antigen, whereas the lymph nodes of ISCOMS-OVA primed mice contained pCTL which could be activated by IL-2 alone. This suggests that OVA in ISCOMS primed a higher frequency of antigen-specific CTL and is also consistent with my findings that Quil A plays an important role in the induction of CTL responses by ISCOMS.

As noted above, ISCOMS appeared superior to PLG microparticles in inducing serum IgG responses, CTL responses and T cell proliferation, despite the fact that a 10-fold lower dose of antigen was used with ISCOMS. However, it cannot be concluded

with certainty that PLG microparticles have no potential for use as vaccine vectors. Their delayed release properties (233) make it likely that they will stimulate long-term memory *in vivo*. In fact, it has been shown already that PLG microparticles evoke long-term antibody responses which persisted for a full year after a single parenteral immunisation (313) and it would now be important to compare the kinetics of the primary and memory responses of all types induced by the PLG microparticles and ISCOMS. Economic factors such as ease and cost of manufacture, as well as practical conditions such as stability and possible toxic side effects, must also be taken into account when designing novel vaccine vectors. Nevertheless, my results suggest that ISCOMS may provide a more suitable vector for use with antigens which are not in plentiful supply, such as recombinant protein antigens or peptides.

In summary, my results highlight that particulate presentation of protein antigens in PLG microparticles greatly increases their immunogenicity for both B and T cells, when administered orally or parenterally. These results suggest that the exceptional adjuvanticity of ISCOMS can be attributed to the combined effects of their particulate structure and the presence of the saponin adjuvant Quil A.

ISCOMS as Oral Vaccine Vectors

The results I have presented in this thesis have extended previous observations on the induction of immune responses by ISCOMS and have highlighted their potential as oral vaccine vectors of the future. I have shown that a number of physico-chemical and immunological factors contribute to the unique adjuvant properties

of ISCOMS which are wider in scope and more effective than other conventional adjuvants.

Recent advances in molecular biology and in the understanding of microbial pathogenesis led to the idea of producing synthetic subunit vaccines, comprising purified protein antigens in an appropriate delivery system. However, as I have described earlier, an effective oral subunit vaccine vector must possess three essential properties. It must be able to withstand the harsh acidic and proteolytic environments of the stomach and small intestine; it must allow incorporated protein antigens to circumvent the normal regulatory mechamisms responsible for oral tolerance; and it must be able to effectively prime all forms of the immune response which would normally be provoked by natural pathogens, including class I MHC-restricted T cells. When taken together with the work of others, my results show that ISCOMS fulfill these requirements.

The first important property of ISCOMS is that they are particulate structures which present protein antigens in multimeric form. This may enhance the immunogenicity of orally administered protein in a number of ways. As well as being able to withstand the harsh environments (166), ISCOMS alter the uptake and processing of protein antigen, by targetting it to M cells overlying the Peyer's patches (241) and avoiding the induction of oral tolerance (229). In this way a relatively high concentration of immunogenic antigen can be achieved in the MALT inductive sites. Whether intact ISCOMS particles reach the systemic circulation after oral administration, either alone or after transport by phagocytic cells is not yet known and further studies using radio- or fluorescent-

labelled ISCOMS are required in order to clarify the precise uptake and processing route of orally administered ISCOMS.

Although important, the particulate nature of ISCOMS is only one factor involved in their potent adjuvant effects. Variable results have been reported on the effectiveness of other particulate vectors such as liposomes when administered orally (314-316) and there has been little investigation of T cell priming using these vectors. In addition, while my own studies on PLG microparticles confirmed other reports on their oral adjuvant properties (137, 234), ISCOMS were more efficient and required fewer feeds.

The main reason that ISCOMS are superior oral vaccine vectors to the other particulate vectors, is the presence of the saponin Quil A. My results showed that most of the adjuvant properties of ISCOMS could be reproduced using free Quil A. However, much higher doses of free Quil A were required in order to achieve equivalent levels of immune priming. In addition, incorporation within ISCOMS avoided the non-specific cytotoxic responses which I and others observed after the administration of free Quil A (192). Thus, physical linkage of adjuvant and antigen within the same particle greatly increased the efficacy and specificity of the immune responses induced.

The most important property of a vaccine vector is its ability to induce relevant immune responses and my results have confirmed that ISCOMS are capable of priming a unique range of local and systemic immune responses when administered orally. This included secretory IgA and systemic IgG responses, local and systemic $CD4^+$ T cell responses and the induction of class I MHC-restricted CTL responses. My findings emphasise the range, strength and dissemination of the immune responses induced by

oral or parenteral immunisation with ISCOMS. In particular, the priming of $CD4^+$ T cell-dependent proliferative responses and both T_h1 and T_h2 cytokine responses indicates the potent adjuvant effect of ISCOMS on $CD4^+$ T cells, which are central to the development of most protective immune responses *in vivo*. This suggests that ISCOMS could form the basis for vaccines against most of the pathogens likely to be encountered by the mammalian host. My findings with ISCOMS contrast with those reported for the other well-studied oral adjuvants, which induce a more restricted set of immune responses.

Cholera toxin (CT), perhaps the best studied mucosal adjuvant, can effectively prime local and systemic humoral immune responses against co-adminstered protein antigens (142, 143). In addition, more recent studies have shown that CT also has potent adjuvant effects on T cells when administered orally, as evidenced by the induction of local and systemic proliferative and cytokine responses (317, 318). Although contrasting evidence has been reported recently (318), it is commonly assumed that CT predominantly stimulates T_h2 type responses, favouring IL-4 and IL-5 production and the production of antibodies of the IgG1 and IgE subclasses (56, 154, 155). This bias may limit the broad applicability of CT as an oral adjuvant, especially in instances where the induction of T_h1 responses is essential for protection, such as intracellular parasitic infections like *Leishmania*, where development of T_h2 responses may actually exacerbate disease progression (42, 319). In addition, although one study has reported that CT can augment the induction of class I MHC-restricted CTL responses against non-viable antigens (320), the ability of CT to induce CTL responses when admininstered orally has not yet been

demonstrated. This activity is a prerequisite before CT can be considered as a potential vector for the induction of immunity against many viral infections.

Similar criticisms may applied to the use of live vaccine vectors such as atttenuated *Salmonellae*. Although these vectors can induce local and systemic humoral immunity against heterologously expressed antigens (110-112), there have been few reports on the induction of class I MHC-restricted CTL responses (321) and further work is required in order to address this issue. Furthermore, studies on the nature of the $CD4^+$ T cell responses primed by oral administration of *Salmonellae* have indicated that they preferentially stimulate the T_h1 subset of $CD4^+$ T cells, based upon the profile of cytokines produced *in vitro* and IgG2a antibodies *in vivo* (56, 115, 322, 323). When the other potential problems with live vaccine vectors, such as reversion to virulence and stability, are also taken into account, then it becomes clear that ISCOMS have several advantages as oral vaccine vectors over the other well studied candidates. In this respect it is also worthwhile to note that, in contrast to many other immunomodulatory strategies that prevent the induction of oral tolerance, oral immunisation with ISCOMS does not induce any intestinal immunopathology, despite the high levels of local and systemic priming elicited (165, 324, 325)

At present, it is premature to claim that ISCOMS represent perfect oral vaccine vectors, as a number of issues on the use of ISCOMS still have to be addressed. A major concern over the use of ISCOMS for human vaccination is the possible toxic side effects. In common with others (326), I found occasional toxicity with different batches of ISCOMS in mice and there is evidence that high

doses of saponins can be highly toxic *in vivo* (194, 201). However, saponins are much less toxic when administered orally and are already accepted as food additives (197, 198). Furthermore, I found ISCOMS much less toxic by the oral route and it is reported that mice are unusually susceptible to the toxic effects of ISCOMS, with larger species being more resistant (326). Indeed, recently, a clinical trial has begun on a human influenza ISCOMS vaccine. The long-term effects of ISCOMS immunisation have yet to be studied, although it is worth noting that in the long-term experiment that I performed, there was no difference in mortality between the ISCOMS primed and control groups for more than 12 months after priming. This problem is also faced by other oral adjuvants, most notably CT, where doses as low as 1-5μg are able to cause severe diarrhoea in humans (147).

These concerns over toxicity have prompted attempts to purify saponin fractions which retain adjuvanticity, but have reduced toxicity. QS-21 is one such saponin purified from crude Quillaja extract, which has potent inherent adjuvant properties (201). Other studies have shown that some of the non-toxic saponin fractions of Quil A retain the ability to form ISCOMS (166, 279, 327), but it remains to be seen whether these "new generation ISCOMS" will prove to be as effective parenteral and oral adjuvants as their predecessors. In addition, QS-21 enhances the adjuvant effects of alum, allowing it to stimulate T cells more efficiently and to induce cytotoxic T cells (298). Thus, non-toxic saponins may prove useful in augmenting the activities of conventional adjuvants. However, the effectiveness of such preparations when administered orally has not yet been investigated.

A further area for possible improvement is the optimisation of methods for incorporating non-hydrophobic nominal antigens in ISCOMS . Initial ISCOM preparations used viral membrane extracts, containing hydrophobic proteins which easily incorporated into lipophilic ISCOMS (159, 167). However, modifications of native proteins, by acidification or palmitification techniques have enabled a wide range of purified proteins including OVA, BSA, HIVgp120, cytochrome c and the C fragment of tetanus toxin to be incorporated within ISCOMS (164, 165, 203, 212). In most cases, these ISCOMS have potent immunogenicity when administered parenterally and the specificity of the immune responses induced, suggests that little epitope destruction or modification occurs after incorporation into ISCOMS. However, incorporation of proteins within ISCOMS can be technically difficult and the retention of immunogenicity after oral immunisation has been studied for only a few antigens.

Another interesting possibility is the idea of incorporating other immunological mediators into ISCOMS to enhance their adjuvant effects. For example, it may be possible to incorporate cytokines within ISCOMS or to incorporate monoclonal antibodies which could target the ISCOMS to DC or Mϕ. Incorporation of CT, or the binding subunit CT-B, within ISCOMS may further enhance their oral adjuvant properties.

In summary, my results have highlighted that ISCOMS have several advantages over existing oral adjuvants by combining the benefits of a stable particulate structure with a built-in adjuvant, Quil A. They induce an unparalleled range of local and systemic immune responses and if concerns over their safety for admininstration to humans can be satisfied then they may form the

basis of future oral vaccines against almost any class of pathogenic organism.

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