Mucosal Immunisation Using the Ovine Nasopharyngeal Route

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Abstract

The mucosal surfaces are a major site of pathogen entry and methods that stimulate the local immune response to provide a barrier to infection are highly desirable. The purpose of this study is to develop a novel intra-nasal vaccination strategy in sheep specifically targeting the mucosal-associated lymphoid tissue (MALT) in the nasopharyngeal tract. Initial studies demonstrated the location and composition of ovine nasal-associated lymphoid tissue, which was shown to be characteristic of an immune inductive site of MALT. Specialised epithelial cells with sparse irregular microvilli were revealed by electron microscopy within the follicle-associated epithelium (FAE). These cells were closely associated with lymphocytes in the underlying tissue and were characteristic of M cells, shown to be involved in the uptake of particulate antigenic material. Attempts to mark these M cells using lectins, alkaline phosphatase activity and antibodies against vimentin and cytokeratins proved unsuccessful. However, uptake of fluorescent microparticles into the epithelium could be demonstrated both *in vitro* and *in vivo*, suggesting that these M cells were functionally active. These initial studies suggested there was potential to stimulate an effective mucosal immune response by targeting ovine NALT with particulate antigen. A particulate delivery system using poly(D,L-lactide-co-glycolide) (PLG), a biodegradable polymer, was then developed to deliver antigen to MALT through the M cells. Firstly, microparticles within the appropriate size range were produced, and protein encapsulation into these microparticles was optimised using BSA as a model protein. Protein encapsulation and release studies were performed on microparticles made from low and high molecular weight PLG polymers, and finally the stability and functionality of encapsulated proteins from *Listeria monocytogenes* were determined. These results allowed the optimal methods for particle preparation to be chosen.

An intra-nasal vaccination trial against *Toxoplasma gondii* was then performed in sheep. Proteins were extracted from toxoplasma tachyzoites and incorporated into PLG microparticles using the methods developed above. Sheep were vaccinated intranasally with soluble or particulate toxoplasma antigen, with blank particles as a negative control, or were infected with toxoplasma oocysts as a

positive control. The potential for the use of cholera toxin as a mucosal adjuvant was also investigated. All sheep were challenged with an oral infection of toxoplasma oocysts at the end of the experiment. Sheep immunised with particulate toxoplasma antigen produced enhanced levels of both local and systemic antigen-specific IgA antibody. Some increase in systemic antigen-specific IgG antibody levels were measured in sheep immunised with particulate toxoplasma antigen and cholera toxin. After challenge with toxoplasma oocysts increased levels of both local and systemic IgG were measured more rapidly in all animals immunised with toxoplasma antigen, suggesting a secondary-type IgG response. Increased cellular immune responses and a corresponding increase in interferon gamma production were measured in sheep immunised with particulate toxoplasma antigens. A slight modification of the febrile response to toxoplasma infection could be observed in animals immunised with particulate toxoplasma antigen and cholera toxin, although none of the immunised animals were protected against the challenge infection. These studies have shown that the intra-nasal route stimulates both local and systemic immune responses, and shows promise as an effective route for mucosal immunisation.

Declaration

The work presented in this thesis was carried out at the Moredun Research Institute, Edinburgh. The experimental work and the interpretation of the results were carried out by the author. Contributions to the work in this thesis by colleagues are fully acknowledged in the text.

This work has not been nor is currently being submitted for candidature for any other degree.

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Abbreviations

αβ T cell	alpha beta T cell
ANOVA	Analysis of variance
AP	Alkaline phosphatase
APS	Ammonium persulfate
BALT	Bronchus-associated lymphoid tissue
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CNS	Central nervous system
CO_2	Carbon dioxide
Con A	Concanavalin A
cpm	counts per minute
CT	Cholera toxin
CTL	Cytotoxic T lymphocyte
DAB	3,3′-diaminobenzidine
DCM	Dichloromethane
DTE	Dithioerythritol
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAE	Follicle-associated epithelium
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FITC-BSA	Fluorescein istohiocyanate bovine serum albumin
g	gravity
G	gauge
GALT	Gut-associated lymphoid tissue
γδ T cell	gamma delta T cell

GI	Gastrointestinal
h	hours
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HBSS	Hank's balanced salt solution
HE	Haemotoxylin and eosin
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IEL	Intra-epithelial lymphocyte
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
IFNγ	Interferon gamma
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgG1	Immunoglobulin G1
IgG2	Immunoglobulin G2
IgM	Immunoglobulin M
IMDM	Iscove's modified Dulbecco's medium
i.p.	intra-peritoneal
kBq	kilo Becquerel
kDa	kilo Dalton
LLO	Listeriolysin O
LPS	Lipopolysaccharide
LT	Heat-labile toxin
M	Molar
M cell	Microvillous cell
Mab	Monoclonal antibody
MadCAM1	Mucosal addressin cellular adhesion molecule 1
MALT	Mucosa-associated lymphoid tissue

Med LN	Mediastinal lymph node
MEM	Minimal essential medium
MHC	Major histocompatibility complex
min	minutes
MLN	Mesenteric lymph node
MW	Molecular weight
ml	millilitre
mm	millimetre
μg	microgram
μl	microlitre
μm	micrometre
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NALT	Nasal-associated lymphoid tissue
NB	Natural blue
NG	Natural green
°C	Degrees Celsius
OD	Optical density
OPD	Ortho-phenylenediamine
OVA	Ovalbumin
PB	Phosphate buffer
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
P-F LN	Pre-femoral lymph node
pIgR	Polymeric immunoglobulin receptor
PLG	Poly(D,L-lactide-co-glycolide)
PMSF	Phenylmethylsulfonyl fluoride
PP	Peyer's patch
PT	Pharyngeal tonsil
PVA	Polyvinyl alcohol
REML	Restricted maximum likelihood
rovIFNγ	Recombinant ovine interferon gamma

RP LN	Retropharyngeal lymph node
rpm	revolutions per minute
RT	Room temperature
RTALT	Respiratory tract-associated lymphoid tissue
SAG1	Surface antigen 1
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide agarose gel electrophoresis
sec	seconds
SI	Stimulation index
SEM	Scanning electron microscopy
sem	Standard error of the mean
SIgA	Secretory immunoglobulin A
SPF	Specific pathogen-free
TCR	T cell receptor
TEM	Transmission electron microscopy
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TGFβ	Transforming growth factor beta
Th cell	T helper cell
TRITC	Tetramethyl rhodamine isothiocyanate
v/v	volume/volume
w/v	weight/volume
ZSF	Zinc salts fixative

CHAPTER 1

General Introduction

1.1 HYPOTHESIS

Intranasal immunisation in sheep can stimulate antigen-specific mucosal and systemic immune responses, involving both cell-mediated and humoral immunity.

1.2 THE MUCOSAL IMMUNE SYSTEM

The mucosal surfaces covering the intestinal, respiratory and urogenital tracts represent the interface between the host and environment, and have a combined surface area of up to $400m^2$ in humans (Brandtzaeg *et al.*, 1999). Most pathogenic organisms either inhabit or penetrate the mucosal membrane surface area, and diseases affecting mucosal surfaces remain the greatest cause of mortality and morbidity in both man and animals. The systemic immune response alone is not adequate to control mucosal infection and the induction of specific immunity at the site of pathogen invasion is desirable. Thus a large part of the immune system is dedicated to protection from infection at these vulnerable mucosal sites. This mucosal immune system is distinct from the systemic or blood-borne immune system, as it is tightly regulated to maintain the integrity of the mucosal barrier, and involves the majority (some 80%) of immunologically active cells. Recent observations support the idea that induction of mucosal immune responses is effective both in preventing infection at mucosal surfaces and in triggering a systemic immune response. The lymphoid tissues of the mucosal immune system are known as mucosa-associated lymphoid tissue or MALT, and are present throughout the mucosal surfaces. This term was introduced by Bienenstock (1978) because the lymphoid tissues of various mucosae possess a relatively uniform morphology and are thought to function in a similar manner. MALT is found along all parts of the gastrointestinal tract, in the oral cavity, along the upper and lower airways, in the urogenital tract, in the mammary glands and in the conjunctiva of the eye. MALT is characterised regionally depending at which mucosal surface it is located; the main MALT are the gut-associated lymphoid tissue (GALT), the bronchus-associated lymphoid tissue (BALT) and the nasal-associated lymphoid tissue (NALT). The following data are largely derived from studies in rodent models or humans, apart from where otherwise specified.

1.3 THE MUCOSAL IMMUNE RESPONSE

The mucosal surfaces are protected by both innate and adaptive immune systems. Innate mechanisms include the trapping of pathogens in mucus, the destruction of pathogens by low pH or enzymatic activity in the GI tract, and the production of proinflammatory cytokines e.g. IL-8 by epithelial cells (Jung *et al.*, 1995). Protection at mucosal sites due to adaptive immune responses is achieved to a large extent by secretory immunoglobulin A (sIgA), the most abundant antibody in the body and the predominant antibody at these locations (Conley & Delacroix, 1987). sIgA is resistant to endogenous protease activity, which makes it well suited to protecting the mucosa (Steward, 1971; Lindh, 1975). The mucosal immune system is separate from the systemic immune system, but antigenic material can be carried by the lymphatics and blood vessels from the mucosae to lymph nodes, spleen and bone marrow, where the appropriate systemic immune response can be initiated. However, specific IgA antibodies that can protect against mucosal challenge when they are present in mucosal secretions can be ineffective against systemic microbial challenge when injected systemically (Michetti *et al.*, 1992, Subbarao & Murphy, 1992).

The first step of a mucosal immune response may be the induction of helper T cells (Bjerke *et al.*, 1988). Once antigen is taken up by specialised epithelial cells termed M cells in the mucosal epithelium (discussed in Section 1.5), it is processed by antigen-presenting cells (APCs) and presented to T cells, which regulate the mucosal IgA response (Dunkley *et al.*, 1990) and stimulate the development of IgA-committed antigen-specific B cells. T cells are required to provide an initial signal to the B cell via cell contact, and by another signal provided by transforming growth factor β (TGF-β). TGF-β in mucosal tissue switches B cells from the expression of surface IgM to surface IgA while suppressing those cells responsible for IgG production (van Vlasselaer *et al.*, 1992; Lebman *et al.*, 1990). The T cell cytokines interleukin 4 (IL-4), IL-5 and IL-6 are necessary for the final differentiation of B cells to IgA-secreting cells in the lamina propria (Beagley, *et al.*, 1988, 1991).

Antigen-specific lymphocytes proliferate locally in mucosal germinal centres, migrate via the bloodstream and eventually 'home' to mucosal sites (McDermott & Bienenstock, 1979) where terminal differentiation into subepithelial plasma cells

occurs (Kraehenbuhl & Neutra, 1992; Mestecky & McGhee, 1987). In this way IgA-committed antigen-specific B cells become plasma cells in the mucosae that produce polymeric IgA antibodies. IgA is not only enriched in the lamina propria, but also secreted across the epithelial surface. Transport of IgA into mucosal secretions occurs via transepithelial transport, a unique selective process mediated by specific polymeric Ig receptors to ensure selective secretion onto mucosal surfaces (Apodaca *et al.*, 1991). Polymeric immunoglobulin receptors (pIgR) are present on the basolateral membranes of mucosal epithelial cells (Mestecky & McGhee, 1987). Dimeric IgA antibodies bind to pIgR, triggering internalisation and transport through the epithelial cell to the surface where the pIgR is cleaved to release the antibody at the mucosal surface. Part of the pIgR remains attached to the antibody and is known as the secretory component and may help protect the antibody from enzymatic cleavage. The majority of IgA released from plasma cells at effector sites is in the form of a dimer, two IgA molecules linked at the constant regions of their heavy chain by the J chain.

Secretory IgA specifically binds to luminal antigens and leads to neutralisation, agglutination and/or opsonisation (reviewed in Brandtzaeg *et al.*, 1989; Neutra & Kraehenbuhl, 1992). sIgA largely functions by binding and preventing contact of the pathogen with epithelial cells, and preventing attachment by blocking microbial surface molecules, a mechanism known as immune exclusion (Mestecky & McGhee, 1987; Kilian *et al.*, 1988; Tomasi, 1983). This is largely achieved by specific IgA against surface antigens or secreted toxins that can cross-link target macromolecules and micro-organisms, thus inhibiting motility and facilitating entrapment in the mucus and clearance by peristalsis in the gut or ciliated cells in the upper and lower respiratory tract. sIgA is highly glycosylated and can also interact with bacteria in a non-specific fashion and can neutralise microbial toxins. Binding of IgA to pathogens in the lamina propria may cause them to be exported out to the epithelial surface as immune complexes (Kaetzel *et al.*, 1994). IgA may also promote phagocytosis by binding to Fcα receptors and may have a role in the intracellular neutralisation of virus in epithelial cells (Mazanec *et al.*, 1992). Despite all these effector functions the induction of sIgA alone may yet be inadequate for protection (Ermak *et al.*, 1998).

T cells are also crucial for the induction of an effective mucosal immune response. Other cell-mediated mechanisms that have been found in MALT and may play a role in mucosal effector sites are cell-mediated cytotoxicity, antibody-dependent cytotoxicity involving IgA, natural killer cells, and functional cytotoxic T lymphocytes (CTLs) (Staats & McGhee, 1996; VanCott *et al.*, 1996; Klavinskis *et al.*, 1996).

1.4 ORGANISATION OF THE MUCOSAL IMMUNE SYSTEM

The mucosal immune system is anatomically and functionally divisible into discrete inductive and effector tissues, demonstrated in Figure 1.1. In simple terms, mucosal inductive sites are where foreign antigens are encountered and mucosal immune responses are initiated, and effector sites are the more diffuse collections of B and T lymphocytes, differentiated plasma cells, macrophages and other antigen-presenting cells in the lamina propria where the immune response is produced.

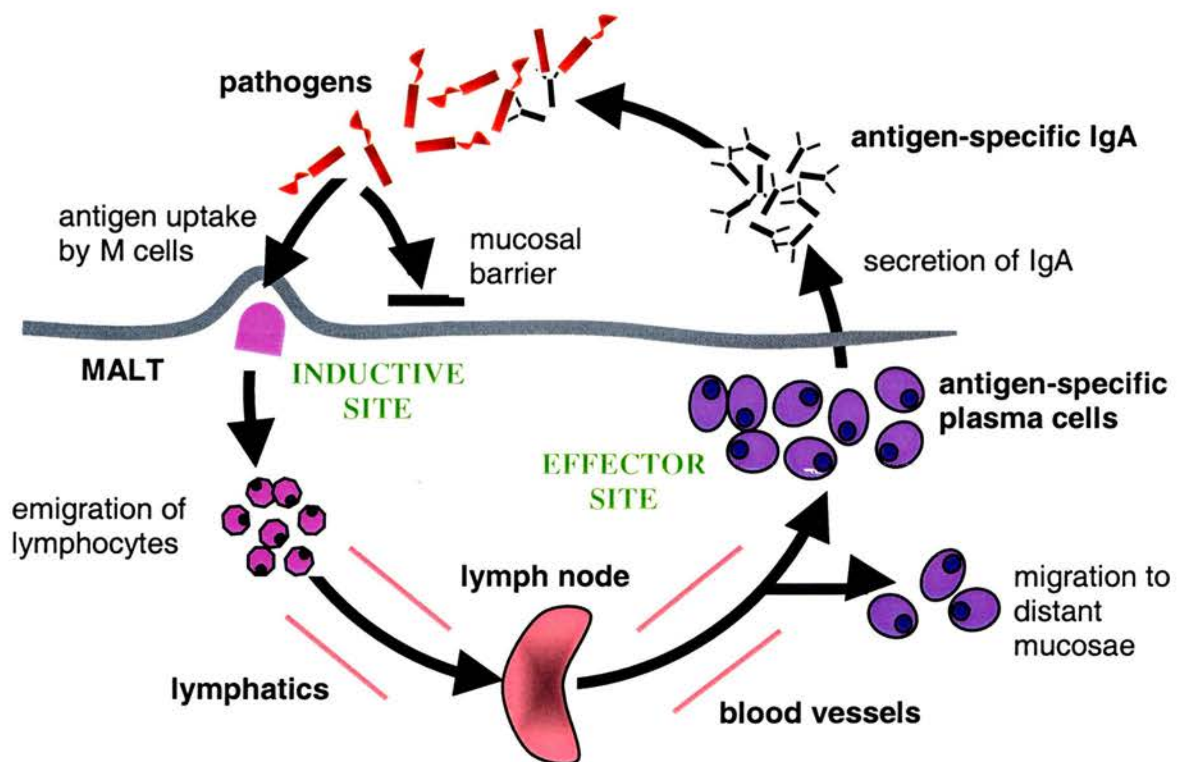


Figure 1.1 *Schematic representation of the mucosal immune system, adapted from Gebert (1997)*

1.4.1 Mucosal Inductive Sites

Inductive sites are localised in the organised lymphoid tissues along mucosal surfaces where antigen is encountered and initial responses are induced, and can consist of solitary or multiple lymphoid follicles, the most defined example of which is the Peyer's patch in the small intestine. The number and location of inductive sites varies among species and may change in individuals over time in response to mucosal exposure to antigens (Owen & Ermak, 1990). Inductive sites contain all cells necessary to induce and regulate immune responses, and have an organisational structure to facilitate the development of both cell-mediated and humoral immunity (reviewed in McGhee *et al.*, 1992; Mestecky & McGhee, 1987).

MALT is covered by an area of specialised epithelium known as the follicle-associated epithelium (FAE), that contains specialised M cells that transport antigen to the underlying lymphoid tissue (Owen & Jones, 1974). These cells are discussed in detail in Section 1.5. Underneath this epithelium, characteristic dome-like accumulations of lymphocytes, macrophages and plasma cells are present, strategically placed to respond to antigen transported to them by M cells. Beneath this dome area groups of lymphoid follicles of varying number lie in the subepithelial lamina propria or submucosa. The follicles are mainly populated by B lymphocytes, and contain follicular dendritic cells and macrophages. Follicles may form around follicular dendritic cells (MacDonald & Spencer, 1990) which function to present antigen to B cells. These distinct B cell follicles contain actively dividing cells in germinal centres, which are the site of expansion of B lymphocyte populations committed to the IgA isotype (Ermak & Owen, 1986). Most B cells in the periphery of the follicle and the corona express IgM surface receptors whereas B cells in germinal centres have switched to the IgA isotype (Lebman & Coffman, 1988; McGhee *et al.*, 1989; Murray *et al.*, 1987). Relatively few plasma cells are present in comparison with similar follicles in lymph nodes and spleen.

T cell-dependent areas containing all major T cell subsets and interdigitating dendritic cells are found surrounding and separating these B cell areas, in the interfollicular or parafollicular regions. In humans the T cells present are mature (contain a TCR/CD3 complex), and over 95% express the $\alpha\beta$ TCR while a small subset exhibit a $\gamma\delta$ TCR. In ruminants a higher proportion of $\gamma\delta$ T cells are present

than in humans or rodents (Hein & Mackay, 1991). Approximately 50-60% of the $\alpha\beta$ T cells are CD4+ cells (T helper cells) while the remainder are CD8+ cells (cytotoxic T cells). CD4+ cells are predominantly found in the dome region between the follicle and the epithelium and function to mediate B cells through cytokines (Coffman *et al.*, 1988; Stevens *et al.*, 1988) while CD8+ T cells are more abundant in the parafollicular zones (Bjerke *et al.*, 1988; Ermak & Owen, 1986).

1.4.2 Mucosal Effector Sites

Effector sites are derived from organised MALT and represent effector and memory lymphocytes that were generated from cells stimulated by antigen in inductive sites. After initiation of the immune response at the inductive site, activated lymphocytes and possibly antigen-presenting cells migrate via efferent lymphatics to local draining lymph nodes where further maturation and expansion of immune responses can occur. Activated 'effector' cells then enter the systemic circulation through the thoracic duct and migrate or 'home' to the submucosa and mucosal epithelium where they are selectively retained and perform their functions. Areas where dispersed and diffuse effector lymphocytes are located are known as effector sites or diffuse MALT, protecting the mucosal surfaces throughout the body (Kraehenbuhl & Neutra, 1992).

Effector cells include lymphocytes and plasma cells dispersed in the lamina propria and interstitial tissues of mucosae and glands, as well as intraepithelial lymphocytes (IELs). T cells are the most frequently isolated cell type seen in the lamina propria (Kanof *et al.*, 1988; Bull & Bookman, 1977), mainly with CD4+ surface characteristics (Kanof *et al.*, 1988), and B cells, represented mainly by IgA-producing plasma cells, are also extremely common. A smaller fraction of plasma cells in the lamina propria also produce IgM and IgG. Macrophages can also be found in significant numbers in the lamina propria and may be involved with antigen processing and presentation at this site.

Intraepithelial lymphocytes (IELs) located in the epithelium on the basolateral side of mucosal epithelial cells may also be effector cells. The precise functions of IELs are continuing to be defined, but evidence suggests that these cells have both effector and immunoregulatory functions and may form the 'first line of

defence' against mucosal infections (Simecka 1998). Most gut IELs from humans, mice, and rats are T lymphocytes, mainly with CD8+ surface characteristics. In nasal and respiratory mucosal surfaces in these species the number of IELs is smaller than in the gut, and CD4+ T helper cells are more abundant than CD8+ cytotoxic T cells (van der Brugge Gamelkoorn *et al.*, 1986; Winther *et al.*, 1987).

1.4.3 The Common Mucosal Immune System

The immune responses that arise as a result of stimulation by the mucosal route do not only induce a response at the induction site, but also bring about immune reactions detectable in the circulation and in the remote mucosal surfaces, due to the proposed existence of the 'common mucosal immune system' (Mestecky, 1987). This is a circular cell redistribution pathway for the dissemination or 'homing' of primed lymphocytes from mucosal inductive sites to widespread effector sites, linking immune responses at different mucosal sites. Hence antibody secretion may be detected at both the site of initial infection and at other mucosal sites, e.g. stimulation of the gut immune system may result in the production and detection of specific antibody in the trachea (Mestecky *et al.*, 1994). It has also been demonstrated that lymphocytes from the PP can repopulate lymphoid tissues of irradiated animals (see evidence in McGhee *et al.*, 1992). Stimulation of MALT is therefore likely to generate immune responses both locally and at more distant mucosal sites.

The migration or 'homing' of primed lymphocytes provides for widespread dissemination of effector and memory cells to distant mucosal surfaces to give body-wide surveillance. Homing occurs by the specific binding of molecules on the surface of the lymphocytes to molecules in the cells of the high endothelial venules (HEVs) and small flat venules in mucosal tissue. Stimulated memory cells express adhesion molecules or "homing receptors" specific for corresponding determinants ("addressins") on endothelial cells in mucosal and glandular tissues and therefore extravasate preferentially at such exocrine sites (Duijvestijn & Hamann, 1989; Salmi & Jalkanen, 1991). In human and mouse MALT this homing is largely mediated by the expression of the $\alpha 4\beta 7$ integrin on the surface of primed mucosal lymphocytes which is the receptor for mucosal addressin cellular adhesion molecule 1 (MadCAM-

1), the specific mucosal vessel adhesin localised on endothelial cells of mucosal capillaries (Streeter *et al.*, 1988). The interaction between $\alpha4\beta7$ and MadCAM-1 has also been shown to support, in the gut, the attraction of naïve B cells in inductive sites and the emigration of primed B cells in effector tissues. Most of the B and T cells stimulated in GALT migrate to distant intestinal lamina propriae due to prominent expression of $\alpha4\beta7$ integrin (Picker 1994; Cepek, *et al.*, 1993), and a certain proportion end up in mucosal tissues and exocrine glands outside the gut. Mucosal homing and extravasation may occur independently of the initial tethering mediated by L-selectin, which is the first step in the emigration of circulating leukocytes (Berlin *et al.*, 1995). L-selectin is the primary receptor used for preferential homing to peripheral rather than mucosal lymph nodes.

1.4.4 Compartmentalisation

There remains debate as to whether a regional specificity characterises this mucosal homing, since primed B cells are thought to migrate preferentially into effector tissues corresponding to the inductive site where they were initially stimulated (Butcher & Picker 1996). This compartmentalisation within the mucosal immune system may be due to the expression of different homing receptors and their ligands at different sites. Some evidence, in mice and pigs, shows a dichotomy between homing in the upper aerodigestive tract and the gut in that migration of NALT or BALT induced B cells to the gut is negligible in terms of generating sIgA antibody (Nadal *et al.*, 1991; Sminia *et al.*, 1989; VanCott *et al.*, 1994). In contrast considerable indirect evidence suggests that dissemination of primed polymeric IgA precursor cells takes place from NALT to regional secretory effector sites (Brandtzaeg, 1999). In general immune responses are stronger at nearby mucosal effector sites or those related in terms of lymph drainage (Moldoveanu *et al.*, 1995).

This regionalisation of the mucosal immune system may be attributed to a disparity in adhesion molecules expressed on the local microvascular endothelium and lymphoid cells primed in different MALT structures, and perhaps by different local chemokine profiles. Mucosal homing determinants appear to be shared among PP, MLN and intestinal lamina propria, but another set of endothelial molecules may be shared between the inductive sites in the upper aerodigestive tract. The $\alpha4\beta7$-

MadCAM-1 interaction has been clearly documented in homing from GALT to the intestinal lamina propria, although it is believed that other adhesion molecules are employed by immune cells primed in BALT and NALT. Some evidence suggests that $\alpha4\beta7$ is not an important homing receptor for lymphoid cells in the airways of humans (Picker *et al.*, 1994), mice (Wagner *et al.*, 1996), sheep (Abitorabi *et al.*, 1996) or cattle (Rebelatto *et al.*, 2000). The urogenital tract might employ similar molecular homing mechanisms as those of the upper aerodigestive tract and appears to receive primed immune cells from NALT, and GALT to some extent, as well. High levels of specific IgA and IgG antibodies are detected in cervicovaginal secretions of mice and Rhesus monkeys after intranasal vaccination with a variety of antigens (Brandtzaeg, 1997). This putative heterogeneity may mean there is compartmentalisation within the common mucosal immune system that must be taken into consideration in the design of the appropriate type of mucosal vaccine.

1.4.5 Follicle-Associated Epithelium

The majority of mucosal epithelia are simple and composed of a single cell layer in which highly polarised epithelial cells are joined by tight junctions, forming a barrier generally effective in excluding peptides and macromolecules with antigenic potential (Madara *et al.*, 1990). In the gut the enterocyte membrane is covered by the thick glycocalyx, a transmembrane coat composed of highly glycosylated, stalked glycoprotein enzymes that can be up to 500nm thick. The glycocalyx acts as the size-selective diffusion barrier, ensuring only soluble antigens are taken up into the epithelium (Frey *et al.*, 1996).

At all locations of MALT, the epithelium that covers the dome area has a modified structure compared to the surrounding epithelium; goblet cells and mucus are largely absent and it is heavily infiltrated by lymphoid cells. This is known as dome epithelium or follicle-associated epithelium (FAE) (Bockman & Cooper, 1973). In these specialised epithelial regions M cells (see Section 1.5) are interspersed among the enterocytes, functioning to take up particulate antigenic material. Certain modifications of the FAE serve to aid M cell function. Mucus overlying normal mucosal epithelia physically holds micro-organisms away from the mucosal surface, trapping particles so they can be removed either by peristalsis or

ciliated epithelial cells. However, the absence of mucus in the FAE means that interaction between pathogens and the epithelium can occur. In addition, FAE cells lack pIgR on their basolateral membrane and therefore do not participate in IgA secretion (Pappo & Owen, 1988). This leads to localised reduction in the capacity for the transport of IgA out of follicles, facilitating the increased uptake of antigens normally excluded from the remainder of the intestinal surface by transported sIgA.

1.5 M CELLS

In order for antigens and micro-organisms to be processed in mucosal lymphoid tissues and elicit a mucosal immune response they must first be transported from the mucosal surface into the mucosa (Owen, 1977; Neutra & Kraehenbul, 1992). For this to occur, intimate contact between MALT and the mucosal membrane is essential and fulfilled by the M cells, specialised epithelial cells that occur exclusively over organised MALT throughout the mucosal surfaces. M cells were first identified in rabbit appendix (Bockman & Cooper, 1973) and named in human Peyer's patches by Owen & Jones (1974), and the term M cell can refer to microfold or membranous cell.

M cells have been detected at all locations of GALT along the digestive tract: above isolated follicles (Rosner & Keren, 1984) and in the PP of the small intestine (Owen & Jones, 1974), in the appendix (Bockman, 1983) and at different locations of lymphoid tissue in the caecum (Gebert & Bartels, 1995), colon (Morfitt & Pohlenz, 1989; Fujimura *et al.*, 1992) and rectum (Liebler *et al.*, 1991). M cells have also been identified in the epithelium of BALT (Gebert & Hach, 1992) and in the crypt epithelium of the palatine tonsils (Olah & Everett, 1975; Gebert, 1995). The proportion of M cells in the FAE depends on the species and the location of the MALT, and can range from 10% in humans and mice, 50% in rabbits and up to 100% in the terminal ileum of pigs and calves (Jepson & Clark, 1998). The population density of M cells is thought to be affected by antigen stimulation and maturation as well as by species differences (Smith & Peacock, 1980; Smith *et al.*, 1987).

1.5.1 M Cell Structure

M cells are epithelial cells: they express large amounts of the cytokeratins 8, 18 and 19, typical for the intestinal simple epithelia (Gebert *et al.*, 1992, 1994; Jepson *et al.* 1992; Rautenberg *et al.*, 1996), and are connected to adjacent epithelial cells by desmosomes and tight junctions (Madara *et al.*, 1984; Gebert & Bartels, 1991). M cells have two key features that allow them to be distinguished from other epithelial cells. They have an irregular apical border consisting of short, scattered microvilli (Owen, 1977) compared to the typical brush border, and a basolateral lymphocyte-containing cytoplasmic pocket (Wolf & Bye, 1984; Jarry *et al.*, 1989). The intracellular lymphocytes are IELs contained within endosomes (Jarry *et al.*, 1989) and are thought to originate from the follicular marginal zone and mantle, and migrate into the FAE to interact with M cells. The phenotypes of the cells in the M-cell pocket have been described in rodents (Jarry *et al.*, 1989; Ermak & Owen, 1986), rabbits (Ermak *et al.*, 1990) and humans (Farstad *et al.*, 1994). CD4+ αβ TCR+ cells are most commonly observed, in close contact with naïve intraepithelial B cells expressing IgM+ and IgD+ surface receptors, and to a lesser extent, dendritic cells and macrophages (Farstad *et al.*, 1994). In this cellular network antigens are likely to be efficiently processed and presented. The other features that can be used to describe M cells are the lack of a rigid internal cytoskeleton, a poorly developed and greatly reduced glycocalyx only 20-30nm thick (Owen *et al.*, 1986), a basally located nucleus and apical cytoplasm rich in pinocytotic vesicles and mitochondria (Borghesi *et al.*, 1996). Figure 1.2 shows a schematic diagram of an M cell, including function.

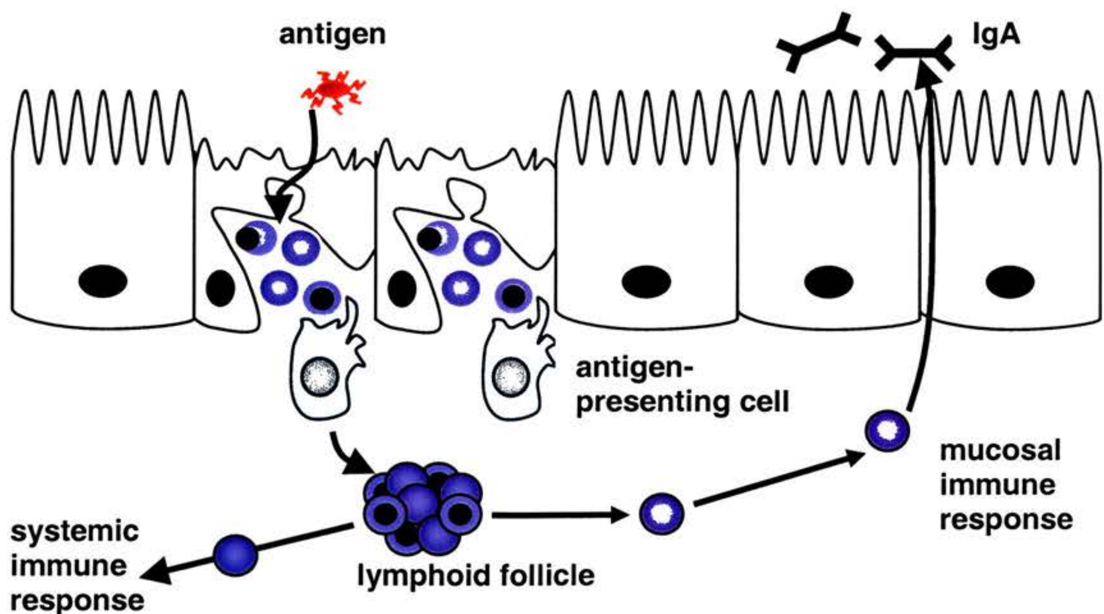


Figure 1.2 *Schematic representation of M cell structure and function, adapted from Gebert* et al. *(1996)*

1.5.2 M Cell Function

Evidence supports the theory that M cells function to transport antigens from the mucosal surface to the underlying antigen-presenting cells and lymphoid tissue, thereby initiating mucosal immune responses without compromising the integrity and protective functions of the mucosal epithelial barrier (reviewed in Neutra, 1998 and Hathaway & Kraehenbuhl, 2000). The passage of antigen through the M cell therefore represents the first necessary step to generate an antigen-specific response at the mucosal level. In this way antigens can be sampled in order to generate specific immune responses even before the pathogen invades the host tissue. M cells can transcytose soluble macromolecules, particles and even entire micro-organisms, and the magnitude of the antibody response to certain antigens in the mucosa has been shown to directly correlate to antigen capability to cross the epithelial barrier (Amerongen *et al.*, 1992). The M cell basolateral pocket diminishes the transcytotic distance antigen has to travel, and therefore makes transcytosis more efficient (Neutra *et al.*, 1996) so that it can occur in as little as 10 min (Neutra *et al.*, 1987).

M cells simultaneously contact cells of the lymphoid system with their basolateral membrane and the lumen with their apical membrane and therefore provide functional openings in the epithelial barrier through vesicular transport activity (Neutra *et al.*, 1996). The selective adherence of antigen to M cell surfaces may be due to two factors: the accessibility of receptors due to the lack of thick coat of complex glycoprotein enzymes, or the presence of unique receptors on the M cell surface, although studies with the cholera toxin B subunit suggest the former (Frey *et al.*, 1996). The initial contact with the M cell apical surface is thought to involve lectin-carbohydrate interactions, and the molecular structure of the M cell glycocalyx is critical for the adhesion of antigen to the cell surface (Neutra *et al.*, 1987). Uptake is then by receptor-mediated adsorptive endocytosis (Frey *et al.*, 1996) via clathrin-coated pits and vesicles (Neutra *et al.*, 1987), fluid-phase endocytosis (Gebert, 1995) in coated (Neutra *et al.*, 1987) or uncoated vesicles (Owen, 1977), or phagocytosis (Gebert, 1995) involving the extension of cellular processes and reorganisation of sub-membrane actin assemblies (Winner *et al.*, 1991). The method of endocytosis is likely to depend on the size and properties of the substance or particles, the charge and the surface receptors present. Following antigen uptake the trafficking of

vesicles or endosomes across the cytoplasm occurs, followed by exocytosis to the invaginated basolateral membrane that forms the intracellular pocket, where uptake by lymphocytes can occur (Owen, 1977; Neutra *et al.*, 1987). The current accepted view is that M cells merely transport and deliver antigens to the underlying lymphoid tissue, not process and present them, and therefore antigens remain intact following M cell transport (Neutra *et al.*, 1996). M cells contain very few lysosomes (Owen, Apple & Bhalla, 1986) and can therefore transport and deliver apparently unaltered micro-organisms to the extracellular pocket. However, rat intestinal M cells have been shown to contain acidic lysosomal-like vesicles in the cytoplasm and express MHCII on their basolateral membrane consistent with a role in antigen processing and presentation (Allan *et al.*, 1993).

M cells are also able to export secretory immunoglobulins to mucosal surfaces for defence (Neutra & Kraehenbuhl, 1992), offer a migration route for lymphoid cells moving from lymphoid tissue to the intestinal lumen (Regoli *et al.*, 1994) and have been shown to release IL-1 (Pappo & Mahlman, 1993). Release of IL-1 may be stimulated by LPS from bacteria as they are transcytosed by M cells. IL-1 can act as a costimulatory signal for T and B cell proliferation in MALT and in this way M cells may aid the immune response. A further additional function may lie in the selective adherence of immunoglobulins and IgA-antigen complexes in the lumen to apical membrane of M cells (Weltzin *et al.*, 1989). It is possible that the re-uptake of these complexes not excluded by sIgA could enhance or sustain the mucosal immune response to pathogens not cleared from the lumen. M cells could also exert a modulating effect in this manner by directing antigens to mucosal cells that display IgA receptors or by masking immunogenic epitopes.

A disadvantage of the mucosal antigen sampling system is that it renders the host vulnerable to invasive pathogens, and some micro-organisms exploit M cells as a route of host invasion to breach the mucosal barrier and establish local and/or systemic infections (Siebers & Finlay, 1996; Owen, 1994). Fourteen different pathogenic and non-pathogenic bacteria and three different viruses have been shown to selectively adhere to M cells, such as *Escherichia coli*, Salmonella, reovirus and poliovirus (reviewed in Neutra *et al.*, 1999). Despite the fact that these micro-organisms are highly immunogenic in the mucosal system, by the time an immune

response is in place pathogens may have spread far beyond the reach of secretory antibodies, e.g. entry of virus into target cells.

1.5.3 Isolating & Marking M Cells

Currently, no methods exist for obtaining cultured cells that exhibit M cell-like properties when grown in isolation from other cell types. M cells cannot be separated from surrounding epithelial cells because they are disrupted during enzymatic and/or mechanical separation due to their fragile structure and close association with lymphocytes and enterocytes (Pappo *et al.*, 1988; Pappo, 1989; Pappo & Mahlman, 1993). In addition, M cells are end-stage differentiated cells and therefore do not divide (Bye *et al.*, 1984) and cannot be cultured.

Several attempts have been made to generate monoclonal antibodies against M cell-specific epitopes, particularly in rabbits, however none of the antibodies produced seem to be specific for M cells or bound to M cells of other species (Roy *et al.*, 1987; Pappo, 1989; Pappo *et al.*, 1991). A number of cytochemical markers have been successfully used to identify M cells in a number of species in a number of locations, detailed in Table 1.1 (adapted from Jepson & Clark, 1998), although no universal 'M cell receptor' has been established so far. At present three main options for marking M cells exist, although there are problems associated with each method. Firstly, in the intestine, the apical membranes of gut epithelial cells contain high levels of digestive enzymes, such as alkaline phosphatase (AP), involved in the absorption of nutrients. M cells are associated with low expression of these brush border enzymes providing a negative M cell marker. However, there is extreme variability in AP content in all FAE cells (Gebert, 1997; Jepson *et al.*, 1993) and often no clear cut-off between positive and negative cells. Other cell types such as goblet cells, also have low alkaline phosphatase activity. This method is not applicable at some sites, e.g. rabbit caecal M cells have higher AP activity than enterocytes, and enterocyte AP activity in mouse caecum and rabbit appendix is generally low. Secondly, M cells demonstrate different apical membrane lectin binding, due to the fact that the M cell glycocalyx is composed of different glycoconjugates than other epithelial cells, and the glycosylation patterns are different. However, this appears to be very variable between species, strains and

sites, suggesting that there are various local adaptations in the molecular composition of M cell membranes. Lectins that label M cells may also label other cell types, although it may be possible to discriminate these cells by their morphology. No lectins have been found that identify with specificity for M cells in PP of rabbit, rat, guinea pig, cat or man (Gebert, 1997). In humans M cells show a more limited range of lectin-binding sites than enterocytes. Thirdly, M cells demonstrate atypical expression of intermediate filament proteins of the cytoskeleton, such as vimentin and cytokeratin, related to both cellular shape and transport function of epithelial cells (Ingber, 1993). The composition of the intermediate filaments closely correlates with the cell type (Moll *et al.*, 1982). For example, vimentin is characteristic of fibroblasts, macrophages and other mesenchymal cells, and may help to retain the integrity of M cells despite the deforming forces from migrating antigen. However the expression of these filaments can be very variable between species and other cell types may be labelled. Intermediate filaments of human M cells appear very similar to enterocytes and therefore this method has not been used successfully in humans. In rats and pigs the expression of the cytokeratins is merely higher than that in the surrounding epithelial cells, not exclusive to the M cells.

Method	**Specific Example**	**Species**	**Sites**	**References**
Enzyme activity	Alkaline Phosphatase	Rabbit Rat Mouse Human?	Peyer's Patches	Gebert, Rothkotter & Pabst, 1996
Lectins	UEA-1, EEA	Mouse	Small intestine	Jepson *et al.*, 1996 Sharma *et al.*, 1996
	BS-I-B4, EEA	Mouse	Caecum/ colon	Clark *et al.*, 1995
	UEA-1, WGA, BS-II	Rabbit	Caecum	Jepson *et al.*, 1993 Gebert & Hach, 1993
	UEA-1, HPA, VVA	Rabbit	Tonsil	Gebert, 1996
	BS-I-B4	Hamster	NALT	Giannasca *et al.*, 1997
Intermediate filament proteins	Vimentin	Rabbit	PP, caecum, BALT, tonsil, etc.	Gebert *et al.*, 1996
	Cytokeratin 18	Pig	PP	Rautenberg *et al.*, 1996
	Cytokeratin 8	Rat	PP	Kucharzik *et al.*, 1998

Table 1.1 *Methods of marking M cells, including location and species*

In humans the anti-sialyl Lewis A monoclonal antibody was shown to recognise most M cells in the PP FAE, labelling both the apical and sub-cellular membranes (Giannasca *et al.*, 1999). The identification of an M cell specific marker would aid their study and if atypically expressed may be a target for mucosal vaccines. Investigation for further M cell specific markers is ongoing, including the demonstration of their functional activity.

1.5.4 M Cell Development

In the gut, M cells originate at the periphery of lymphoid follicles from surrounding crypts that also supply enterocytes and goblet cells to adjacent villi (Bhalla & Owen, 1982). Restriction of M cells to FAE sites is likely to be due to the inductive influence of cells and/or secreted factors from the organised lymphoid tissues on epithelial differentiation, and it is generally accepted that local factors associated with MALT trigger M cell development. However, there remains some controversy concerning whether M cells are derived from fully differentiated absorptive enterocytes within the FAE upon interaction with immunocompetent cells (Smith & Peacock, 1992) or whether they are derived from a separate cell lineage and immature pre-programmed precursor cells originate from follicle-associated crypts that supply epithelial cells to both FAE and adjacent villi (Siebers & Finlay, 1996; Gebert *et al.*, 1996; Savidge, 1996) (Bye *et al.*, 1984; Fujimura *et al.*, 1990).

Gebert *et al.* (1999) demonstrated that certain types of crypts were specialised to produce epithelial cells for domes including M cells, suggesting that M cells represented a separate cell line deriving directly from undifferentiated crypt stem cells. Other studies have indicated that differentiated absorptive enterocytes can be induced to convert into M cells under appropriate conditions such as contact with lymphocytes or antigenic stimulation. Exposure to some bacteria, e.g. *Streptococcus pneumoniae* R36a (Meynell *et al.*, 1999) or *Salmonella typhimurium* in germ-free mice (Savidge *et al.*, 1991) can increase the number of M cells, showing that this phenotype is inducible. Kerneis *et al.*, (1997) showed that co-culture of a Caco-2, a human intestinal epithelial cell line with lymphocytes caused epithelial cells to acquire M cell like characteristics, including re-organisation of the brush border, and enhanced transport of inert particles and *Vibrio cholerae.* The M cell phenotype does

appear to be extremely plastic since a continuum of phenotypes between differentiated enterocytes and M cells has been observed (Kerneis & Pringault, 1999).

1.6 THE MUCOSAL IMMUNE SYSTEM IN SHEEP AND OTHER RUMINANTS

Most of the findings discussed above originate from studies in laboratory animal models. It is generally assumed that similar systems operate in large animals such as ruminants, although studies characterising the mucosal immune system in sheep and other ruminants are more limited. The main difference in the ruminant mucosal immune system is that IgG1 plays an important role in mucosal defence in addition to IgA (Butler, 1998), whereas the primary immunoglobulin for defence of mucosal tissues in mice and humans is IgA. Many of the biological functions attributed to IgA, such as neutralisation of virus, bacteria and toxins, are shared by IgG (Brandtzaeg, 1984). Although IgG molecules do not have a selective mechanism for secretion onto mucosal surfaces similar to IgA, serum derived and locally produced IgG may reach the mucosal surfaces by passive diffusion between epithelial cells or leakage through minor breaks in the mucosal epithelium (Brandtzaeg, 1984).

The ovine small intestine contains two distinct types of PP that differ in their ontogeny, cell composition and physiology (reviewed in Griebel & Hein, 1996). The continuous ileal PP are very different from classical MALT, and are not thought to function in mucosal immunity but to be a primary lymphoid organ, responsible for the primary generation of B cells and the antigen-independent diversification of the immunoglobulin repertoire (Reynaud *et al.*, 1995). The jejunal PP, on the otherhand, are the major site for the induction of mucosal immunity in the ovine gut. Jejunal PP are analagous to PP in rodents and humans (Larsen & Landsverk, 1986; Hein, Dudler & Mackay, 1989). They contain many CD4+ T lymphocytes in the follicles and numerous CD4+ and CD8+ T cells in the interfollicular areas (Aleksandersen *et al.*, 1990) and the FAE contains M cells shown to be functionally active in the uptake of bacilli (Momotani *et al.*, 1988) and ferritin (Paar *et al.*, 1992) in cattle. Vaccine vector delivery to jejunal PP results in both mucosal and systemic immune responses,

whereas delivery to ileal PP results in only systemic immune responses, showing that only the jejunal PP are effective as mucosal inductive sites (Mutwiri *et al.*, 1999).

In the sheep NALT distinct lymphoepithelium has been described in the pharyngeal tonsil, the bronchioles, and the nasopharynx (Chen *et al.*, 1989). The lymphoid tissue of the ovine pharyngeal tonsil and its associated epithelium are morphologically ready to cope with antigens in the extra-uterine environment at birth, but their full development and maturation appear to be dependent on postnatal antigen stimulation (Chen *et al.*, 1991). The amount and location of BALT and the presence of lymphoepithelium has been shown to be dependent on age, and may be influenced by antigenic stimulation (Anderson *et al.*, 1986). IgA has been shown to be the major immunoglobulin in the respiratory tract of sheep (Smith *et al.*, 1975). There are a large number of IgA plasma cells populating the mucosa (Scicchitano *et al.*, 1984) and the bulk of IgA, approximately 81%, is locally derived (Scicchitano *et al.*, 1986). However, the ovine respiratory tract is a poor source of IgA precursors, suggesting that the IgA plasma cell population may originate from distant mucosal sites. Thus the ovine respiratory tract is qualitatively similar to the intestine with respect to immunoglobulin synthesis. A smaller but significant local contribution of IgG1 and IgG2 also occurs, but most IgM and IgG are derived from the plasma.

1.7 MUCOSAL IMMUNISATION

Since the vast majority of pathogens establish infection in the host by initiating colonisation or invasion of a mucosal surface there is great interest in developing vaccination directly on these surfaces to induce local protective immune responses. Although most currently available systemic or parenteral immunisation strategies can clear systemic infections, they generally fail to elicit sIgA responses or local cell-mediated immunity and hence fail to protect mucosal surfaces (Mestecky, 1987). Immunisation via mucosal routes can however induce both systemic and mucosal immunity and protect against mucosal infections (McGhee *et al.*, 1992), clearing organisms, and preventing colonisation and invasion of mucosal surfaces and replication within the mucosal epithelium. Mucosal vaccines can also be used to treat systemic inflammatory diseases through the induction of antigen-specific mucosal tolerance (Eriksson & Holmgren, 2002). Furthermore, evidence suggests

that immunisation at one mucosal surface can generate sIgA production in the local as well as distal mucosal surfaces due to the existence of the common mucosal immune system. However the potential existence of subcompartmentalisation suggests that delivery of vaccine to the site of infection may be required to generate the most effective local immunity (Van Ginkel *et al.*, 2000) and may have a major impact on the choice of route for immunisation. The correct inductive site must be stimulated to induce effective and optimal immune responses at a particular effector site (Wu & Russell, 1997).

The success of mucosally administered vaccines is best exemplified by the human Salk and Sabin oral poliovirus vaccines (Salk & Salk, 1977; Sabin, 1984) that have been responsible for the near worldwide eradication of this disease. However, despite the clear advantages of mucosal immunisation almost all currently marketed vaccines are administered parenterally and only very few mucosal vaccines are commercially available at present. The limited success of mucosal immunisation is largely attributed to suboptimal mucosal stimulation (Holmgren, 1991), and many mucosal vaccines are limited due to poor long-term efficacy and the requirement for multiple doses to stimulate and maintain an immune response. Furthermore due to the complexities of mucosal immune regulation efforts to stimulate immunity may inadvertently induce tolerance instead.

Subunit vaccines are perceived to be safer and are favoured over live or attenuated vaccines but they tend to be less immunogenic when delivered mucosally, possibly because of their poor immunogenicity, low absorption efficiency or the induction of tolerance. Typically mucosal immunisation requires higher doses of antigen than systemic immunisation. The main barriers to effective immunisation are the enzymatic degradation of antigens, mechanical clearance of antigens from the mucosal surfaces, and low uptake efficiency of antigens by APCs. Enzymatic degradation leads to alterations in antigenic structure due to the loss of critical epitopes and irreversible conformational changes, and is a particular problem in oral vaccination, resulting in precipitation, loss of binding affinity to M cell surfaces, or presentation of inactive epitopes.

For successful mucosal immunisation antigen must be protected and delivered effectively to a mucosal immune inductive site by selective, efficient M

cell transport. A regime and route of immunisation must be chosen to induce appropriate protective responses at the desired mucosal site and preferably systemically as well. Induction of long-term immunological memory is also highly desirable. Several new approaches to mucosal immunisation have been proposed to address these issues, particularly for subunit vaccines. Most strategies combine the use of an efficient delivery vehicle to facilitate antigen transfer across membranes, with an effective mucosal adjuvant to enhance immune responses, given through the optimal route of administration.

The focus of this project is mucosal immunisation through the intranasal route, although numerous studies in mice and humans have shown that protective immunity can be elicited by immunisation through the oral, intranasal, intravaginal and intra-rectal routes of delivery (reviewed in Chen, 2000; Ogra *et al.*, 2001). More recent studies have focused on immunisation by mixed routes or a combination of mucosal and parenteral routes. Most attention has been paid to the mucosal delivery of protein antigens but more recently DNA vaccines are also being developed for administration at mucosal surfaces, and intranasal delivery in mice leads to rapid and even distribution of plasmid DNA throughout the body (Oh *et al.*, 2001).

1.7.1 Intra-Nasal Immunisation

The nasal mucosa is often the first point of contact for inhaled antigens, and organised lymphoid tissues are present in the upper respiratory tract of all species, either as NALT or tonsils (Lemoine *et al.*, 1998). When viewed in terms of its structural organisation and cell phenotype NALT in rodents bears a resemblance to GALT and BALT tissues (Kuper *et al.*, 1992), and recent studies have shown that it is a mucosal inductive site for humoral and cellular responses in the upper respiratory tract (Zuercher *et al.*, 2002). In addition, NALT in rodents and in human tonsils are covered with a specialised FAE containing M cells morphologically and functionally comparable to those found in the FAE overlying organised MALT in the gut (Kuper *et al.*, 1992; Karchev & Kabakchiev, 1984; Fujimura, 2000). NALT is therefore clearly a potential target for mucosal vaccination, and nasal delivery of vaccines offers the following key advantages over oral delivery.

- The nasal mucosa is a common portal of entry for many infectious agents and topical application of antigen may be the most efficient way of inducing a protective local immune response at the site of pathogen entry. Numerous studies have shown a correlation of protection against respiratory disease with high levels of specific IgA antibodies in nasal secretions (Wilkie, 1982; Hjerpe, 1990).
- The micro-environment of the nasal mucosa is less harsh on antigens compared with the oral route (Zhou & Po, 1991). It has a less acidic pH and a lower level of proteolytic enzymes compared to the gastrointestinal tract. This is particularly relevant in the ruminant system due to the harsh conditions of the four stomachs, particularly the rumen, that could potentially interfere with the integrity of orally delivered vaccine antigens.
- NALT is easily accessible and has a much smaller surface area and restricted location than the GALT, therefore the uptake of antigens is less problematic and lower doses of antigen are required, perhaps with less adjuvant. Antigen contact with NALT is also likely to occur much more rapidly than with GALT.
- The intra-nasal route has been shown to induce immune responses in a broader range of distant mucosal sites than the oral route, including the upper respiratory tract, the genital tract and the GI tract (Almeida & Alpar, 1996; Lemoine *et al.*, 1998; McGhee *et al.*, 1999), and nasal vaccination has emerged as the optimal vaccination route for the induction of genital antibody responses. This may be due to the fact that B cells generated after intranasal immunisation express a greater variety of homing receptors (including L-selectin, $\alpha 4\beta 7$ integrin and CD44) than B cells induced after oral or rectal immunisation (Quiding-Jarbrink *et al.*, 1995, 1997). The concept of the common mucosal immune system is thus supported and potential exists to develop nasal vaccines against infections occurring at distant mucosal sites.
- The nasal mucosa is highly vascularised (Watanabe *et al.*, 1980) and may facilitate absorption of antigens into the blood circulation, which may induce systemic immunity (Neutra *et al.*, 1996) as well as mucosal immunity.

The recent review by Davis (2001) lists some of the more important studies on the nasal administration of vaccines in recent years. Numerous studies have

shown that intranasal administration of various antigens, particularly with adjuvants, is an efficient way of inducing both mucosal IgA responses (saliva, nasal, tracheal, gut and vaginal washings) and systemic IgG responses (Lemoine *et al.*, 1998). Nasal vaccine delivery is superior to oral delivery in inducing specific IgA and IgG responses in the upper respiratory tract (Rudin *et al.*, 1999). In addition, Wu & Russell (1997a, b) have shown that intranasal vaccination is more effective than intragastric immunisation at generating earlier and stronger mucosal and systemic immune responses. Moreover, NALT may retain long-term memory, and intranasal priming in mice has been shown to induce a strong primary IgA antibody secreting cell (ASC) response in the NALT and an immunological memory that mediated a strong secondary type ASC response following intranasal boosting (Asanuma *et al.*, 1998). Most studies have been conducted in rodent models, where the administration of different vaccine formulations has established methods for increasing absorption via the nasal epithelium, protecting antigens from proteolytic degradation and inducing the desired type of immune response (McGhee *et al.*, 1992; Partidos, 2000). Many mouse studies show data in which the immune responses to nasally applied vaccine particles are almost equivalent to injections (Ugozzoli *et al.*, 1998; Greenway *et al.*, 1998). Some studies have been carried out in humans, but to date only a very few have utilised large animal models.

There is very little information available concerning the fate of antigens and the mechanisms by which local and systemic immunity are induced after intranasal administration. It is thought that antigen is taken up by M cells or APCs overlying NALT in rodents or the tonsils in humans, leading to the differentiation of NALT lymphocytes into IgA-secreting cells (Wu *et al.*, 1996; Quiding-Jarbrink *et al.*, 1995; Hata *et al.*, 1996; Aggerbeck *et al.*, 1997). FITC-labelled *Salmonella typhimurium* can be detected in mouse NALT 24 h after nasal immunisation, and viral mouse mammary tumour virus particles can efficiently reach the lymphoid cells of NALT and initiate infection after nasal administration (Hopkins *et al.*, 1998). Specific IgA and IgG secreting cells then appear in local lymph nodes, presumably due to the migration of either APCs or antibody-forming cells (Wu & Russell, 1993; Wu *et al.*, 1997). In rodents, specific IgA-secreting cells have also been detected at remote sites, such as the salivary glands or the lamina propria of the small intestine, and

specific IgG-secreting cells have been found in the spleen (Wu & Russell, 1993; Wu *et al.*, 1996; Wu *et al.*, 1997). In humans IgA-secreting cells do not enter intestinal mucosa suggesting there is some compartmentalisation of the human mucosal immune system (Quiding-Jarbrink *et al.*, 1995). The tonsil does play a role in generation of systemic immune responses as specific IgG secreting cells can be found in the serum following intranasal immunisation. Thus it appears there is a dichotomy in the degree of compartmentalisation of the mucosal immune system between rodents and humans. The degree of compartmentalisation of the ruminant mucosal immune system is unknown.

1.7.2 Mucosal Delivery Systems

Mucosal delivery systems have intrinsic adjuvant activity and are used to enhance the binding, uptake and half-life of antigens, as well as target the vaccine to mucosal surfaces, thereby increasing immune responses. They can be manipulated to incorporate variable amounts of antigen and/or adjuvants, used to protect antigen and minimise degradation and chemically modified at the surface to improve site specificity. Delivery systems may also be designed to provide sustained antigen release following uptake, resulting in prolonged stimulation compared to a single dose of antigen (Eldridge *et al.*, 1991; Maloy *et al.*, 1994).

Current delivery systems largely rely on improving antigen uptake by M cells, as this is thought to be a crucial step in the development of an effective mucosal immune response appears. Numerous living and non-living antigen delivery systems have been designed that incorporate antigens into particles (Michalek *et al.*, 1999; Ryan *et al.*, 2001) because M cells are more efficient in the endocytosis of particulate antigens than soluble antigens. It is also thought that the efficiency of mucosal vaccines can be improved by targeting to M cell-specific surface components. Living delivery systems include the use of live attenuated and commensal organisms as vectors for encoded antigens. Non-living vehicles used for improving the efficiency of mucosal vaccines include liposomes (Gregory *et al.*, 1986; Wachsmann *et al.*, 1986), ISCOMs (Mowat *et al.*, 1993) and biodegradable microspheres (O'Hagan *et al.*, 1989, 1991a,b; Eldridge *et al.*, 1991). Such non-replicating delivery systems not only enhance M cell transport, but protect the

antigen from degradation, and promote the appropriate type of immune response required for protection. The results discussed below relate to mouse models unless otherwise stated, and as far as possible examples using the intranasal route for vaccination have been used.

1.7.2.1 Replicating Delivery Systems

Recombinant bacteria or viruses genetically engineered to express protective proteins or epitopes from other pathogens can be utilised for mucosal delivery. These live vectors are attenuated to render them avirulent, but retain the ability to populate and invade mucosal surfaces and replicate *in vivo* to produce a large persistent immunogenic dose. The key advantage is that antigen is presented in the context of a live virus or bacterial infection, and protective immunity is produced against both the carrier organism and the pathogen for which it codes. In addition, these organisms may bind specifically to mucosal epithelial cells through receptor-ligand interactions. The main disadvantage is that it is difficult to construct stable live vectors that are safe in terms of non-reversion to more virulent organisms. Bacterial vectors used include Salmonella strains, *Mycobacterium bovis,* Bacille Calmette-Guerin (BCG), Streptococci, Lactobacilli, and Yersiniae (reviewed in Medina & Guzman, 2001). BCG of *Mycobacterium bovis* has received considerable attention as a vector system for intranasal immunisation (Stover *et al.*, 1991, Langermann *et al.*, 1994a,b), and more recently, a highly attenuated *Shigella flexneri* vector was used to deliver intracellularly plasmid-DNA-encoding measles proteins after intranasal immunisation (Fenelly *et al.*, 1999). The viral vectors most often used for mucosal delivery include vaccinia, adenovirus, pox viruses, yellow fever and poliovirus (reviewed in Morrow *et al.*, 1999; Olszewska & Steward, 2001). Adenoviruses show the most promise and have been shown to induce long-lasting memory cytotoxic T-cell responses in mucosal tissues (Gallichan & Rosenthal, 1996) and to enhance serum and sIgA responses to the encoded antigen (Gallichan *et al.*, 1993) after intranasal immunisation.

1.7.2.2 Non-Replicating Delivery Systems

The main non-replicating delivery systems used for mucosal vaccination are discussed below. Unless otherwise specified all have utilised specific proteins for immunisation. Other systems that have been used include proteosomes, meningococcal outer membrane protein vesicles that form hydrophobic complexes with antigens, and the use of surfactants or bioadhesive molecules to enhance contact of antigens with mucosal surfaces by altering the mucus layer or tight junctions.

1.7.2.2.1 Biodegradable Polymeric Particles

One approach to the mucosal delivery of vaccines involves the encapsulation of protein antigens into polymeric devices. Polymeric microparticles are designed to enhance the efficacy and immunogenicity of mucosally administered vaccines. They can protect antigens from degradation, concentrate them in one area of the mucosal tissue for better absorption, extend their residence time in the body, or target them to specific sites of antigen uptake. It is also possible to adsorb DNA onto positively charged microparticles for DNA vaccination at mucosal surfaces (Singh *et al.*, 2000). The ability of microparticles to enhance immune responses to entrapped antigens delivered by mucosal routes is considered to be a consequence of their targeting to MALT and uptake into M cells (Eldridge *et al.*, 1989). Particles may also deliver antigen directly to APCs. Factors controlling the absorption of particles include size, nature of polymer, zeta potential, vehicle, and coating with lectins or other adhesion factors (Delie, 1998).

A number of studies have been undertaken to quantify the optimum particle size for uptake into MALT. Most of these studies have examined the uptake of polystyrene particles in the rodent PP, and it is hoped that these results are applicable for other types of microparticle in other locations of MALT. However, binding and uptake of particles by M cells and enterocytes may have been over-estimated in many intestinal loop instillation experiments (reviewed in Delie, 1998). In the gut a number of cells may be capable of particle uptake using a variety of mechanisms but the majority of evidence suggests that the MALT of the PP are the predominant site of uptake for nanoparticles and microparticles (Hillery *et al.*, 1994; Desai *et al.*, 1996). The upper size limit for M cell uptake in PP to induce strong mucosal immune

responses has been suggested to be 10μm in diameter (Beier & Gebert, 1998; Eldridge *et al*., 1990; O'Hagan, 1998). More recent studies (Tabata *et al*., 1996; Jenkins *et al*., 1995) suggest that larger particles may translocate, but immune responses are usually induced against smaller particles, and particle size may determine the type of immune response produced. Smaller particles (<1μm) may induce both mucosal and systemic responses because they disseminate to systemic lymphoid tissue in the MLN and spleen, whereas larger particles 3-5μm may be retained in the PP and induce predominantly a mucosal immune response (Eldridge *et al*., 1990; Jenkins *et al*., 1995). Recently however, consensus has emerged that the optimum particle size for M-cell absorption should ideally be less than 1μm in diameter (Brayden & Baird, 2001). 600-750nm fluorescent latex are rapidly transported into rabbit PP by M cells and released into lymphoid area of dome (Pappo & Ermak, 1989; Jepson *et al*., 1993c; Ermak *et al*., 1995). The number of nanoparticles (10-1000nm) which cross the intestinal epithelium is greater than the number of microspheres, and both M cells and conventional epithelial cells may be involved in the transport (Desai *et al*., 1996; McClean *et al*., 1998; Jung *et al*., 2000).

The most appropriate microparticle size for intranasal administration remains to be determined (Rebelatto *et al*., 2001b) but there is good evidence to indicate that following intranasal delivery and absorption, microparticle translocation to local immunoresponsive tissues may occur (Kuper *et al*., 1992). Following intranasal administration in rats the uptake of polymeric particles has been demonstrated (Alpar *et al*., 1994), including the uptake of 1.0μm fluorescent polystyrene latex and 1.7μm PLG microspheres into NALT and draining cervical lymph nodes (Carr *et al*., 1996; Ridley Lathers *et al*., 1998). The transport of nanoparticles across the nasal membrane is due mainly to transcellular transport mechanisms by NALT, especially the M-cells, but also some paracellular transport (Brooking *et al*., 2001). Smaller particles (20nm) showed greater uptake than larger particles (1000nm). Nothing is currently known about the potential for uptake of microparticles in the ovine nasal cavity.

Polymer composition also has an effect on particle uptake and in general the uptake of particles prepared from hydrophobic polymers seems to be higher than particles made with more hydrophilic surfaces (Eldridge *et al*., 1990). Hydrophobic

particles have a higher association with M cells and adhere much more avidly to M cells than to enterocytes (Jung *et al.*, 2000).

To further improve particle uptake, strategies to specifically target particles to M cells may be employed. Lectin-binding studies have suggested that M cells display regional and species variation in their glycosylation state (Clark *et al.*, 1993, 1995; Giannasca *et al.*, 1994). Hence the lectin-conjugation of proteins or lectin-coating of particles or liposomes can enhance M cell transport and subsequent immune responses (Jepson *et al.*, 1996; Chen *et al.*, 1996; Giannasca *et al.*, 1997; Ermak & Giannasca, 1998). This has been demonstrated in the mouse PP (Giannasca *et al.*, 1994; Clark *et al.*, 1995; Foster *et al.*, 1998) and in hamster NALT (Giannasca *et al.*, 1997). An alternative approach is to use adhesive molecules that are used by bacteria for mucosal colonisation. For example fibronectin-binding protein I, which is responsible for mediating the binding of *Streptococcus pyogenes* to epithelial cells, enhances immune responses to conjugated ovalbumin after intranasal immunisation (Medina *et al.*, 1998). M cell specific monoclonal antibodies have also been used to increase uptake of fluorescent polystyrene microspheres by M cells in intestinal loops in rabbits (Pappo *et al.*, 1991). sIgA also exhibits selective adherence to M cells (Weltzin *et al.*, 1989) and may be used as a vaccine delivery vector for foreign epitopes (Corthesy *et al.*, 1996).

Many different types of biodegradable polymers have been studied for mucosal delivery, but in recent years, the principal polymers used for the preparation of microencapsulated vaccines have been the aliphatic polyesters, the poly(lactide co-glycolides) (PLGs). PLG polymers are biodegradable and biocompatible, and have been used in humans for many years as suture material and as controlled release drug delivery systems (Yamaguchi & Anderson, 1993), and offer the following advantages for mucosal vaccination:

- The PLG polymeric sheath forms a protective coating for the antigen from the harsh external mucosal environment.
- PLG polymers undergo non-enzymatic hydrolysis producing lactic and glycolic acids without causing any inflammation, facilitating controlled release of the antigen.

- Different ratios of polylactide and polyglycolide or different molecular weight polymers can be used to manipulate the release rate of the entrapped antigen to provide a controlled release delivery system and reduce frequency of vaccination required.
- The size can be easily manipulated and used to favour rapid uptake across epithelium or prolonged retention over the mucosal lymphoid tissue.
- Immunogenicity studies using encapsulated viruses (Marx *et al.*, 1993), proteins (Eldridge *et al.*, 1991), peptides (Partidos *et al.*, 1996; Men *et al.*, 1996), or DNA (Jones *et al.*, 1997) have confirmed the immunopotentiating properties of PLG microparticles. Immune responses are often protective against re-infection.
- PLG microparticles can induce cell-mediated immunity as well as humoral immunity following mucosal immunisation with proteins. A Th1 response is preferentially induced (Michalek *et al.*, 1999).

The intranasal delivery of antigens entrapped in particles has been more successful than oral immunisation, perhaps due to the diluting effects of the gut, and has been shown to induce protective immunity against pathogen challenge. Following intranasal administration microparticle-delivered antigens appear to activate the NALT and draining lymph nodes in rodents (Heritage *et al.*, 1998). Intranasal administration of tetanus toxoid adsorbed onto or encapsulated in PLG microspheres resulted in enhanced mucosal and systemic antibody responses to the antigen (Almeida *et al.*, 1993; Alpar & Almedia, 1994). Protective immune responses to pathogen challenge have been produced via intranasal administration of PLG encapsulated human parainfluenza type-3 virus (Ray *et al.*, 1993), *Bordetella pertussis* (Cahill *et al.*, 1995; Shahin *et al.*, 1995), ricin toxoid (Yan *et al.*, 1996) and *Streptococcus pneumoniae* (Trolle *et al.*, 2000). The induction of strong cell mediated immunity was demonstrated using a lipidated HIV-1 gp120 peptide (Moore *et al.*, 1995) or ovalbumin (Simmons *et al.*, 1999). The long-term *in vivo* release of protein from PLG microparticles has been demonstrated after a single nasal immunisation of glutathione S-transferase of *Schistosoma mansoni* which resulted in sustained antigen-specific IgG and IgA responses (Baras *et al.*, 1999). A comparative study using gD2 from herpes simplex virus with different adjuvants and delivery systems concluded that the intranasal immunisation with proteins entrapped in PLG

microparticles was the most effective way of enhancing specific antibody responses in various mucosal secretions, while also inducing strong systemic antibody responses (Ugozzoli *et al.*, 1998). Furthermore, recent developments in the aerosolisation of PLG microparticles offer the potential of delivery of microencapsulated vaccines to the lungs (Edwards *et al.*, 1997; Masinde & Hickey, 1993).

The problems associated with the use of PLG microparticles mainly concern the harsh production methods. PLG only dissolves in organic solvents, and exposure of antigens to these solvents during microencapsulation may result in antigen denaturation. Vaccine antigens may also be exposed to high shear, aqueous-organic interfaces, cavitation and localised elevated temperatures during microencapsulation. However, despite these harsh conditions a number of proteins have been successfully entrapped in PLG microparticles with full maintenance of structural and immunologic integrity (O'Hagan, 1998). The efficacy of microparticle uptake by the mucosal epithelium may also be a problem, and PLG microspheres are known to have an inherent weak capability to bind intestinal PP M cells (McClean *et al.*, 1998). Finally, stability of entrapped antigen during storage or production of sterile preparations for human use may also be of concern. Current research efforts tend to focus on the modification of PLG particles to improve uptake and combine the use of particles with mucosal adjuvants.

1.7.2.2.2 Other Particulate Delivery Systems

A number of water-soluble biodegradable polymers have been devised to circumvent the problems of using organic solvents in the antigen encapsulation process. These include chitosan (Illum, 1998), starch (Artursson *et al.*, 1986), dextran (Schroder & Stahl, 1984) and alginate (Bowersock *et al.*, 1999). Lipid particles including liposomes and ISCOMs have also been successfully used for the mucosal delivery of antigens. Liposomes are spherical vesicles made of concentric lipid bilayers encasing an aqueous core, made from cholesterol and phospholipids and have been shown to increase the effectiveness of mucosally delivered protein and peptide antigens (Vadolas *et al.*, 1995). However they are generally less effective than microspheres, and coadministration of antigens and adjuvants in liposomes can

significantly improve immune responses (Baca-Estrada *et al.*, 1999; Watarai *et al.*, 1998; Harokopakis *et al.*, 1998; Okada *et al.*, 1997). ImmunoStimulating COMplexes (ISCOMs®) (Morein *et al.*, 1984) are typically negatively charged, symmetrical colloidal particles with an open cage-like structure in the size range of 30-100nm composed of Quil A (a saponin extract from *Quillaja saponaria* Molina bark), cholesterol, phospholipids and associated antigen. ISCOMs are efficient mucosal adjuvants that induce strong cytotoxic T-cell responses typical of the Th1-type response (Mowat & Donachie, 1991). The nasal route seems to be the most effective and promising for mucosal vaccination using ISCOMs (Hu *et al.*, 2001).

1.7.2.2.3 Transgenic Plants/ Edible Vaccines

Among the newest technologies for the mucosal delivery of antigens are the edible vaccines. This strategy involves the synthesis of transgenic plants, by incorporating genes encoding antigens from pathogenic organisms into plant species, first introduced by Mason *et al.* (1992). The incorporation of vaccine into feed remains an attractive strategy for the oral vaccination of domestic animals.

1.7.3 Mucosal Adjuvants

Most antigens tend to induce poor immune responses when given mucosally and mucosal adjuvants or immunostimulants are often required to boost mucosal and systemic immunity, and to prevent the induction of tolerance. An effective delivery system may be combined with an adjuvant to optimise mucosal immune responses, and an alternative to targeting antigen-loaded particles to M cells is to formulate antigen-loaded particles with adjuvants, or to co-entrap an adjuvant with the protein in an untargeted or targeted particle. The most recent mucosal adjuvants have been designed based on bacterial toxins and their derivatives, CpG-containing DNA, and different cytokines and chemokines, and are discussed below. There are a number of other potential mucosal adjuvants, such as plant lectins. Mistletoe lectin 1 is a strong mucosal adjuvant, perhaps due to its receptor-binding specificity combined with its high immunogenicity and immunomodulatory activity (Lavelle *et al.*, 2001).

1.7.3.1 Bacterial Enterotoxins

In experimental animal models the most potent mucosal adjuvants under investigation are the major enterotoxins, cholera toxin (CT) from *Vibrio cholerae* and heat-labile toxin (LT) from *Escherichia coli.* Both are multi-subunit macromolecules composed of two structurally, functionally and immunologically separate A and B subunits (Spangler, 1992; Gill, 1976; Gill *et al.*, 1981). The CTB subunit consists of 5 identical 11.6kDa peptides that bind to GM1 ganglioside receptors on the apical surface of mammalian epithelial cells (van Heyningen *et al.*, 1976; Frey *et al.*, 1996). Following B subunit binding the single toxic A subunit reaches the cytosol where it exerts ADP-ribosyltransferase activity (Spangler, 1992; Rappuoli *et al.*, 1999) leading to the elevation of cAMP resulting in diarrhoea (Field *et al.*, 1989). *V. cholerae* is known to bind to M cells resulting in efficient sampling by the mucosal immune system and a strong secretory immune response (Jertborn *et al.*, 1986; Svennerholm *et al.*, 1984).

Both enterotoxins are strong mucosal immunogens and act as effective adjuvants to mucosally co-administered antigens by enhancing antigen-specific serum IgG and serum and mucosal IgA antibody responses (Dickinson & Clements, 1995) and helper and cytotoxic T cell responses (Partidos *et al.*, 1996, 1999; Rappuoli *et al.*, 1999). Particular attention shall be paid to CT which has been used widely for intranasal vaccination with protein antigens, bacterial components, viruses or virus-related peptides, inducing mucosal IgA responses and/or protection (Yamamoto *et al.*, 1997, 1998; Imaoka *et al.*, 1998; Kurono *et al.*, 1999; Reuman *et al.*, 1991). The adjuvanticity of CT has been demonstrated with antigens such as KLH, *Helicobacter pylori*, *Campylobacter jejuni*, *Pseudomonas aeroginosa*, ovalbumin, and BSA.

The mechanism of adjuvanticity is thought to be a multistep phenomenon resulting from the interaction of the toxin with different cell types in the mucosa. The first critical step is binding of CT to mucosal epithelial cells, which increases the mucosal barrier permeability (Lycke, 1997) and allows CT to enter the submucosa where it exerts immunomodulatory effects on different cell types. CT is able to induce the maturation of DCs and stimulate antigen presentation (Anastassiou *et al.*, 1990; Bromander *et al.*, 1991) and IL-1 production by APCs (Bromander *et al.*,

1991). CT leads to altered regulation by T cells, stimulation of B-cell switching to IgA and IgG and possibly enhancement of B cell clonal expansion (Holmgren *et al.*, 1994; Snider, 1995). Both mucosal and systemic immune responses are thought to be mediated via a Th2 cell-dependent pathway perhaps due to the ability of CT to inhibit IL-12 production (Gagliardi *et al.*, 2002).

A major concern with the use of CT and LT, particularly in humans, is their innate toxicity, and consequently much effort has focused on dissociating their adjuvanticity from their toxicity. The first approach is to exploit the use of the non-toxic B subunit alone. The B subunit is likely to function as more than a simple carrier protein (Dertzbaugh & Elson, 1993) and mucosal exposure of native CT results in antibodies that are almost entirely restricted to CTB (Elson & Ealding, 1984). However both CTB and LTB subunits have been shown to be relatively poor adjuvants compared with the holotoxins (Lycke *et al.*, 1992). CTB has been reported to act as an adjuvant in some cases (McKenzie & Halsey, 1984; Tamura *et al.*, 1988) but not in others (Czerkinsky *et al.*, 1989; Liang *et al.*, 1988; Lycke & Holmgren, 1986). These conflicting results may be due to the antigen used, the method of conjugation, the dosage and route of immunisation, and whether CTB preparations were contaminated with trace amounts of holotoxin.

An alternative approach is the genetic detoxification of CT and LT, which has been relatively successful. Site-directed mutagenesis has permitted the generation of CT mutants that have reduced toxicity but which retain significant adjuvanticity (Pizza *et al.*, 2001). In general, these mutants contain single amino acid substitutions in the active site of the A subunit resulting in loss of ADP-ribosylating activity (Douce *et al.*, 1995). Such mutants of CT have been found to exert an adjuvant effect to intranasally co-immunised pneumonococcal surface protein A, resulting in protection (Yamamoto *et al.*, 1998). However, there is concern that the ADP-ribosylating activity is important for adjuvanticity (Agren *et al.*, 1999) and that it can add to the potency of the toxins as adjuvants for the induction of mucosal immune responses (Douce *et al.*, 1999). In general the use of enzymatically inactive mutant toxins and B subunits alone is more effective following intranasal than oral immunisation (Russell *et al.*, 1999). To further enhance adjuvanticity of toxin

mutants or B subunits, proteins may be coupled to the adjuvant or administered in association with a delivery system.

1.7.3.2 CpG Motifs

CpG motifs are components of bacterial DNA that have been shown to induce T-cell independent B cell proliferation, and to activate monocytes, macrophages and dendritic cells (Davis *et al.*, 1998; Hartmann *et al.*, 1999). They are specific single-stranded oligonucleotide sequences containing unmethylated cytosine-phosphate-guanosine dinucleotides. The adjuvanticity of CpG-DNA results from the binding of the CpG-rich DNA to the Toll-like receptor 9, and is associated with the induction of both pro-inflammatory and Th1-inducing cytokines and chemokines, and the induction of MHC and costimulatory molecules on APCs. The resulting immune responses in mice are Th1 dominated with high levels of CTLs, IFNγ production and IgG2a antibody production (Eriksson & Holmgren, 2002). CpG motifs can enhance both antigen-specific serum IgG and sIgA responses when administered nasally with recombinant hepatitis B surface antigen (McCluskie & Davis, 1998), or formalin-inactivated influenza virus (Moldoveanu *et al.*, 1998).

1.7.3.3 Cytokines

The cytokine environment at the site of antigen delivery plays a critical role in the induction of immune responses, and activation of the appropriate phenotype of CD4+ T-cells (Th1 or Th2) after intranasal immunisation is an important priority. Therefore instead of adding an adjuvant to induce the appropriate Th1 or Th2 cytokines for immunity a new approach is the direct addition of the desired type of cytokine, either as protein or coding DNA. IL-12 is most commonly used for Th1 responses, and IL-6 for Th2 responses (Mosmann & Coffman, 1989).

1.8 MUCOSAL TOLERANCE

The major obstacle to mucosal immunisation is that mucosally delivered antigens may induce tolerance rather than the desired protective immune response. Mucosal tolerance is the specific systemic hyporesponsiveness that arises after mucosal administration of an antigen, resulting in a diminished capacity to develop

an immune response when re-exposed to the same antigen (Challacombe & Tomasi, 1980). It is an important immunological phenomenon that prevents the body from overreacting to harmless antigens encountered at the mucosal surfaces and is considered a useful approach to prevent or treat autoimmune diseases.

Mucosal tolerance is an active immunological process and is mediated by more than one mechanism. It is characterised by a lack of peripheral immune responses upon subsequent systemic challenge with the same antigen, but mucosal immune responses are usually intact. Tolerance appears to affect most arms of immunity but cell-mediated immunity and IgE responses are most easily tolerised (Simecka, 1998). Three primary mechanisms have been proposed: clonal anergy or deletion of antigen-specific cells, resulting in functional or actual elimination of cells, or active suppression of immune responses mediated by regulatory T cell populations. Low doses of antigen are thought to favour the induction of active cellular regulation, probably due to the restricted distribution of antigen in an immunoregulatory environment, whereas high doses of antigen may be spread in APCs throughout the body favouring the induction of anergy or deletion. Clonal deletion involves the physical elimination of antigen-specific cell populations by apoptosis (Chen *et al.*, 1995). Anergy is a state of unresponsiveness likely to result from ignorance of antigen by the immune system, due to the absence of costimulation or inflammatory signals, or proliferation at the time of initial antigen encounter (Friedman, 1996). Active suppression results from the induction of antigen-specific regulatory T cells that are capable of suppressing the development and activity of effector cells. This can result not only in the suppression of naïve T cells but other antigen-reactive cells in the same microenvironment, irrespective of their specificity, a phenomenon known as bystander suppression (Garside *et al.*, 1999, MacDonald, 1999). CD4+ T cells are suggested to be the principal regulatory T cell population (Groux *et al.*, 1997), largely due to the production of TGFβ and IL-10, cytokines with well-documented suppressive anti-inflammatory effects (Garside & Mowat, 2001).

The balance between active immunity and tolerance greatly depends on the nature of the antigen and its interaction with mucosal inductive sites, as well as on the dose, frequency of antigen administration and the genetic background and

immunological status of the host (Partidos, 2000). Therefore induction of tolerance may be a reflection of how antigen in the mucosa is processed and presented to T lymphocytes in inductive sites, and the factors that modulate and regulate this response. Mechanisms that determine whether an immune response or tolerance develops are still poorly understood and there are several possible sites where antigen presentation and T cell activation could occur. In general soluble antigens are known to be poor mucosal immunogens that are likely to induce tolerance (Waldo *et al.*, 1994). Soluble antigens are likely to be taken up by enterocytes which are thought to have the capablity to present antigen directly (express MHCII) to CD4+ T cells in the epithelium or lamina propria. However, although enterocytes have been shown to present antigen to CD4+ T cell hybridomas *in vitro* they do not express the costimulatory molecules CD80 or CD86 (Hershberg & Mayer, 2000). Antigen presentation without costimulation would result in tolerance of CD4+ T cells (Bland & Warren, 1986). Particulate antigens are thought to promote active immunity because they are predominantly taken up by M cells and delivered to APCs in inductive sites (Neutra, 1998). The most vigorous stimulators of T cells are dendritic cells (DCs), and there are phenotypically distinct populations of DCs in the PP that are proposed to undergo two distinct differentiation pathways depending on the nature of antigen encountered. Innocuous antigens are thought to lead to Th3 responses dominated by IL-10 and TGFβ production, whereas antigen encountered in the context of microbial stimulus or an inflammatory signal leads to DC maturation and migration to interfollicular T cell regions, where IL-12 secretion and T cell stimulation occurs (Kelsall & Strober, 1996a,b; Iwasaki & Kelsall, 1999a,b). The outcome of antigen encounter is therefore likely to depend on the presence of some kind of inflammatory stimulus (Garside & Mowat, 2001).

Recent studies in pigs and mice have suggested that tolerance to mucosal antigen may be regulated at the effector stage rather than the inductive stage (Bailey *et al.*, 2001). This theory stems from the observation that secretory IgA responses accompany tolerance (Challacombe & Tomasi, 1980) suggesting the presence of primed effector T cells, which could differentiate into effector or regulatory cells depending on whether a 'damaged' or stable microenvironment is present in the lamina propria. Tolerance could also be converted to active immune responses if the

conditions in the microenvironment changed. Control of the balance in the lamina propria in this model is likely involve secretion of cytokines such as IL-4 for effector function and IL-10 for regulatory function, and apoptosis is essential to prevent excessive accumulation of T cells in the mucosa. TGFβ is also likely to be involved.

Tolerance is a mechanism that is not yet completely understood. Most research is based on oral tolerance, little is known the induction of tolerance in NALT, and direct comparisons of nasal versus oral tolerance have not been performed. Although there are differences the same basic principles appear to apply to both routes in terms of the generation of regulatory cells and immune responses that favour IL10 and TGFβ (Khoury *et al.*, 1992; Duchmann *et al.*, 1996). There appears to be less involvement of both anergy and clonal deletion in the nasal mucosa, which may be due to differences in regulation or reflect the limited amount of antigen in the nose. The lymph nodes that directly drain the nasal mucosa are instrumental in the induction of mucosal tolerance (Wolvers *et al.*, 1999), and the balance between the activation in the posterior or superficial cervical lymph nodes is thought to determine the outcome of a NALT stimulation (Hameleers *et al.*, 1991; Kuper *et al.*, 1992; Wu & Russell, 1993). Intranasal immunisation has been shown to induce specific systemic tolerance, e.g. OVA in rodents (Sedgwick & Holt, 1985) or KLH in humans (Waldo *et al.*, 1994). However, it is hoped that the use of particulate delivery systems in combination with well-defined mucosal adjuvants will prevent the development of tolerance to an intranasally delivered antigen.

1.9 THE POTENTIAL FOR INTRANASAL IMMUNISATION IN THE SHEEP

The results of the various intranasal immunisation studies that are discussed here are fairly encouraging, in that mucosal and systemic antibody and cell-mediated responses have been produced, and these responses are often protective. However most of this work has been carried out using mouse models, and it is unclear whether this data can translate to the situation in large animals or humans. Human NALT is present as tonsils rather than the organised NALT organ found in rodents, and little is known about ovine NALT. Thus it is unclear whether the principles of intranasal administration of antigens in rodents can be transferred to other systems.

Another problem is that some reports describing nasal administration in mice may involve other mucosal surfaces. Large volumes administered into the nose may be subsequently swallowed, leading to oral delivery. Moreover intranasal vaccines are often administered to anaesthetised mice, which may result in the delivery of a large proportion of the vaccine to the lung (McGhee *et al.*, 1999). In the lung the vaccine has relatively easy access to the systemic lymphoid tissue leading to the induction of systemic immunity.

Large animal models such as the sheep, offer several key advantages over rodent models. Sheep are outbred animals and show similar inter-individual differences to those expected in human populations. The immune system of large animals such as sheep is likely to be more similar to that in humans than rodents, and the results may offer a more realistic reflection of the situation in humans. In addition lymphatic cannulation studies may be performed in sheep to continuously monitor the local immune responses produced following immunisation.

Mucosal vaccination strategies are desirable in domestic animals, and the intranasal route seems the most viable in ruminants to avoid antigen passage through the harsh environment of the rumen. To date only a handful of mucosal vaccination studies have been conducted in ruminants. The intranasal delivery of inactivated parainfluenza 3 virus in sheep did not result in protective immunity unless animals were given an initial intra-muscular injection of inactivated virus (Smith, 1975), and the intra-vaginal immunisation of sheep with an antigen from influenza virus haemagluttinin in degradable starch microspheres did not result in the induction of enhanced levels of antibodies in serum or vaginal wash samples (O'Hagan *et al.*, 1993). However alginate microspheres <10μm containing colloidal carbon have been shown to attach only to the FAE in the GALT, and after 2 hours colloidal carbon was visible within the lymphoid follicles of the PP (Kim *et al.*, 2002). More recently intranasal delivery of ISCOMATRIX® adjuvanted influenza vaccine in sheep induced antibody responses in both serum and nasal secretions that surpassed the levels obtained with unadjuvanted vaccine administered subcutaneously (Coulter *et al.*, 2003).

In cattle the oral administration of OVA in alginate microspheres resulted in a mucosal immune response in the respiratory tract, enhanced by subcutaneous

priming (Bowersock *et al.*, 1998). The intranasal immunisation with soluble *Limulus* haemocyanin with cholera toxin adjuvant induced a weak LH-specific serum IgA response and lymphocyte proliferative responses (Rebelatto *et al.*, 2001a). A further study showed that intranasal immunisation with pig serum albumin encapsulated into alginate microparticles induced strong antigen-specific systemic and mucosal humoral immune responses with IgG1 being the predominant antibody isotype generated in the serum, nasal secretions and saliva (Rebelatto *et al.*, 2001b). The results from these studies are encouraging, and suggest that intranasal vaccination in farm animals may offer a new strategy for the control of livestock diseases.

1.10 *TOXOPLASMA GONDII*

To determine whether immunisation through the intra-nasal route in sheep leads to the production of an effective immune response the use of a disease challenge model is more useful than a model antigen. In this way the protectivity of immune responses produced by immunisation against re-infection can be assessed. The model disease to be used is the obligate intracellular protozoan parasite, *Toxoplasma gondii*.

Toxoplasma gondii is a coccidian that causes abortion in sheep. Approximately 28% of diagnosed cases of abortion in the UK are caused by *T. gondii*, resulting in losses of £15-17 million per year (D. Buxton, personal communication). Animal toxoplasmosis is also a public health risk. Tissue cysts in the muscles of domestic farm livestock represent a very large reservoir of the parasite, consumption of which can lead to human toxoplasmosis. There are good assays available to monitor both cellular and humoral immune responses to *T. gondii*, established at the Moredun Research Institute (Edinburgh, UK) and a well-defined febrile response occurs following infection that can be used to assess whether these responses are protective (McColgan *et al.*, 1988; Buxton *et al.*, 1991).

Sheep are thought to become infected following ingestion of sporulated toxoplasma oocysts in contaminated food and water. Sporozoites are released from oocysts in the gastrointestinal tract, invade the intestinal epithelium and disseminate throughout the body. Here they become tachyzoites which multiply until the host cell ruptures, causing acute infection. In most cases infection with *T. gondii* induces a

rapid and effective immune response which will protect the host but not eliminate the parasite. A persistent infection becomes established as the parasite accumulates in tissue cysts as bradyzoites. Complications of the disease only arise in pregnant ewes, where it causes abortion (Buxton, 1998).

Adoptive transfer experiments in mice have shown that protection can be transferred by immune T cells but not serum (Pavia, 1986). The major mechanism for protection against Toxoplasma is considered to be systemic cell-mediated immunity (Khan *et al.*, 1988; 1994) involving IFNγ (Subauste & Remington, 1991), produced by both CD4+ and CD8+ T cells. IFNγ limits the amount of iron available to the parasite, thereby inhibiting *T. gondii* replication. CD4+ T lymphocytes are thought to be important in establishing immunity during early stages of infection (Araujo, 1991). CD8+ T cells are the major effector T lymphocyte, through the production of IFNγ or direct lysis of infected cells, but their induction and optimal activity is dependent on the production of Th1 cytokines by CD4+ helper T (Chardes *et al.*, 1993; Denkers *et al.*, 1993). In the sheep IFNγ appears in the lymph at day 4 post-infection (Innes *et al.*, 1995a). Initially the predominant population responding to infection is CD4+ T cells, a switch then occurs at day 9-10 post-infection to CD8+ T cells, coinciding with the disappearance of parasite from the lymph (Innes *et al.*, 1995b; Innes & Wastling, 1995).

Infection with *T. gondii* most commonly occurs via the oral route (Frenkel *et al.*, 1969) and the site of penetration into the animal body is the intestinal mucosa. The parasite is thus in intimate contact with the cells of the mucosal immune system. Defined mucosal immunity can be elicited following toxoplasmosis and *T. gondii* immunogens are potent inducers of a local immune response via IgA production, T-cell stimulation and cytokine production: IFNγ, IL-5 and IL-6 (McLeod *et al.*, 1988). IgA has been shown to inhibit the infection of host cells by *T. gondii* parasites (Mack & McLeod, 1992; Mineo *et al.*, 1993) and is therefore protective against subsequent oral infection. In the mouse, IELs (mainly CD8+ αβ+ Thy-1+) also play a major role at the mucosal surfaces as a first line of defence (Chardes *et al.*, 1994) and when transferred to naïve mice can cause a reduction in tissue cysts.

There is a clear need for a veterinary vaccine to prevent animal toxoplasmosis and as a result, human toxoplasmosis. Unlike many other parasitic diseases, primary

exposure to *T. gondii* results in complete resistance to secondary challenge (Araujo, 1994; Alexander *et al.*, 1996) and there is thus possibility to control the disease by vaccination. Toxovax (Intervet) is a successful commercial vaccine that exists for sheep. It is a live attenuated vaccine derived from non-cyst forming mutant strains of *T. gondii* that induces good long-lasting immunity in sheep after only one injection, without the persistent infection seen in naturally infected sheep (Buxton, 1993). However the brief seasonal demand, high production costs and the short (2-3 weeks) shelf-life of the vaccine present logistical problems in commercial production. The vaccine also has the potential to cause human infection because it is live. A non-living vaccine for use in farm animals and in man is highly desirable, but no further vaccination studies have taken place in sheep.

Vaccination against the fast-replicating tachyzoite stage of the life-cycle should prevent the acute phase of the infection. Several studies in mice have shown that immunisation with whole *T. gondii* tachyzoite extracts or specific native antigens or excretory-secretory antigens can confer protection against tissue cyst or tachyzoite challenge as assessed by reduction in a number of brain cysts (reviewed in Jenkins, 2001). Protective immunity obtained after a natural infection with *T. gondii* points to the importance of developing a vaccine that stimulates mucosal defences to control further oral toxoplasmosis and a number of studies in mice have shown that there is potential to stimulate protective immunity via the mucosal route (Bourguin *et al.*, 1991, 1993; Chardes *et al.*, 1993, Debard *et al.*, 1996). However, mice do not mimic infection in humans and domestic animals particularly well, and clinical disease in pregnant ewes offers a more useful model of the human infection. Successful development of a killed vaccine for use in sheep would permit its wider use and bring a vaccine for the prevention of human toxoplasmosis very much closer.

1.11 AIMS OF THIS THESIS

The basic aim of this thesis was to determine whether the intranasal route of immunisation could be used to produce specific immune responses in the sheep, and to determine whether these responses were protective. The model infection to be used for this purpose is *T. gondii*. Sheep and other ruminants appear to have an organised mucosal immune system, similar to that in rodents and humans, and initial

studies using the intranasal route of vaccination in ruminants have produced interesting and promising results that require further investigation. However, studies on NALT and mucosal vaccination in sheep are very limited, despite the fact that there is a clear need for alternative vaccination strategies to control the diseases of domestic livestock. In addition, large animal models offer many advantages over rodent models, such as the potential to use lymphatic cannulation techniques to monitor localised responses in both afferent and efferent lymph. It is also hoped that large animal models may provide a more realistic reflection of the results that may be expected in humans. The list of mucosal immunisation studies conducted in mice and other rodents is exhaustive, but very few studies report success using the mucosal route of immunisation in humans.

One large vaccination trial was conducted as part of this study and a number of variables were chosen, such as the antigen, delivery system and mucosal adjuvant. The purpose of this work was the "proof of principle" to act as a basis for further development and optimisation of the technique.

The primary aims of the work presented here were as follows:

1. To characterise mucosal-associated lymphoid tissue present in the ovine nasopharyngeal tract, specifically examining the location, structure and composition to determine whether it is an immune inductive site of the mucosal immune system comparable to NALT in other species. In addition close examination of the follicle-associated epithelium will determine whether M cells with the structural and functional capabilities described in other species are present. In this way the potential to stimulate a mucosal immune response using the intranasal route of immunisation may be assessed.

2. To develop an appropriate delivery system to target protein antigens to M cells and NALT. Work shall focus on the biodegradable PLG polymer microparticles due to their success in other mucosal immunisation studies. Methods of optimising a number of variables including protein encapsulation, protein release, and particle size for M cell uptake shall be developed. In addition if possible the functionality and antigenicity of encapsulated proteins shall be investigated.

3. To choose an appropriate antigen from *T.gondii* tachyzoites for use in immunisation studies, and to produce sufficient quantities of this antigen, and encapsulate it into the optimal PLG delivery system developed as part of this study, with and without the mucosal adjuvant cholera toxin.

4. To conduct an extensive intranasal immunisation trial in sheep using the antigen from *T. gondii* and the delivery system developed. This will determine not only whether this is a viable strategy for immunising sheep, but whether this route of immunisation could offer potential for the development of a new vaccine against *T. gondii*. Both humoral and cell-mediated responses shall be assessed, as well as the level of protectivity afforded by these responses.

CHAPTER 2

Characterisation of Ovine Nasal-associated Lymphoid Tissue and M Cells

2.1 INTRODUCTION

The aim of the research described in this chapter was to characterise mucosal-associated lymphoid tissue present in the ovine nasopharyngeal tract. The respiratory mucosa, particularly in the upper respiratory tract and nasal cavity, is in constant contact with airborne antigenic material. Like other mucosal surfaces the nasal mucosa is invested with, and immunologically protected by, NALT, which belongs to the mucosal immune system (Spit *et al.*, 1989; Kuper *et al.*, 1992). NALT plays a crucial role in trapping particulate matter and providing the first line of defence against airborne pathogens, primarily with a protective mucosal IgA response, the characteristic effector mechanism of the mucosal immune system (Neutra & Kraehenbuhl, 1992).

Relatively few studies have focused on MALT in the respiratory tract in comparison to GALT. In humans NALT is present as the Waldeyer's ring consisting of the lingual, palatine and pharyngeal tonsils located in the posterior pharynx (Howie, 1980; Karchev & Kabakchiev, 1984; Brandtzaeg & Halstensen, 1992) whilst in rodents NALT is situated on both sides of the septal opening to the pharyngeal duct (Spit *et al.*, 1989; Kuper *et al.*, 1992). NALT has been described in various other species including monkeys and the horse (Loo & Chin, 1974; Harkema *et al.*, 1987; Mair *et al.*, 1988). The NALT in the Waldeyer's ring in humans is composed of primary B cell follicles and extrafollicular T cell areas, mainly consisting of the CD4+ phenotype (Brandtzaeg & Halstensen, 1992). Nasal-associated lymphoid cell populations have been described in the rat and other rodents and are of similar structure (Spit *et al.*, 1989; Koornstra *et al.*, 1991, 1993; Kuper *et al.*, 1990). In general NALT has a similar structure to GALT, but some regional differences do exist, e.g. NALT has a greater proportion of T cells than the PP in the mouse, with a greater percentage of CD4+ compared to CD8+ cells, but similar numbers of mature IgM+ IgA- B cells (Heritage *et al.*, 1997). Both human and rodent NALT are now considered to be immunologically fully equipped and active mucosal lymphoid organs, similar to the GALT in the PP. Recent studies have proved that mouse NALT is a mucosal inductive site for humoral and cellular responses in the upper respiratory tract. Following intranasal reovirus infection the development of germinal centres, the expansion of IgA+ and IgG2a+ B cells and the presence of virus specific CTL

could be demonstrated in NALT, as well as local IgA production in the upper respiratory tract and systemic IgG2a production (Zuercher *et al.*, 2002).

In addition, the epithelium overlying NALT can be distinguished from the surrounding respiratory epithelium by the presence of a specialised follicle-associated epithelium. M cells have been identified overlying the nasopharyngeal lymphoid tissue of humans (Karchev & Kabakchiev, 1984) and shown to be similar to the M cells in the GALT both morphologically and functionally, by the demonstration of HRP particle uptake (Fujimura, 2000). M cells have also been identified overlying rodent NALT (Spit *et al.*, 1989), and the nasal absorption of fluorescent polystyrene particles has been observed, suggesting the mechanism of solid particle uptake by the nasal mucosa is similar to that found in the gut (Alpar *et al.*, 1994). M cells are thus thought to be to be essential for the uptake of antigen and the initiation of immunity in the upper aerodigestive tract.

Studies on MALT in the ovine nasopharyngeal tract are relatively scarce. The distribution and gross morphological features of ovine respiratory tract-associated lymphoid tissue (RTALT) including NALT have been previously described (Chen *et al.*, 1989). Lymphoid aggregations have been observed throughout the respiratory tract, and are found most frequently in the pharyngeal tonsil, the mid-nasopharynx and around the opening to the auditory tube, but only the ovine pharyngeal tonsil has been studied further. MALT in the ovine pharyngeal tonsil is able to take up intra-nasally administered colloid carbon (Chen *et al.*, 1989), and the mucosal epithelium overlying the ovine pharyngeal tonsil consists of predominantly non-ciliated cells, the majority of which possess microvilli or microfolds of varying number, height and density on their surface and are ultrastructurally similar to the M cells described in other mucosal sites in other species (Chen *et al.*, 1991). However, the cellular structure and composition of the lymphoid follicles in the ovine nasal region have not been studied in any detail, and the nature of local immune responses and the role of NALT in these responses have also yet to be determined. This study investigates the structure and composition of lymphoid tissue in the ovine nasopharyngeal tract, including the distribution of B- and T-cell subsets and the presence of M cells in the mucosal epithelium, as a prelude to future studies on intranasal vaccination.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Greyface cross Suffolk sheep, aged 0.5-1 year, were housed in a conventional loose box, fed on hay and concentrates, and given free access to fresh water.

2.2.2 Collection and Preparation of Lymphoid Tissue

Sheep were stunned with a captive bolt and exsanguinated, and their spinal cords severed. Each head was removed and sagitally sectioned at the atlanto-occipital joint. The nasal septum was then removed, exposing both halves of the nasopharyngeal cavity. In a pilot study, lymphoid nodules were located macroscopically, in the nasopharyngeal cavity of two sheep, with 70% glacial acetic acid (Cornes, 1965; Chauhan & Singh, 1970). The tissue containing nodules was subsequently removed from the remaining experimental sheep. Where required jejunal PP and pharyngeal tonsil were also removed. Blocks of tissue containing nasal lymphoid nodules (area $1cm^2$) were fixed by immersion in glutaraldehyde 3% v/v in 0.1M phosphate buffer (PB), pH 7.4 for scanning electron microscopy (SEM) and individual nodules were fixed by immersion in glutaraldehyde 2.5% v/v in 0.1M PB, pH 7.4 for transmission electron microscopy (TEM). The remaining tissue samples were then placed in a non-formaldehyde zinc salts fixative (ZSF) as described by Gonzalez *et al.* (2001). Briefly, tissues were immersed in ZSF solution (0.1M Tris base buffer with 0.05% Ca acetate (pH 7-7.4), containing 0.5% Zn acetate and 0.5% Zn chloride) for up to 72 h at room temperature. Following fixation, tissues were transferred to 70% ethanol for at least 30 min, then processed by routine methods and embedded in paraffin wax. Tissue sections (5 μm) were cut with a microtome (Jung Biocut 2035, Leica Instruments GmbH, Germany), mounted on glass slides pre-coated with poly-L-lysine and dried overnight. Tissue sections were then dewaxed, hydrated and stained with haematoxylin and eosin (HE) by conventional methods, and examined for the presence of lymphoid tissue. Serial sections from appropriate blocks containing lymphoid tissue were then sectioned further for immunohistochemical analysis.

2.2.3 Demonstration of Reticulin

Slides were stained for reticulin using the Gordon & Sweet method detailed in Bradbury & Rae (1996).

2.2.4 Immunohistochemistry

Specific binding of antibody was identified with the Envision Plus HRP System (Dako, Ely, UK). Briefly, dewaxed sections were incubated with a peroxidase "block" (0.03% hydrogen peroxide) for 5 min at room temperature, and then in 25% normal goat serum for 30 min at room temperature to prevent non-specific labelling of tissue antigens. The slides were then incubated with 100µl of the appropriate monoclonal antibody dilution overnight at 4°C. Controls were provided by replacing the primary antibody with the equivalent concentration of an IgG fraction from normal mouse serum for the same length of time. The secondary antibody (peroxidase-labelled polymer conjugated to goat anti-mouse immunoglobulins) was then added to tissue sections for 30 min at room temperature. The sections were finally incubated with substrate chromogen for 7.5 min at room temperature, washed in distilled water, counterstained with haematoxylin, dehydrated in graded alcohols, cleared and mounted. Tris buffered saline (TBS) (0.05M Tris HCl, 0.15M NaCl, pH 7.2-7.6) was used to wash tissue sections between each incubation and to prepare the normal goat serum and antibody dilutions. The monoclonal antibodies employed, and their specificity, source and dilution, are shown in Table 2.1 (see also Gonzales *et al.*, 2001). Tissue sections were examined and photographed using an Olympus BX50 microscope (Olympus, London, UK).

2.2.5 Electron Microscopy

2.2.5.1 TEM

After fixation for 48 h, the tissue samples were placed in 0.1M PB for 15 min. The PB was replaced by 2% osmium tetroxide for 2 h, and the tissue was then dehydrated in graded water-acetone mixtures and embedded in an Epon-Araldite mixture. After polymerization at 60°C for 48 h, ultrathin sections were cut and

Table 2.1 *Details of the monoclonal antibodies used to identify ovine cell populations*

Monoclonal antibody designation	Specificity (ovine/ bovine)	Cellular expression	Material	Dilution* (1 in)	Source
36F	CD2	$\alpha\beta$T cells	Ascites	5000	Basel Institute for Immunology
CC15	WC1	$\gamma\delta$ T cells	Ascites	12 800	Institute for Animal Health, Compton
17D	CD4	T helper cells	Supernate	50	Basel Institute for Immunology
SBUT8	CD8	Cytotoxic T cells	Supernate	50	University of Melbourne
CC21	CD21	Follicular dendritic cells, mature B cells	Ascites	25 000	Institute for Animal Health, Compton
VPM32	CD14	Macrophages	Ascites	3000	University of Edinburgh
CC20	CD1	Dendritic cells	Ascites	5000	Institute for Animal Health, Compton
SBUII49-1	MHCII	B cells, activated T cells, macrophages	Supernate	50	University of Melbourne
73B	CD45R	All lymphocytes	Supernate	100	Basel Institute for Immunology
VPM13	IgM	B cells	Ascites	100 000	University of Edinburgh
2F1	IgE	IgE+ B cells, mast cell surface	Supernate	800	CSIRO, Prospect, NSW, Australia
K84.2F9	IgA	IgA+ B cells	Supernate	20 000	Serotec Ltd, Oxford
Not known	IgG_1	IgG_1+ B cells	Supernate	700	CSIRO, Prospect, NSW, Australia
Not known	IgG_2	IgG_2+ B cells	Supernate	400	CSIRO, Prospect, NSW, Australia

*Dilutions were as recommended from titration studies on lymph node.
CD = cluster designation
WC-1 = workshop cluster-1

stained with uranyl acetate and lead citrate and examined with a Jeol JEM 1200EX transmission electron microscope (Jeol UK Ltd., Herts, UK) operated at 80 kV.

2.2.5.2 SEM

After fixation for approximately 48 h the tissue was washed in 0.1M PB and post-fixed in osmium tetroxide 1% w/v in 0.1M PB, by a method incorporating the enhancement of osmium penetration with thiocarbohydrazide (Malick & Wilson, 1975). After post-fixation, the specimens were rinsed in several changes of PB, dehydrated through a series of graded ethanols (to 100%), and placed in acetone. The specimens were then subjected to critical point drying with liquid carbon dioxide, attached to aluminium stubs by means of silver conductive paint, sputter coated with gold and examined in a Stereoscan S250 Mark III scanning electron microscope at 10-20 kV.

2.2.6 Marking Ovine M Cells

For the following methods 5μm tissue sections of both pharyngeal tonsil and nasal lymphoid nodules were used. All tissues were fixed in ZSF, embedded in paraffin wax, processed and sectioned as described in Section 2.2.2. Following the labelling procedure sections were examined using an Olympus BX50 microscope (Olympus, London, UK).

2.2.6.1 FITC-Lectins

Slides were incubated in sufficient 3,3' diaminobenzidine (DAB) (Sigma Fast™ DAB Tablet, Sigma, Dorset, UK) to cover the surface of each tissue section (approximately 100μl per slide) in order to quench background fluorescence, and then washed in water. Slides were incubated in a 10μg/ml solution of each FITC-lectin in phosphate buffered saline pH7.4 (PBS) for 1 h at room temperature. The slides were then washed well in PBS, mounted in Citifluor (Citifluor Ltd., London, UK) and examined using a blue (NB) filter at 470-490nm for evidence of lectin binding to the epithelial surface of the tissues. A panel of lectins with differing binding specificities purchased from Vector Laboratories (Peterborough, UK) was employed, detailed in Table 2.2.

Number	Lectin	Abbreviation	Specificity
1	Concavalin A[1,2]	CON A	α-man, α-glc
2	*Dolichos biflorus* agglutinin[1,2]	DBA	α-galNAc
3	Peanut agglutinin[1,2]	PNA	β-gal(1→3)galNAc
4	*Ricinus communis* agglutinin 120[1,2]	RCA 120	β-gal
5	Soybean agglutinin[1,2]	SBA	galNAc
6	*Ulex europaeus* lectin I[1,2]	UEA I	α-L-fuc
7	Wheat germ agglutinin[1,2]	WGA	(glcNAc)$_2$, NeuNAc
8	*Griffonia (Bandeiraea) simplicifolica* lectin I[1,2]	GSL I	α-gal, α-galNAc
9	*Lens culinaris* agglutinin[1,2]	LCA	α-man
10	*Phaseolus vulgaris* erythroagglutinin[1,2]	PHA-E	oligosaccharide
11	*Phaseolus vulgaris* leucoagglutinin[1,2]	PHA-L	oligosaccharide
12	*Pisum sativum* agglutinin[1,2]	PSA	α-man
13	*Sophora japonica* agglutinin[1,2]	SJA	β-galNAc
14	Succinylated wheat germ agglutinin[1,2]	Succinylated WGA	glcNAc
15	*Griffonia (Bandeiraea) simplicifolica* lectin II[1,2]	GSL II	glcNAc
16	*Datura stramonium* lectin[1,2]	DSL	(glcNAc)$_2$
17	*Erythrina cristagalli* lectin[1,2]	ECL	β-gal(1→4)glcNAc
18	Jacalin[1,2]		α-gal→OMe
19	*Lycopersicon esculentum* (tomato) lectin[1,2]	LEL	(glcNAc)$_3$
20	*Solanum tuberosum* (potato) lectin[1,2]	STL	(glcNAc)$_3$
21	*Vicia villosa* agglutinin[1,2]	VVA	galNAc
22	*Amaranthus caudatus* lectin[1]	ACL, ACA	?
23	*Bauhinia purpurea* lectin[1]	BPL	β-gal(1→3)galNAc
24	Succinylated Concavalin A[1]	SConA	α-man, α-glc
25	*Eunonymous europaeus* lectin[1]	EEL	α-gal(1→3)gal
26	*Galanthus nivalis* lectin[1]	GNL	non-reduc, α-man
27	*Griffonia simplicifolica* lectin I – isolectin B$_4$[1]	GSLIB$_4$	α-gal
28	*Lotus tetragonolobus* lectin[1]	LTL	?
29	*Maackia amurensis* lectin I[1]	MAL1	sialic acid
30	*Maclura pomifera* lectin[1]	MPL	α-gal, α-galNAc
31	*Sambucus nigra* lectin[1]	SNA, EBL	α-NeuNAc(2→6)gal, galNAc
32	*Wisteria floribunda* lectin[1]	WFA, WFL	galNAc
33	*Anguilla anguilla* lectin[2]	AAA	α-L-fucose

Table 2.2 *Panel of lectins used and their specificities*

glcNAc = N-acetylglucosamine
galNAc = N-acetylgalactosamine
fuc = fucose
man = mannose
murNAc = N-acetylmuramic acid

[1]FITC-lectin
[2]biotinylated lectin

2.2.6.2 Biotinylated Lectins

Slides were incubated in 0.5% hydrogen peroxide in methanol for 15 min to remove endogenous peroxidase activity. The slides were then washed well in water and incubated in a 10μg/ml solution of the lectin-biotin complex in PBS for 1 h. The biotinylated lectins used are shown in Table 2.2. Slides were then washed thoroughly in PBS and incubated in a 1:500 dilution of a conjugate of streptavidin with horseradish peroxidase (HRP) (Dako, Ely, UK) for 30 min. Slides were then washed again in PBS, and incubated in DAB (Sigma Fast™ DAB Tablet, Sigma, Dorset, UK) for 5 min to visualize the substrate. The slides were then washed in water, counterstained with haematoxylin, dehydrated in a sequential series of alcohols, cleared in xylene and mounted. Omitting the primary antibody incubation step provided a negative control, and a positive control was provided by incubating sections of mouse Peyer's patch with UEA I or AAA lectin.

2.2.6.3 Vimentin

Endogenous peroxidase activity was blocked by incubation with 0.5% hydrogen peroxide in 100% methanol at room temperature for 15 min. The slides were then rinsed in water and incubated in 4% bovine serum albumin (BSA) in PBS for 15 min to reduce non-specific labelling. Sections were then incubated in anti-vimentin monoclonal antibody (clone V9, Sigma, Dorset, UK) diluted to a concentration of 1:40 in PBS for 1 h at room temperature. Slides were washed in PBS and incubated in a 1:300 dilution of a rabbit anti-mouse monoclonal antibody conjugated to horseradish peroxidase (Dako, Ely, UK). Slides were rinsed again in PBS and developed in DAB (Sigma Fast™ DAB Tablet, Sigma, Dorset, UK) for 5 min, then washed in water, counterstained with haematoxylin, dehydrated in a sequential series of alcohols, cleared in xylene and mounted. Omission of the primary antibody was used to provide a negative control.

2.2.6.4 Cytokeratins

Four different monoclonal antibodies specific for different cytokeratin peptides were used to label tissue sections. The antibodies were chosen on the basis of wide species cross-reactivity and previous publications that showed specific

labelling of M cells. The first antibody used was a mouse anti-pan cytokeratin made from a mixture of monoclonal antibodies. The other three antibodies were monoclonal antibodies specific for cytokeratin peptides 4, 8 and 18. All antibodies were purchased from Sigma (Dorset, UK). The antibody signal was amplified with the Envision Plus HRP System (Dako, Ely, UK) using the methods detailed in section 2.2.4. Antibody dilutions (in TBS) are shown in Table 2.3 below.

Antibody	Clone	Isotype	Dilution Used At
pan cytokeratin	C-11, PCK-26, CY-90, KS-1A3, M20, A-53-B/A2	IgG1 / IgG2a	1:5000
cytokeratin 4	6B10	IgG1	1:5000
cytokeratin 8	M20	IgG2b	1:650
cytokeratin 18	CY-90	IgG1	1:5000

Table 2.3 *Antibodies and dilutions used for anti-cytokeratin labelling*

2.2.6.5 Alkaline Phosphatase

Two methods were used to investigate the presence of alkaline phosphatase within the epithelium. The first method used a kit designed for demonstrating alkaline phosphatase in leukocytes in blood smears (85L-1, Sigma, Dorset, UK). Briefly, a Fast Blue RR salt capsule was dissolved in 48ml of deionised water, and 2ml of a Naphthol AS-MX phosphate alkaline solution was added and mixed well. The slides were incubated in the mixture for 30 min whilst protected from direct light, rinsed for 2 min in deionised water and stained with Mayer's haematoxylin solution for 10 min. The slides were then rinsed for 3 min in deionised water and mounted in an aqueous mountant and examined. For the second method, an immunohistochemical technique, tissue sections were treated with a 1:50 dilution of an anti-alkaline phosphatase antibody (Sigma, Dorset, UK) using the methods detailed in Section 2.2.6.3, using a 1:300 dilution of a goat anti-rabbit secondary antibody conjugated to HRP (Dako, Ely, UK).

2.2.7 Demonstration of Functional Activity of M Cells

2.2.7.1 Organ Culture

The culture system used was based on the methods devised by Jackson *et al.* (1996) to culture respiratory tissue. A sterile 3.5cm Petri dish was placed within a

sterile 6cm diameter Petri dish (Corning Costar, Bucks, UK), and 4ml of minimal essential medium (MEM) (Gibco, Invitrogen, Paisley, UK) supplemented with penicillin (50 IU/ml), streptomycin (50μg/ml), and gentamicin (50μg/ml) were pipetted into the outer Petri dish. A strip of sterile filter paper 5mm × 8cm was soaked in MEM and manipulated with sterile forceps so that each end adhered to the base of the inner dish to act as a wick to draw the medium from the outer Petri dish to the underside of the tissue (demonstrated in Figure 2.1). Epithelial tissue containing lymphoid nodules or epithelium from the pharyngeal tonsil was dissected into 3-4mm squares with a thickness of 1-2mm, and a single tissue square was placed, ciliated surface upwards, on the centre of the filter paper strip. Organ cultures were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide for a period of 0-4 h. Tissue was fixed after the 4 h culture period, fixed in 4% paraformaldehyde, sectioned and stained with HE to assess viability and tissue morphology.

2.2.7.2 Application of Microparticles to Tissue Explants

A plastic isolation cylinder 2mm in diameter was pressed firmly on top of each tissue explant to create a seal. 10μl of a 1:100 dilution of yellow-green fluorescent latex microparticles (Polysciences Inc., Eppelheim, Germany) were pipetted onto the ciliated surface of each tissue explant within the isolation cylinder as shown in Figure 2.1.

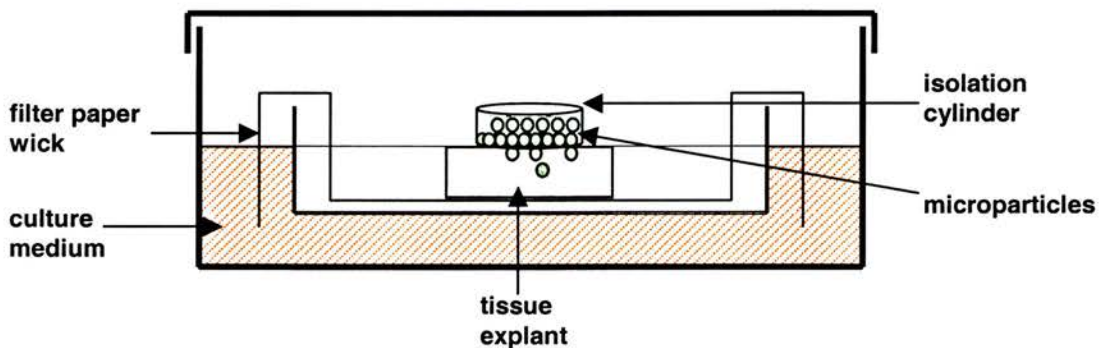


Figure 2.1 *Schematic representation of the application of fluorescent microparticles to ovine NALT using the explant culture system*

Microparticles of 0.1μm, 0.2μm, 0.5μm, 0.75μm or 1.0μm diameter were used. Tissue explants were incubated with the microparticles for time periods of 30 min, 45 min, 1 h, 2 h or 3 h at 37°C in a humidified atmosphere containing 5% CO_2. After this time each explant was washed thoroughly 3 times in PBS at 37°C and fixed in modified Bouins for confocal microscopy, or 4% paraformaldehyde for conventional microscopy. Some explants were examined immediately under a fluorescent stereomicroscope (Zeiss Stemi SV6, Carl Zeiss Ltd., Herts, UK) to determine the surface distribution of the microparticles. The tissues were then cryoprotected in 25% sucrose, and frozen in OCT™ cryo-embedding matrix (CellPath, Wales, UK) in liquid nitrogen. Sections between 5μm and 25μm thick were cut using a Cryostat (Jung Frigocut 2800E, Leica Instruments GmbH, Germany), counterstained with a 1:5000 dilution of propidium iodide, mounted in Citifluor (Citifluor Ltd., London, UK) and examined under a natural blue filter 470-490nm using an Olympus BX50 microscope (Olympus, London, UK) to determine the location of the microparticles.

2.2.7.3 *In vivo* Application of Microparticles

A crude intra-nasal spray was fashioned from a syringe and a length of tubing in which a series of tiny holes had been punched and the end had been blocked. The effectiveness of this spray was tested by inserting the catheter to a depth of 16-18cm into the nasal tract of a dead sheep, and spraying 1ml of Evan's blue dye laterally onto the epithelium. Following removal and sagittal sectioning of the head, the dye was observed to stain both the pharyngeal tonsil and the area containing lymphoid nodules. The spray was then used to apply 1ml of a 1:1000 dilution of fluorescent latex microparticles (sizes 0.5μm, 0.75μm and 1.0μm) (Polysciences Inc., Eppelheim, Germany) to both nostrils of 6 sheep *in vivo* (2 sheep for each size of microparticle). The sheep were left for 45 minutes before being euthanised, and the area of nasopharynx containing lymphoid nodules and the pharyngeal tonsil were then removed, fixed in 4% paraformaldeyde and cryoprotected in 25% sucrose. Small serial blocks of epithelial tissue (approximately 2mm thick) were then frozen in OCT™ cryo-embedding matrix (CellPath, Wales, UK) in liquid nitrogen, sectioned, counterstained and mounted as before, and examined using a natural blue filter 470-490nm with an Olympus BX50 microscope (Olympus, London, UK).

Tissue sections found to contain particles within the epithelium were further examined by confocal microscopy. Fluorescent images were acquired using an MRC-600 confocal laser scanning microscope (CLSM, Bio-Rad Laboratories,) mounted on an Axiovert 100 inverted microscope equipped with Plan-Apochromat® objective lenses (Carl Zeiss, Herts, UK). Fluorophores were excited using the 488nm (FITC) lines from a 15mW Kr/Ar laser (Bio-Rad, Hemel Hempstead, Herts, UK).

2.3 RESULTS

2.3.1 Localisation and organisation of nasal lymphoid tissue

After fixation with acetic acid lymphoid nodules appeared macroscopically as opaque white spots under the mucosal surface, located in the nasopharynx posterior to the opening of the Eustachian tube leading to the inner ear, as demonstrated in Figures 2.2-2.4. This is at a depth of approximately 16-18cm into the nasopharynx from the nostril, depending on the age and size of the animal. While the majority of nodules tended to be clustered in this area, individual nodules were observed throughout the nasopharyngeal mucosa. The number, location and size of nodules varied between animals, apparently regardless of age.

Sections stained with HE revealed that the lymphoid tissue was unencapsulated and had a conventional follicular structure, consisting of tightly packed cells in a germinal centre surrounded by a network of reticular cells and more dispersed lymphocytes. A characteristic dome-like accumulation of lymphoid cells was observed beneath the epithelium, demonstrated in Figure 2.5a. Serial sections showed that at the periphery of the nodule the lymphoid material was discrete from the epithelium (Figure 2.5b), but at the centre the epithelium became modified (Figure 2.5a). This 'lymphoepithelium' (FAE) was attenuated, non-ciliated and heavily infiltrated by cells with the appearance of lymphocytes, which may have been trafficking out of the nodule itself. In comparison, normal pseudostratified columnar epithelium was observed overlying the edge of the nodules and the surrounding tissue (Figure 2.5c). A Gordon and Sweet stain revealed that each lymphoid follicle was surrounded by a network of cytoskeleton and reticulin, a typical feature of lymphoid follicles (Figure 2.5d).

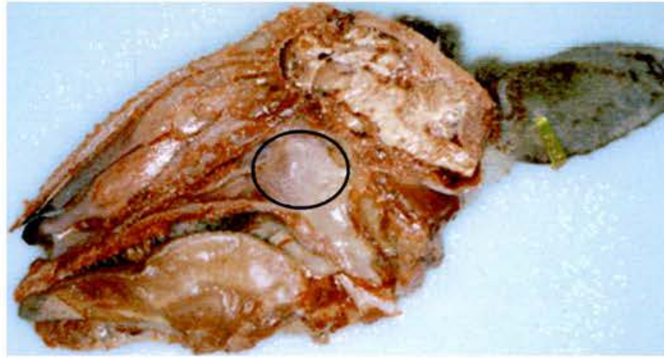


Figure 2.2 *Sheep's head that has been sagitally sectioned to show approximate location of lymphoid nodules in nasopharyngeal tract*

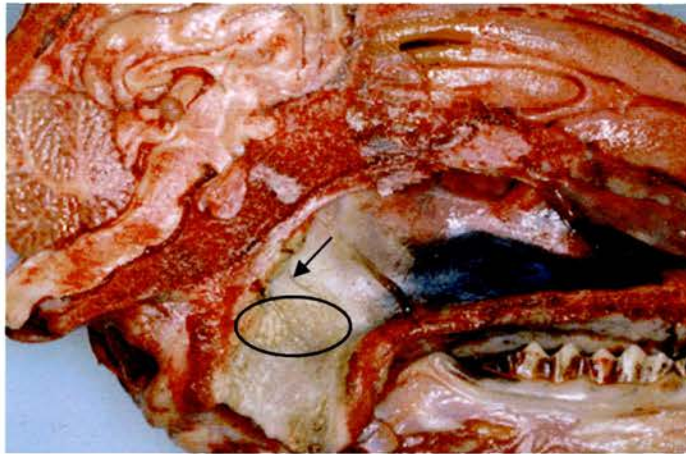


Figure 2.3 *Acetic acid fixation of nasopharyngeal tract. Lymphoid nodules are visible as opaque white spots in the circular area. The arrow shows the location of the opening to the Eustachian tube*

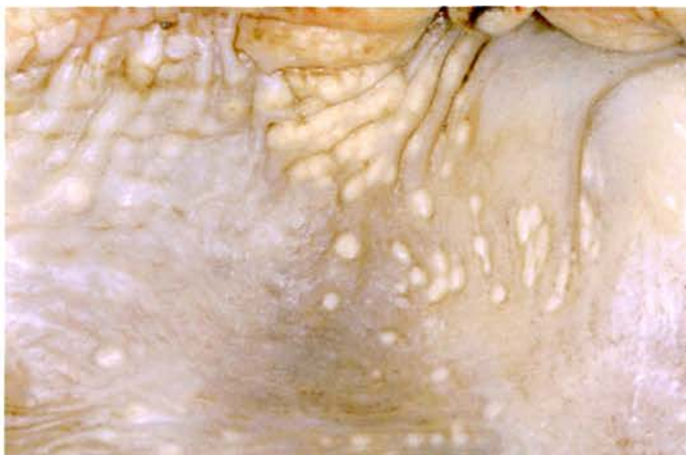


Figure 2.4 *Close-up view of the nasal lymphoid nodules after acetic acid fixation*

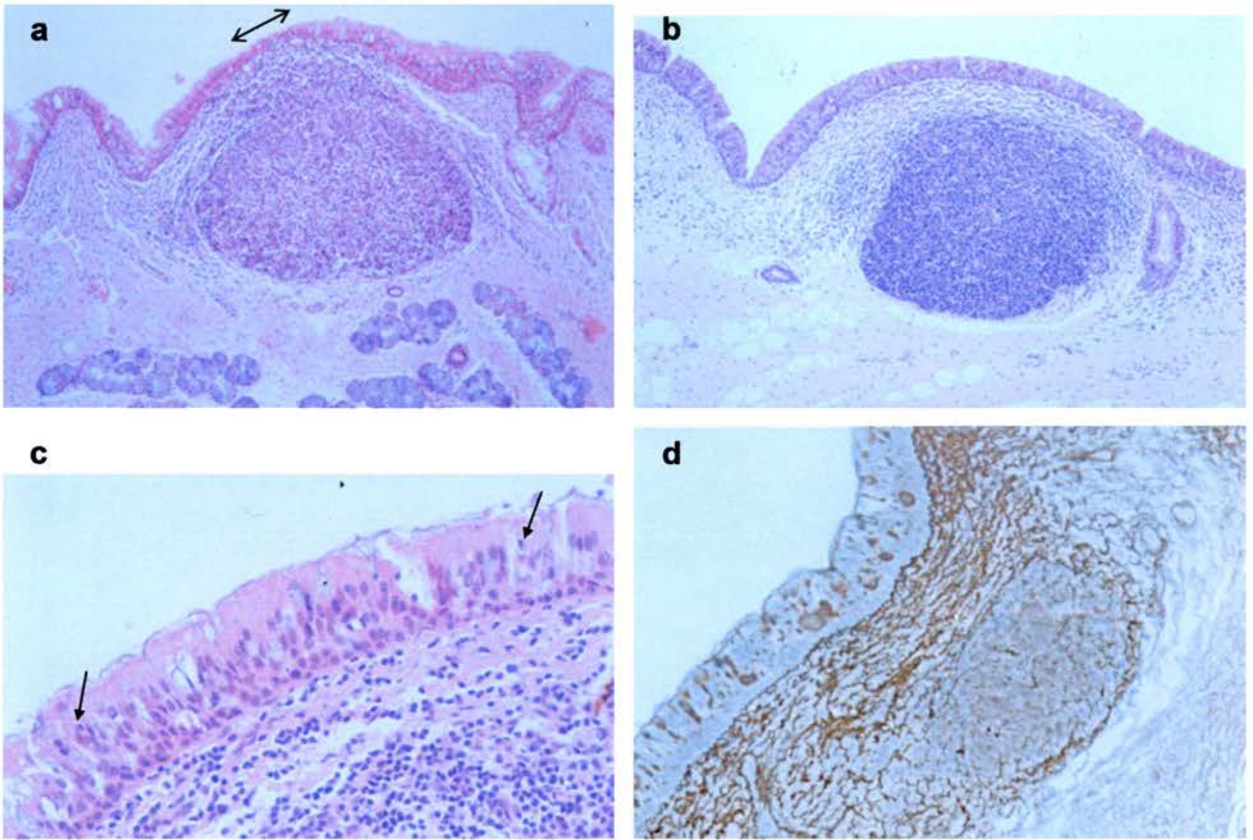


Figure 2.5a-d *Morphological characteristics of ovine NALT. a) Centre of nodule where epithelium becomes attenuated and infiltrated with lymphocytes 'lymphoepithelium', ×54 magnification. b) Edge of lymphoid nodule, where follicle remains discrete from the epithelium, ×54 magnification. c) Typical pseudostratified columnar epithelium observed overlying the edge of lymphoid nodules (arrows show possible lymphocytes within epithelium), ×216 magnification. d) Gordon and Sweet stain revealing conventional network of reticulin around lymphoid nodule, ×108 magnification*

2.3.2 Immunohistochemistry

All antibodies employed showed immunoreactivity, while no labelling was observed in negative control sections. The section chosen for demonstration of serial analysis with different monoclonal antibodies in Figures 2.6-2.8 appeared to have 2 follicular areas present. Sections appeared to be approaching the centre of the larger follicle where dome-like accumulations of lymphocytes could be observed, whilst simultaneously approaching the edge of the smaller follicle, allowing comparison of cell populations in such areas. In general a similar distribution of cell populations could be observed in lymphoid tissue in the nasopharyngeal tract and in the pharyngeal tonsil, unless otherwise specified.

The nasal lymphoid nodules had the characteristics of an organised lymphoid tissue structure, consisting of well-defined follicular B cell-containing areas that included germinal centres, and parafollicular areas containing minor populations of CD4+, CD8+ and $\gamma\delta$ T cells. Numerous lymphocytes were observed in close association with the epithelium, and frequently within the epithelium, particularly at the centre of the dome. Strongly positive labelling by monoclonal antibody for MHC class II and CD45R (Figure 2.6g) was observed on numerous small, round cells throughout the lymphoid nodules. B lymphocytes and plasma cells, labelled for IgM, were very numerous and clearly concentrated in the follicles, as demonstrated in Figure 2.6a. Based on the appearance of the cells, B lymphocytes were concentrated in the follicle and the immediate parafollicular area, whereas plasma cells were scattered throughout the non-follicular areas.

B lymphocytes and plasma cells labelled for IgE, IgA, IgG_1 and IgG_2 were observed in varying numbers in the centre of the follicles, demonstrated in Figure 2.6. Those labelled for IgA were predominant in the centre of the follicle, and a population of IgA+ plasma cells was disseminated throughout the lamina propria (Figure 2.6b). IgE was also present on large cells, scattered throughout the nasal lymphoid tissue (Figure 2.6c), which had the distribution and morphological appearance of mast cells (J.F. Huntley, personal communication). Interestingly in the pharyngeal tonsil, isotype switching to IgE appeared to have occurred in the germinal centres (Figure 2.6d). This was not the case in the nasal lymphoid nodules.

Interstitial labelling for IgA, IgG_1 and IgG_2 was observed throughout the tissue, and for IgA on the surface of the epithelium.

Immunohistochemical analysis of serial sections revealed numerous $\alpha\beta$ T cells surrounding B-cell areas (Figure 2.8d). CD4+ T cells were typically concentrated around one pole of the follicle and in the immediately surrounding parafollicular area (Figure 2.7a), while CD8+ T cells were apparently clustered in smaller numbers in the parafollicular area (Figure 2.7b). $\gamma\delta$ T cells were scattered around the follicles and often seen within the overlying epithelium (Figure 2.7c). There were significant numbers of all three T cell subsets, all of which shared polarisation to some extent towards the epithelium. Dendritic cells (CD1+) were scattered in small numbers around the follicles and appeared in particularly high numbers in the smaller follicle that had been sectioned at the periphery (Figure 2.8a). Large numbers of CD21+ cells were present in the central region of the follicle, representing follicular dendritic cells and mature B lymphocytes (Figure 2.8b). Macrophages were predominant in the centre of the follicular areas (Figure 2.8c).

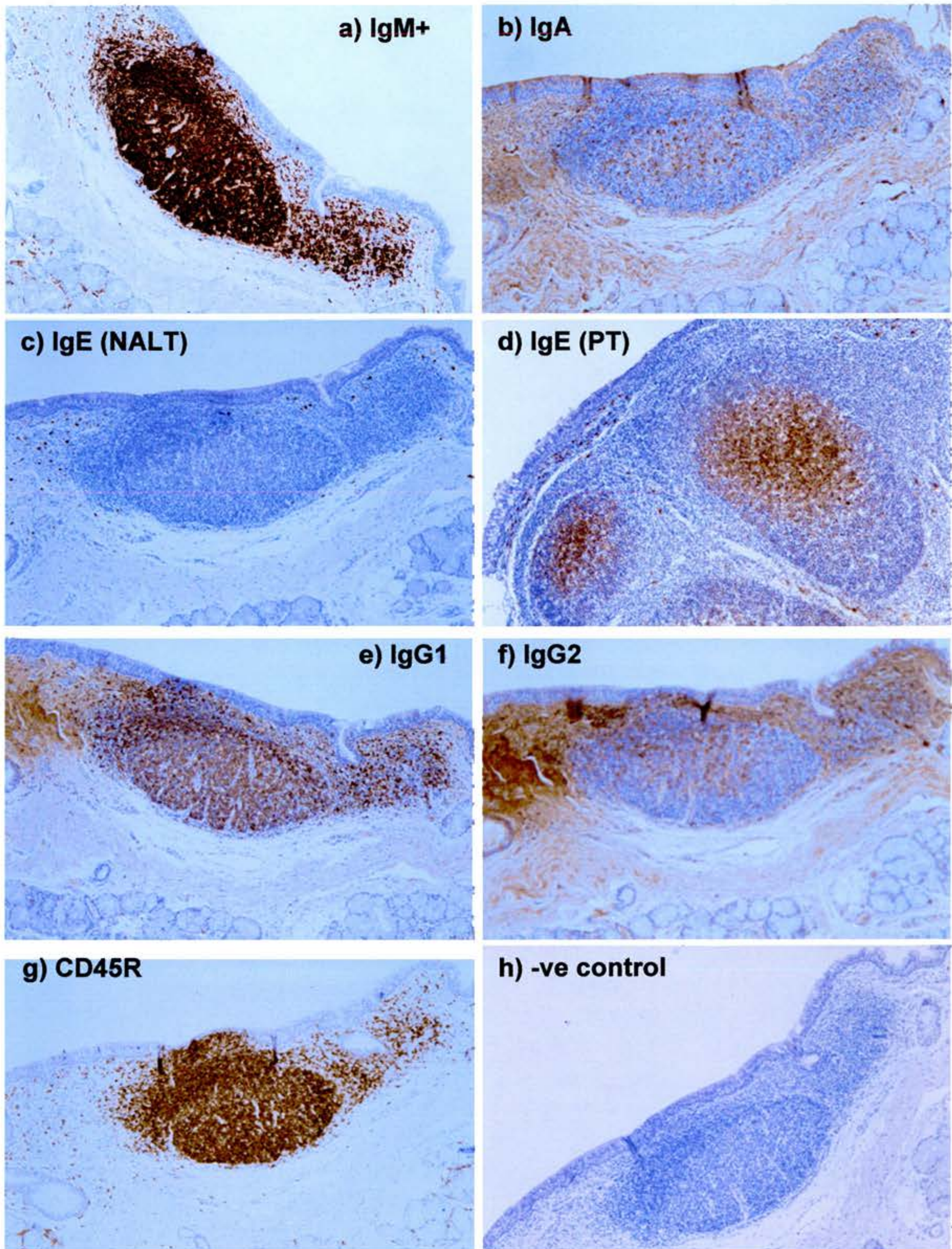


Figure 2.6a-h *Serial sections of an ovine nasal lymphoid nodule labelled for the different immunoglobulins (IgM, IgA, IgE, IgG1, IgG2), and CD45R (mature B cells and naïve T cells), pharyngeal tonsil (PT) labelled for IgE, and negative control slide ×50 magnification*

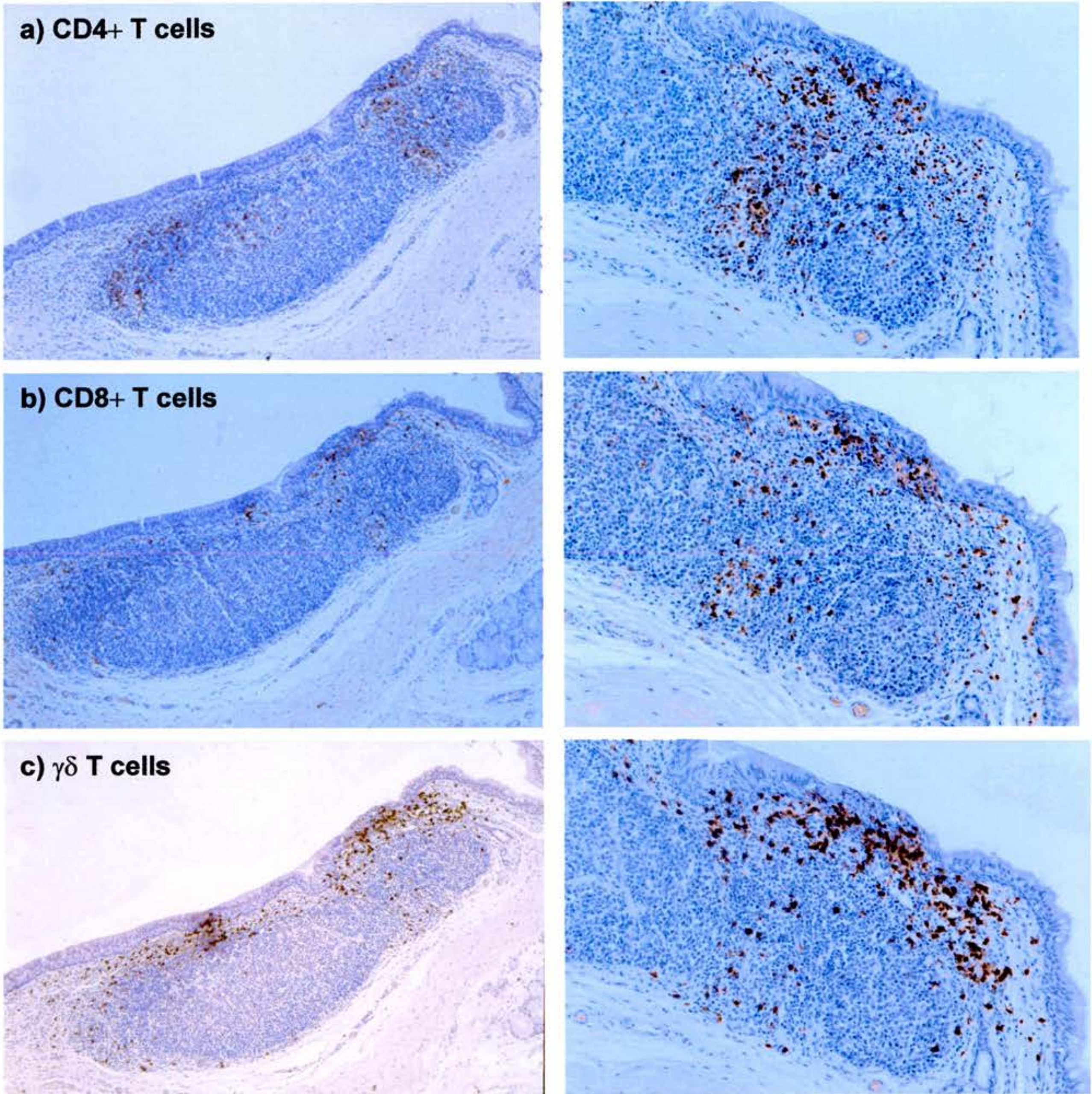
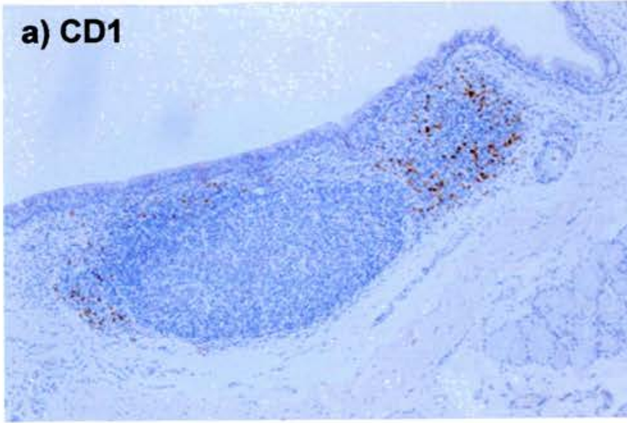
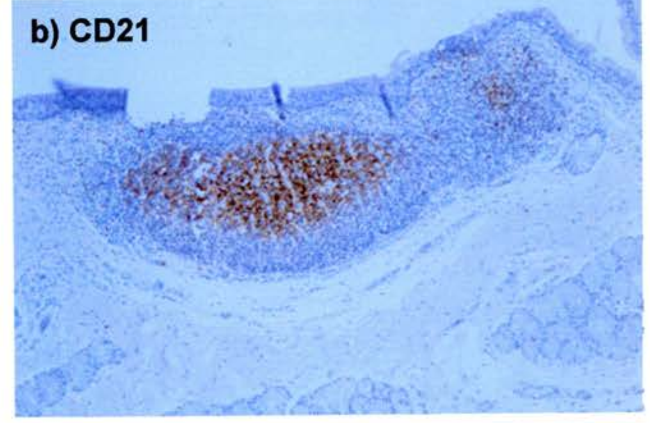
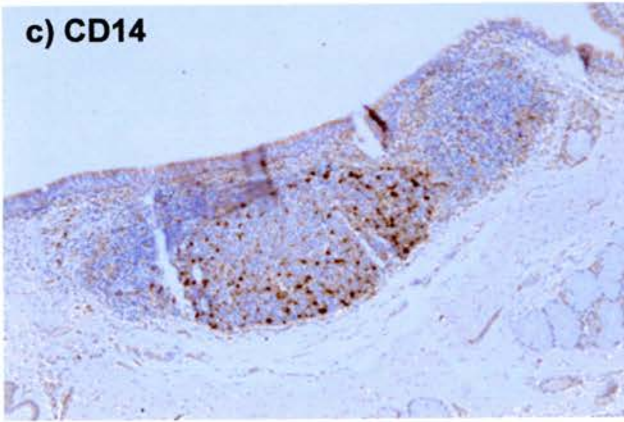


Figure 2.7a-c *Immunohistochemical labelling for different T cell subsets in serial sections of an ovine nasal lymphoid nodule. The left hand figures show the distribution of T cells in the whole nodule at ×56 magnification, while the right hand figures show cells surrounding a follicle at ×112 magnification*







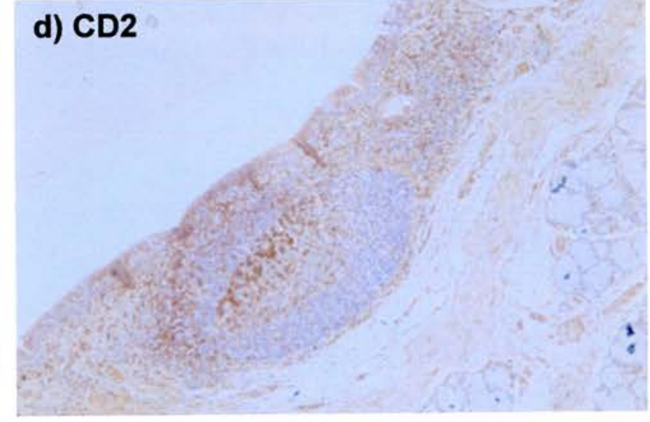


Figure 2.8a-d *Immunohistochemical labelling for dendritic cells (CD1), follicular dendritic cells (CD21), macrophages (CD14), and* $\alpha\beta$ *T cells (CD2) in serial sections of a nasal lymphoid nodule,* $\times$*56 magnification*

2.3.3 Ultrastructure of the lymphoepithelium

SEM showed that both non-ciliated and ciliated cells were present in the follicle-associated dome epithelium. Non-ciliated cells had varying numbers of short, irregular microvilli or microfolds, which were sometimes flattened to form irregular ridge-like microplicae. In the central area of the dome, a region of flattened, relatively smooth epithelium was present; where this had been disrupted large numbers of lymphocytes were seen, apparently extruding from the surface (Figures 2.9a,b). Outside this area (Figures 2.9c,d) the epithelium was composed of ciliated cells, microvillous cells and goblet cells in varying proportions. Ciliated respiratory epithelial cells and microvillous cells were approximately equal in number in the region directly surrounding the flattened area, with microvillous cells often clustered together (Figure 2.9d). Towards the edge of the dome epithelium, a higher proportion of ciliated cells were present, with a few isolated microvillous cells scattered amongst them (Figures 2.10a,b).

TEM showed that the epithelial cells overlying the NALT were ultrastructurally heterogeneous. Respiratory epithelial cells with long, regular cilia were interspersed with cells with mucus-producing cells and epithelial cells displaying stumpy, irregular, fused microvilli. These microvillous cells were more electron-dense and appeared darker than the respiratory epithelial cells and were frequently seen in close association with lymphocytes in the underlying lymphoid tissue (Figures 2.11 and 2.12). Tangential sections showed that desmosomes formed tight junctions between both types of epithelial cell, and that lymphocytes were present within or in close association with the more electron-dense cells. It seems probable that these lymphocytes were contained within pockets of the microvillous cells. Microvillous cells were notably absent from non-lymphoid associated areas.

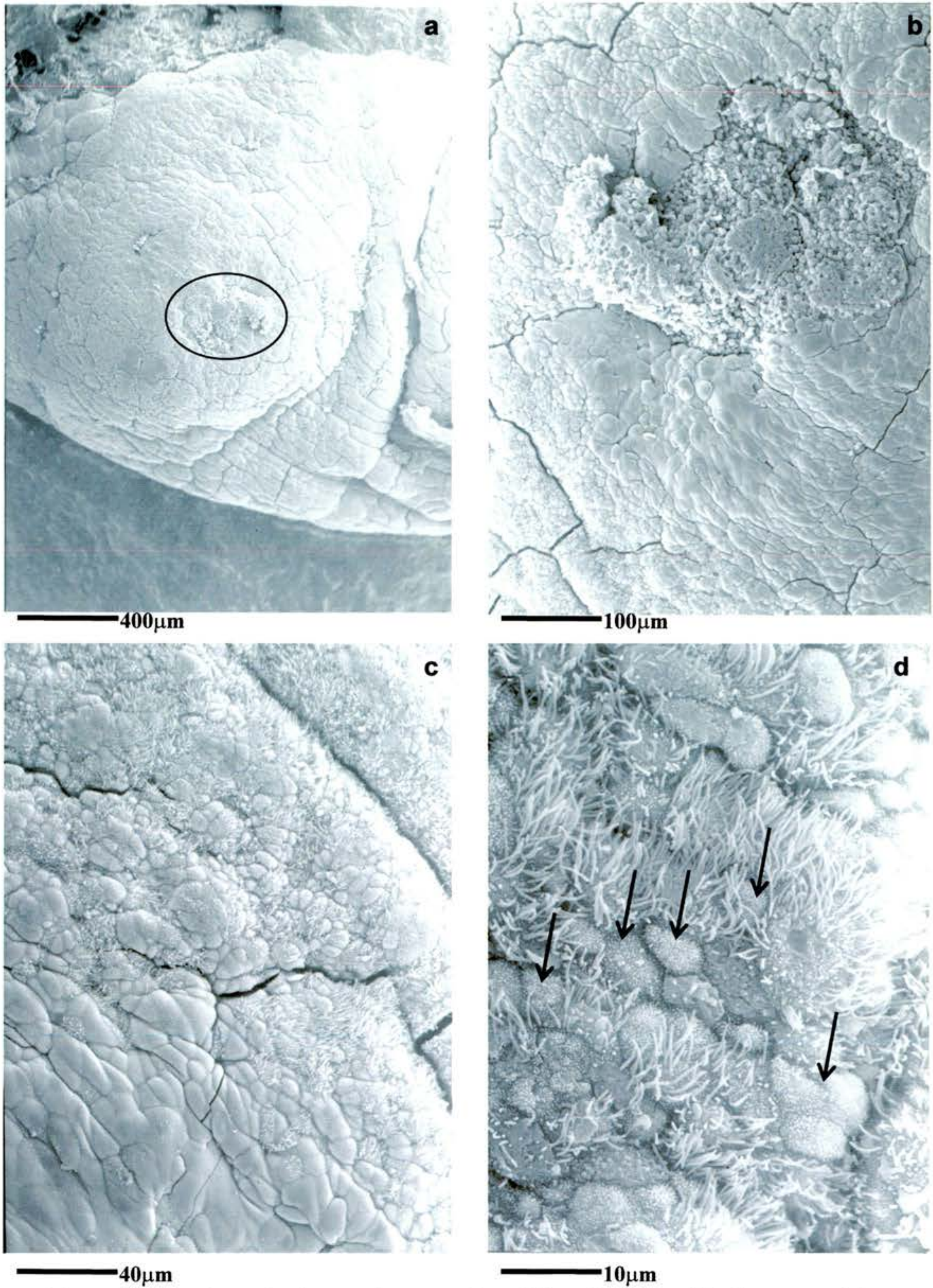
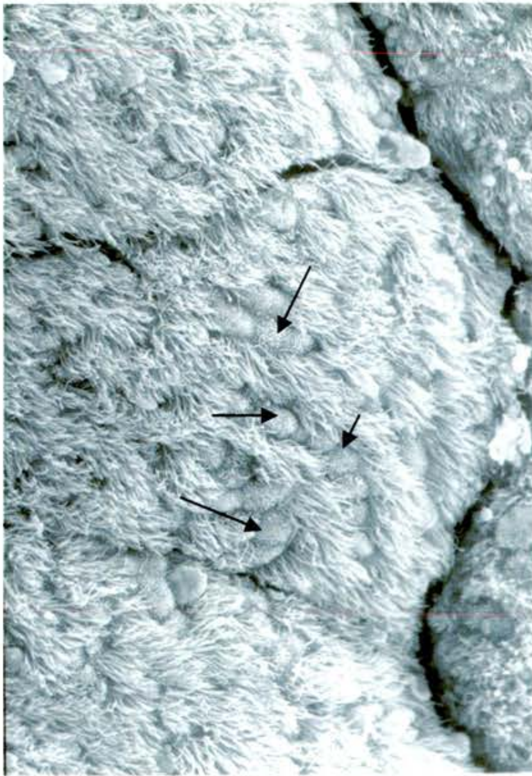


Figure 2.9a-d *Scanning electron micrographs of the dome epithelium overlying nasal lymphoid nodules. a) gross morphology of the dome; b) lymphoepithelium at the top of the dome circled in a), showing close relationship between epithelial and lymphoid cells; c) transitional area between lymphoepithelium and ciliated epithelium; d) microvillous cells (arrows) and ciliated cells*



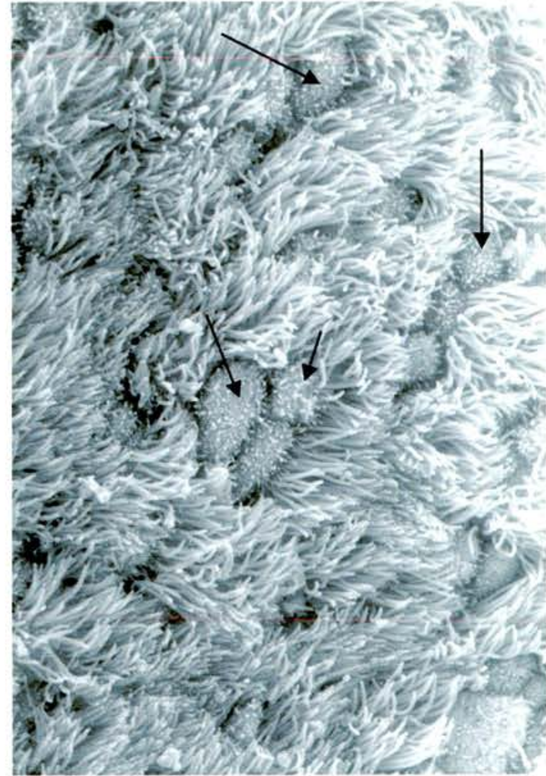


Figure 2.10 *Scanning electron micrographs showing areas at the edge of the dome epithelium where microvillous cells (arrows) are interspersed amongst characteristic ciliated respiratory epithelial cells*

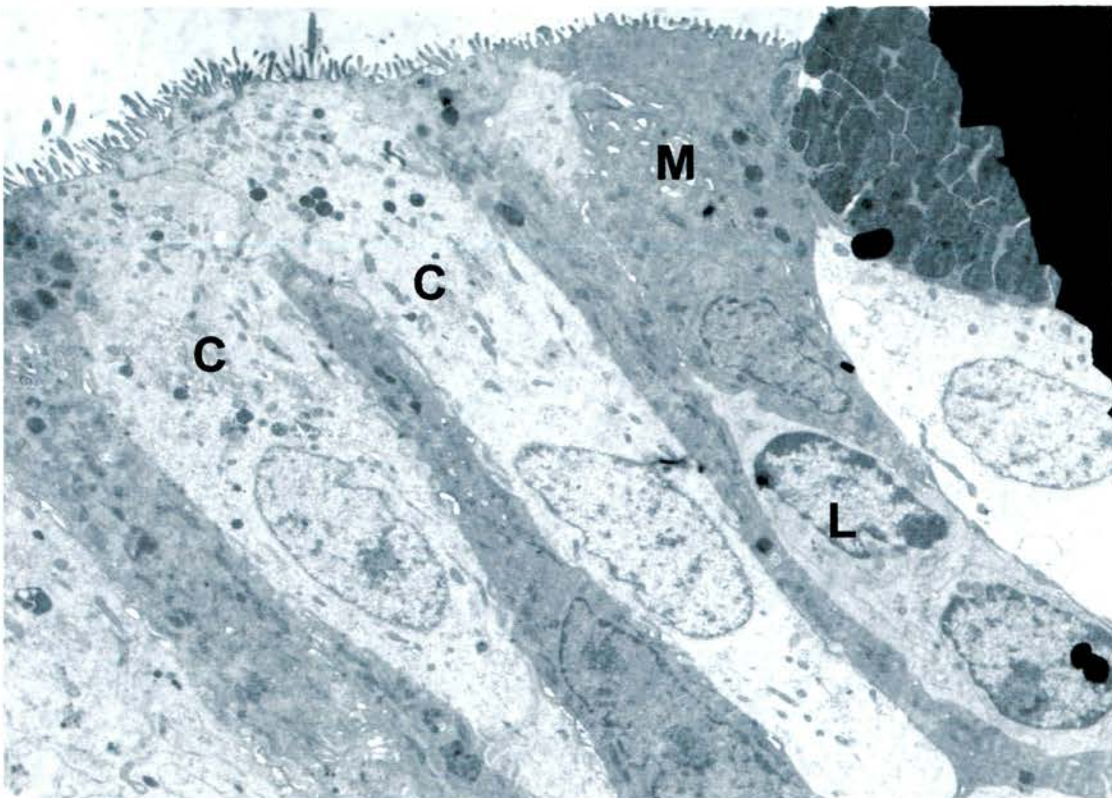
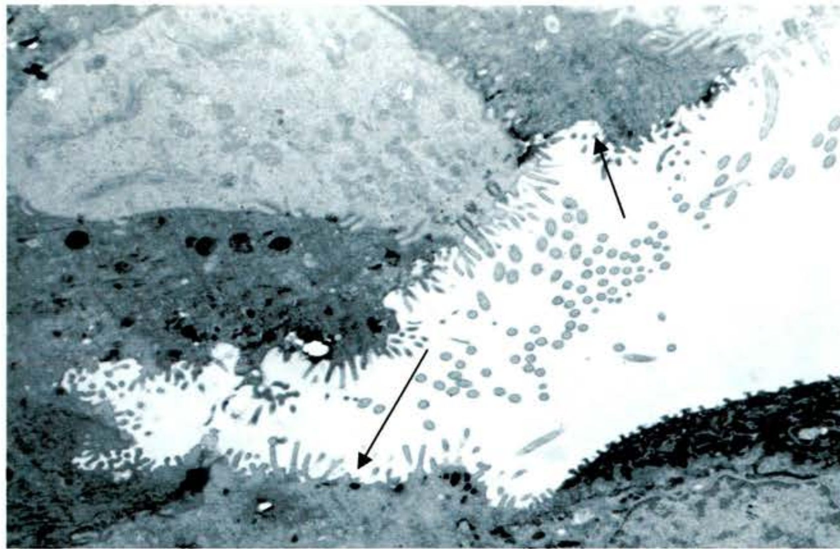
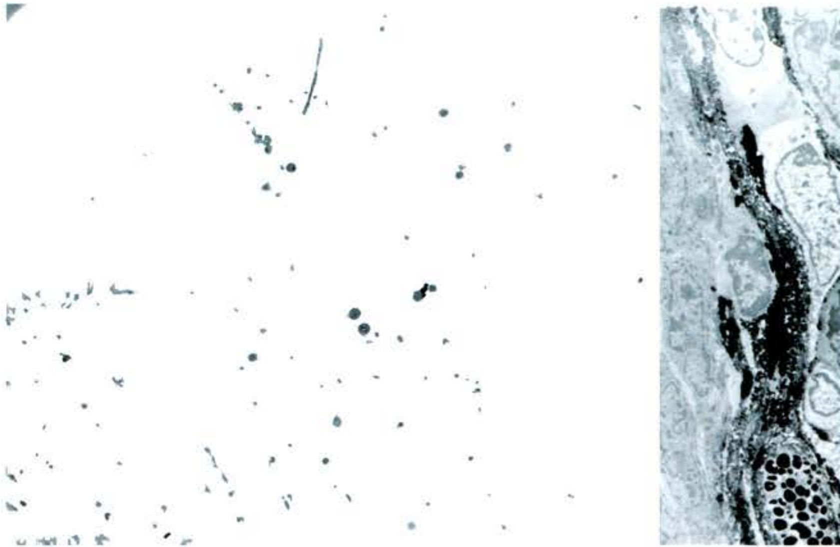


Figure 2.11 *Transmission electron micrograph showing epithelium overlying NALT showing both microvillous cells (M) and ciliated respiratory epithelial cells (C) ×1800. Note the lymphocytes (L) in close association with the M cell.*





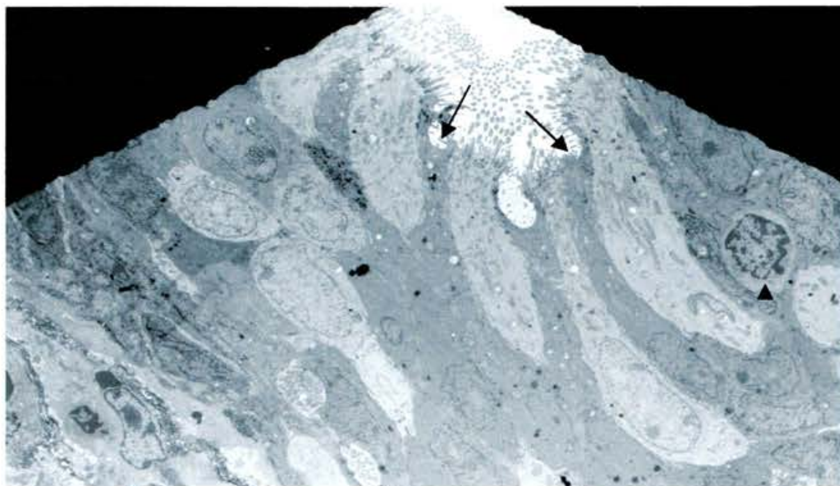


Figure 2.12 *Further transmission electron micrographs showing ultrastructure of the lymphoepithelium. Both ciliated and microvillous cells (arrows) are present. The microvillous cells are more electron-dense and are closely associated with underlying lymphocytes (arrowhead) ×1500 and ×800 magnification*

2.3.4 Marking Ovine M Cells

2.3.4.1 Lectins

The general patterns of lectin-labelling observed in the nasal lymphoid nodules are summarised in Table 2.4. However, these represent generalised results, as the labelling pattern of the different lectins was very variable, both between animals and even between different locations within the same animal. Lectin-labelling did appear to be specific in that the dilution only affected the intensity and not the pattern of labelling, and similar patterns were observed for a lectin whether it was biotinylated or fluorescently labelled. None of the lectins applied specifically marked individual cells in a pattern that could be described as resembling the expected distribution of M cells in the nasopharyngeal tract region of the sheep. Labelling was either too general or not related to the epithelium. Labelling patterns were very different in the nasal lymphoid nodules, pharyngeal tonsil, and Peyer's patches, but no lectin could be described to specifically identify M cells in any of these regions. In the mouse PP tissue UEA I bound to the surface of individual cells along the dome epithelium overlying the Peyer's patches and AAA identified individual cells in their entirety along the dome epithelium. This is comparable to the pattern of M cells previously described for these lectins in this type of tissue and provided a useful positive control.

Lectin	Staining Pattern
CON A	Mucus, whole epithelium, much of underlying tissue
DBA	Virtually no labelling, occasionally mucus
PNA	Mucus and goblet cells
RCA 120	Faint general epithelial binding
SBA	Mucus and goblet/globular cells
UEA I	General epithelium and mucus
WGA	General epithelium and mucus
GSL I	No epithelial labelling
LCA	Strong general epithelial labelling, some mucus and goblet cells
PHA-E	Stains most of epithelium and mucus
PHA-L	Patchy epithelial labelling, including mucus
PSA	General epithelial labelling, goblet cells and mucus
SJA	Labelling some cells along epithelium, not restricted to epithelium overlying lymphoid tissue
Succinylated WGA	Mainly goblet cells and mucus
GSL II	Patchy labelling along epithelium
DSL	General faint epithelial labelling
ECL	Patchy epithelial labelling, mucus and goblet cells
Jacalin	General epithelium, goblet cells, mucus
LEL	General epithelium and mucus
STL	General epithelium
VVA	Mucus
ACL, ACA	All epithelial surface, goblet cells
BPL	Nothing, no epithelial binding
SConA	No epithelial labelling, everything in tissue
EEL	Nothing, no epithelial labelling
GNL	No epithelial labelling, faint mucus staining
GSLIβ_4	No epithelial binding
LTL	No epithelial labelling
MAL1	Very faint general epithelial labelling
MPL	Surface of goblet cells
SNA, EBL	Mucus and goblet cells
WFA, WFL	Faint general epithelial labelling
AAA	General epithelial labelling

Table 2.4 *General lectin labelling patterns observed in ovine nasal epithelium*

2.3.4.2 Vimentin

Some specific labelling for vimentin was apparent within nasal lymphoid follicles, but vimentin was not present in the epithelium overlying either lymphoid or non-lymphoid tissue in the ovine nasopharynx. The epithelium above both ileal and jejunal Peyer's patches was also completely negative for vimentin.

2.3.4.3 Cytokeratins

The anti-pan cytokeratin antibody clearly identified all epithelial cells in the nasal mucosa regardless of the type of epithelium or whether it was present over lymphoid or non-lymphoid tissue. There was no labelling apparent within the underlying tissue and no non-specific background staining. The wide variety of cytokeratin peptides recognised by this mixture of monoclonal antibodies meant that the whole epithelium was labelled, and therefore more specific antibodies were used.

The anti-cytokeratin peptide 4 antibody showed strong labelling of the epithelium in specific areas, however this appeared to be unrelated to areas overlying nasal lymphoid follicles and pharyngeal tonsillar tissue. Some cells within the epithelium in these areas were more clearly identified than others but this phenomenon was also observed in non-lymphoid associated areas. This peptide appeared to be present in a variety of cells, and no specificity to M cells or lymphoid tissue associated areas was observed.

No positive labelling was observed for the anti-cytokeratin peptide 8 monoclonal antibody in this location in the sheep, although faint background staining was observed throughout the tissue sections. The anti-cytokeratin peptide 18 monoclonal antibody showed different labelling profiles in different animals. In tissues from some sheep the whole epithelium was very strongly and specifically labelled, whilst in tissues from other sheep a very similar binding pattern to that observed with the anti-cytokeratin peptide 4 antibody was demonstrated. This peptide appears to be patchily distributed and varies in quantity between animals, but is not specific to M cells, or epithelial areas overlying lymphoid tissue.

2.3.4.3 Alkaline Phosphatase

Immunolabelling with the anti-alkaline phosphatase monoclonal antibody resulted in a dark brown reaction product along the whole epithelium overlying ovine jejunal Peyer's patches including lymphoid and non-lymphoid associated tissue. All epithelial cells appeared to contain alkaline phosphatase. In the nasal epithelium very faint brown colouration was visible above both lymphoid and non-lymphoid tissue, but it was difficult to distinguish individual epithelial cells. With the alkaline phosphatase kit very dark, bright purple colouration was visible along the whole

epithelium above the Peyer's patches, demonstrated in Figure 2.13a. This colour reaction was continuous above the lymphoid tissue, and no negative cells indicative of M cells were observed. In the nasal region, no purple colouration was visible along the epithelium overlying lymphoid or non-lymphoid associated areas, demonstrated in Figure 2.13b.

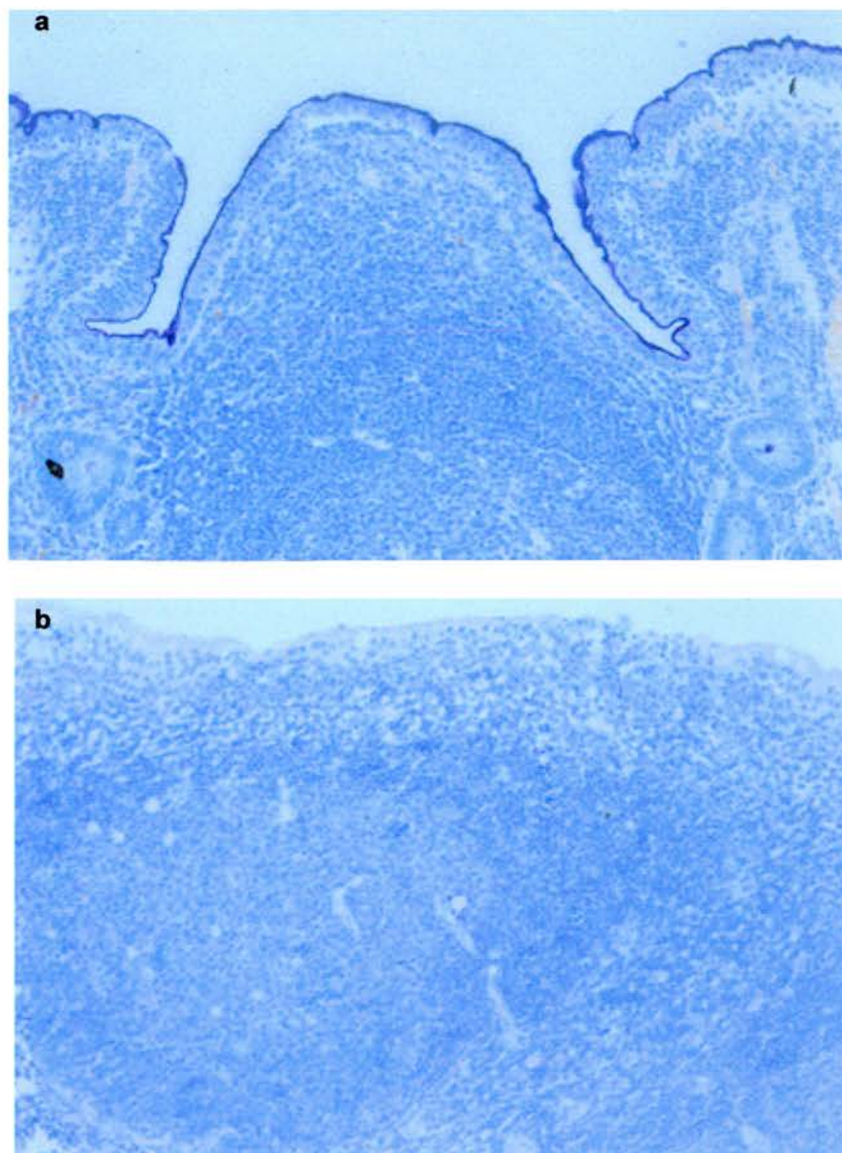


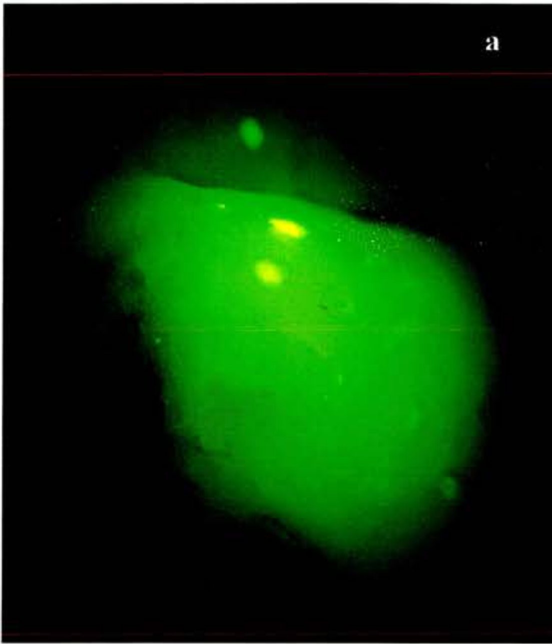
Figure 2.13a-b *Ovine jejunal PP (a) and nasal lymphoid follicle (b) treated to demonstrate alkaline phosphatase activity, ×147 magnification. Nasal epithelium is clearly negative for this enzyme, whilst PP is strongly positive*

2.3.5 M Cell Functional Activity

2.3.5.1 *In vitro* Application of Fluorescent Microparticles

No visible signs of degeneration or shedding of the epithelium were observed in epithelial tissue within the 4 hour culture period. Tissue sections stained with HE provided further evidence that little or no degeneration had occurred during incubation. Following the topical application and incubation with fluorescent latex microparticles, tissue explants were examined under a stereomicroscope, and aggregations of particles could be observed on the surface of the epithelial tissue. Unfortunately with the magnification range of this microscope it was difficult to determine the morphology of the tissue, and the position of dome epithelial areas overlying lymphoid tissue, so it was difficult to relate these aggregations to areas where M cells may be present. Interesting patterns of binding were often observed, with particles clearly concentrated in a circular area that may have been the dome epithelium overlying follicles where M cells were located, demonstrated in Figure 2.14. Particles were often clearly trapped in the mucus overlying the tissue. This was more problematic in tissue that had been cultured for longer periods of time (>2 h) where more mucus was observed on the epithelial surface.

No microparticles could be observed in sections from cultured tissues that were fixed and processed routinely, but this was probably due to the dissolution of the particles in xylene. However, microparticles were clearly visible on sections from the tissue that had been pre-fixed, cryoprotected and frozen in liquid nitrogen. Large numbers of particles were commonly found bound to the surface of the epithelium and within the epithelium due to the large quantities that had been placed on the surface of the explants, demonstrated in Figure 2.15. This particle uptake appeared to be specific to areas of epithelium overlying mucosal lymphoid follicles where M cells are present, and no uptake could be demonstrated in areas separate from lymphoid areas. Particles of all sizes were clearly taken up into the FAE, but quantification and comparison of uptake was difficult and no one size of particle appeared to be optimally transcytosed. Following uptake most particles were present within the epithelium, but occasionally a small proportion of the particles could be observed in the underlying tissue.



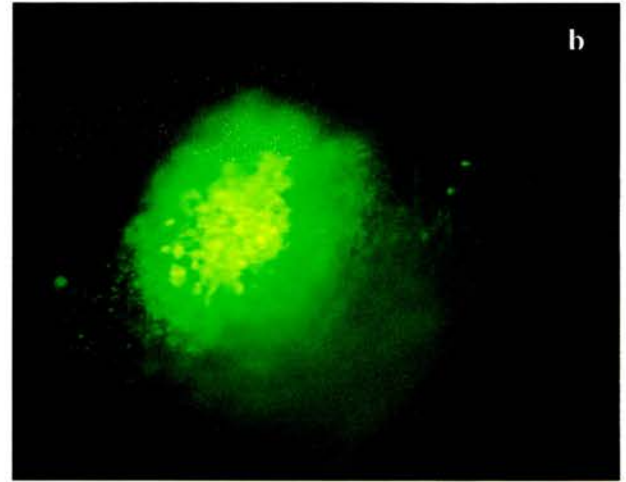


Figure 2.14 *Stereomicroscope images of NALT surface demonstrating potential dome areas where the binding and uptake of microparticles is concentrated (1.0μm particles a) ×8 magnification b) ×27 magnification*

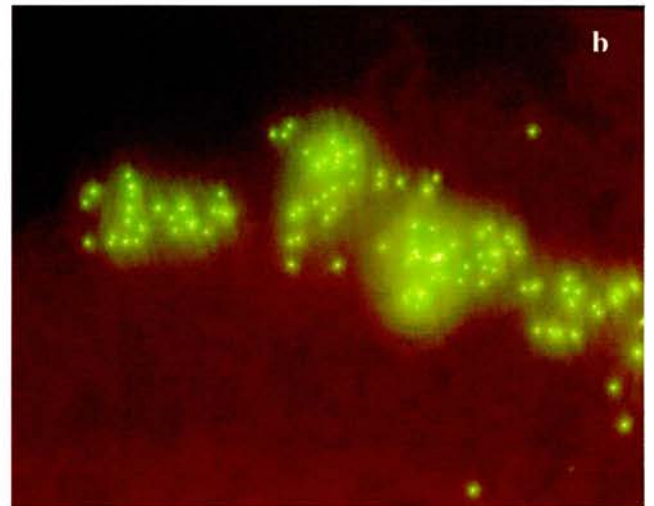
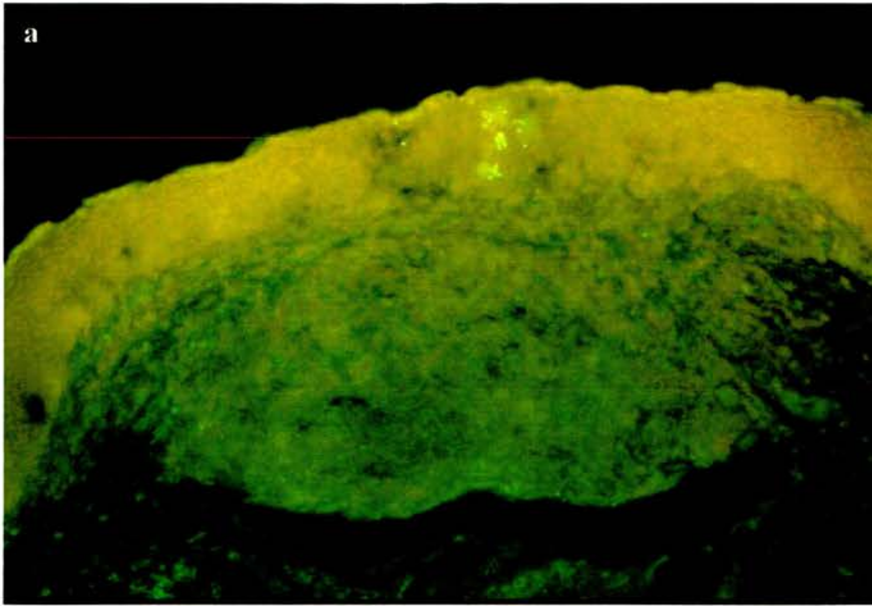
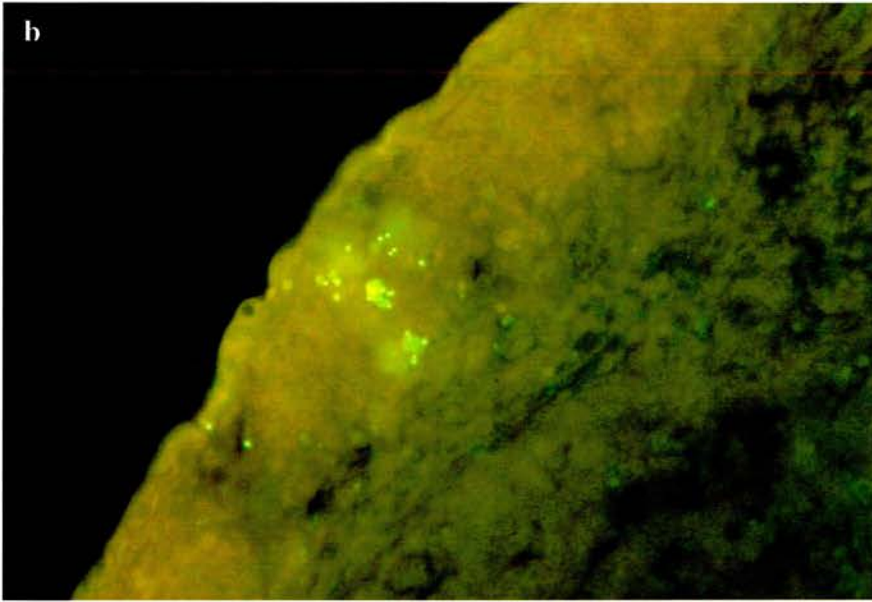


Figure 2.15 *Uptake of fluorescent latex microparticles following* in vitro *application to NALT tissue explants. a) 0.75μm particles, ×112 magnification, b) 0.5μm particles, ×224 magnification*





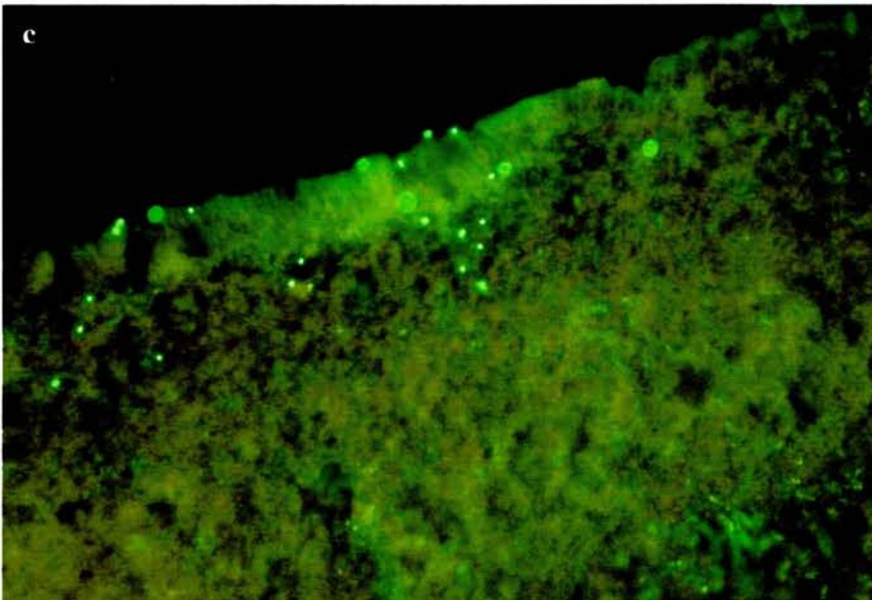


Figure 2.16 *Uptake of fluorescent latex microparticles into FAE overlying NALT following* in vivo *application. a) 0.5μm particles, ×155 magnification, b) 0.5μm particles, ×309 magnification, c) 1.0 μm particles ×155 magnification*

It was not possible to distinguish individual cells that contained particles, and therefore potential M cells could not be positively identified. The different lengths of time of incubation of the tissue explants with the particles did not appear to greatly alter the distribution of particles within the epithelial tissue. Particle uptake into the epithelium could be demonstrated as early as 30 min, and particles were more often observed at a greater depth in the tissue following longer incubation periods.

2.3.5.2 *In vivo* Application of Fluorescent Microparticles

Following the intra-nasal application of fluorescent latex microparticles *in vivo*, particle uptake could be clearly demonstrated within the FAE overlying follicular areas, demonstrated in Figure 2.16. It was not possible to determine specific cells responsible for particle uptake or to identify potential M cell candidates. Examination of tissues using confocal microscopy demonstrated that the particles were internalised within cells, and were not associated with the surface of the tissue sections. This observation indicated that the microparticles were being actively transported rather than being dragged through the section with the microtome blade. However, the distribution of areas where particles had been taken up was very patchy and only small areas of particle uptake could be demonstrated throughout the area of nasopharynx that contained lymphoid nodules. Encouragingly, no particle uptake was observed in non-follicular areas. Sections of pharyngeal tonsil revealed that the epithelium overlying follicles in this organ was also capable of particle uptake, but areas where this could be demonstrated were also relatively scarce. The infrequency of observed particle uptake was likely to be due to the dilution effect of applying a small volume of microparticles to such a large area, and made the comparison of uptake of different sizes of particles difficult. However, uptake of all sizes of particles between 0.5-1.0μm in diameter could be demonstrated within the epithelium. Particles that had been taken up penetrated the entire depth of the epithelial cells and were occasionally observed within the dome area. Particles often reached a greater depth into tissue than was observed when particles were applied to tissue explants *in vitro*.

2.4 DISCUSSION

The present study confirmed that lymphoid tissue found in the ovine nasopharynx is an organised lymphoid tissue characteristic of the MALT, and may be referred to as NALT. The concepts of mucosal immune protection can therefore be applied to ovine NALT, which contains all the elements necessary to function as a potent antigen sampling site. Ovine NALT, together with the pharyngeal tonsil, is the main component of defence in the ovine upper respiratory tract and, due to its strategic location and ability to trap, process and respond to inhaled antigens may play an important role in the development of successful nasal vaccines.

In the sheep, the distribution of ovine NALT was relatively consistent between animals. The nasopharyngeal lymphoid nodules were clustered posterior to the opening to the Eustachian tube, in the proximity of the pharyngeal tonsil. Lymphoid tissue has been observed in this location in many species, including the horse (Mair *et al.*, 1988), where it may help guard against infection spreading from the pharynx towards the inner ear. This strategic location also allows immuno-surveillance at the point where inhaled air, laden with antigenic material enters the pharyngeal duct, the first tubular structure of the respiratory tract. The results presented here are therefore in accord with the studies of Chen *et al.* (1989), which detailed the distribution of lymphoid tissue throughout the ovine respiratory tract.

Immunohistochemical studies have demonstrated that the lymphoid nodules in the ovine respiratory tract are characteristic of the simple follicular areas traditionally described in mucosal lymphoid tissue (Hein, 1999), and are similar in structure to the secondary lymphoid follicles observed at other mucosal sites in the sheep, such as jejunal Peyer's patches (Landsverk *et al.*, 1991) and rectal lymphoid follicles (Sedgmen *et al.*, 2002). The general composition of the lymphoid nodules resembles that of a predetermined lymphoid organ, demonstrating that NALT in sheep is a highly structured and organized secondary lymphoid tissue that has specific functions within the mucosal immune system. Ovine NALT consists of discrete B- and T- cell areas, as in rodent and human NALT (Kuper *et al.*, 1990; Brandtzaeg & Halstensen, 1992; Koornstra *et al.*, 1993), and like rat NALT appears to contain more B than T cells and more T helper cells than T suppressor/cytotoxic cells (Koornstra *et al.*, 1991). As described in other species mature B lymphocytes

are common in the central area of the follicle, rather than the periphery or corona. IgA antibody was clearly visible both within the tissue and on the epithelial surface, where it may have been secreted, and it appeared that switching of B cells to IgA+ may have occurred at the centre of the follicles. There are large numbers of γδ T cells present in ovine NALT when compared to MALT in rodents or humans, which is likely to be a reflection of the larger number of γδ T cells present in the ruminant immune system (γδ T cells may constitute up to 50% of all T cells) (Hein & Mackay, 1991). γδ T cells are thought to play a major role in the early defence of mucosal surfaces against infectious agents (Hein & Mackay, 1991), and in ruminants are capable of much greater diversity in antigen receptors and therefore may play a much more important role than in other species (Evans *et al.*, 1994; Hein & Dudler, 1997).

The cell populations described in ovine NALT are typical of those defined in mucosal inductive sites. Mouse NALT, a well-defined inductive site, is similarly composed of "unswitched" B cells and naïve T helper cells that have the capacity to provide help for B-cell maturation and differentiation, and to maintain immune memory (Wu *et al.*, 1996), and recent studies have shown that mouse NALT is a mucosal inductive site for both cellular and humoral immune responses (Zuercher *et al.*, 2002). Evidence suggests that organised MALT in mucosal inductive sites, such as NALT and Peyer's patches, are responsible for the initiation of antigen-specific responses, characteristically involving IgA responses (Neutra & Kraehenbuhl, 1992). The antigen-stimulated lymphocytes leave these tissues and migrate to mucosal effector sites (diffuse MALT) where the immune response is expressed (Mestecky & McGhee, 1987; Wu *et al.*, 1997).

Ovine nasal lymphoid tissue is covered by a specialized epithelium consisting of ciliated and non-ciliated cells, in which the cilia are replaced by short, irregular microvilli. This modified lymphoepithelium can be easily distinguished from the adjacent epithelium, both topographically and ultrastructurally, and has previously been noted in related areas in many species, including the nasopharynx of the horse (Mair *et al.*, 1987) and overlying the nasopharyngeal tonsils of humans (Karchev & Kabakchiev, 1984; Fujimura, 2000). A specialised lymphoepithelium is also seen overlying lymphoid nodules in the caprine respiratory tract (Kahwa & Purton, 1996). The epithelial cell types, proportions and distribution are similar to those described in

a study of the ovine pharyngeal tonsil (Chen *et al.*, 1991). Based on their morphological features and relationship to underlying lymphoid tissue, these non-ciliated microvillous cells resemble the antigen-sampling cells occurring in MALT in other species, which are commonly known as M cells (Owen & Jones, 1974; Bye *et al.*, 1984; Wolf & Bye, 1984; Bienenstock, 1985). The close contact and communication between the epithelium and the lymphocytes afforded by the lymphocyte-containing pockets in these microvillous cells are crucial to their role in antigen uptake and processing. The uptake of colloid carbon has been demonstrated in MALT in the ovine pharyngeal tonsil (Chen *et al.*, 1989). It seems probable that the microvillous cells in ovine NALT are fully capable of taking up particulate antigens, and demonstration of their functional activity is required to confirm that these cells are functionally similar to M cells described in other species and in other locations.

However, there are a number of problems associated with the identification of M cells using electron microscopy. TEM only focuses on very small areas and small numbers of cells, and misleading assumptions may be made regarding the larger cell population (Jepson & Clark, 1998). SEM can only characterise the epithelial surface and no information on the uptake of material can be provided. Therefore a marker for ovine M cells would be very useful for further analysis of many aspects of their distribution, function and development, and as a prerequisite for M cell culture to provide reliable *in vitro* models. It may also provide a means for specifically targeting ovine nasal M cells for mucosal vaccination. However, despite the wide range of lectins, cytoskeletal intermediate filaments and enzymes tested in this study, it appears that at present there is no specific marker for ovine nasopharyngeal M cells. None of the published methods for other species were specific for M cells overlying ovine nasal lymphoid nodules or pharyngeal tonsil. No reliable markers have been discovered for human M cells and it appears that each marker is only applicable to the limited range of species and or sites identified (Jepson *et al.*, 1996; Brinck *et al.*, 1995; Sharma *et al.*, 1996). In the nasopharyngeal region in other species, UEA I has been shown to selectively label rabbit tonsillar M cells (Gebert, 1997), GS I-B4 is a specific marker for M cells in rat NALT (Takata *et al.*, 2000) and hamster NALT M cells can be distinguished from other epithelial cells by the

expression of glycoconjugates possessing terminal α(1-3)-linked galactose (Giannasca *et al.*, 1997). These lectins did not display the required specificity in ovine NALT providing further evidence that species specificity does exist.

None of the other lectins used in this study could be demonstrated to specifically bind to the surface or label M cells in this location in the sheep, and there may be a number of reasons to explain the lack of specificity for M cells. Firstly, the thick overlying mucus layer in the nasal region may prevent contact between the lectin and the epithelial surface, even though it may be largely absent from the FAE. The majority of lectins bind to mucus itself because it has a carbohydrate rich milieu, which may exclude access to M cells. Secondly, most lectins are capable of recognising a hierarchy of related carbohydrate structures and therefore label a number of different cell types with different intensities. Thirdly, it has only been possible to utilise a limited number of the available lectins on ovine NALT and the appropriate lectin for targeting ovine M cells may not have been included.

The problems of transferring lectin targeting data between species, and even between different regions in the same species are well recognised. The large variability in lectin binding indicates site and species specificity for M cell receptors, and it may not be surprising that difficulties have been experienced in finding a lectin that specifically targets ovine M cells. Attempts to find a lectin that specifically targets glycoconjugates on the surface of human M cells have also been unsuccessful (Sharma *et al.*, 1996; Kucharzik *et al.*, 1999). Both humans and sheep consist of large outbred populations, and large inter-individual differences in the expression of surface glycoproteins may exist that do not permit specific lectin binding. However, epitopes on the apical surface of M cells in the equine nasopharyngeal tonsil are reactive with *Griffonia simplicifolica* lectin I – isolectin B_4 (GSLI- B_4) specific for α-linked galactose (Kumar *et al.*, 2001).

The reduction in expression of brush-border alkaline phosphatase in M cells has successfully been used as a means of marking M cells in rat and mouse PP (Owen & Bhalla, 1983; Smith *et al.*, 1987, 1988). This study demonstrates that it is not possible to use alkaline phosphatase as a marker for M cells in the ovine nasopharyngeal tract. A comparison of the demonstration of this enzyme in the ovine nasal region with the PP clearly demonstrates that this enzyme is either absent in the

nasopharyngeal tract or present in very low quantities that cannot be detected. It is clear that alkaline phosphatase is present in large quantities in the gut epithelium, suggesting that it has a function in digestion, which is not required in the nasal region. Alkaline phosphatase has been previously used to distinguish M cells in ovine jejunal Peyer's patches (Press *et al.*, 1991), but in this study all PP epithelial cells appeared to contain alkaline phosphatase.

The expression of vimentin in M cells in rabbit GALT has been previously demonstrated (Gebert *et al.*, 1992, Jepson *et al.*, 1992). However, the presence of vimentin could not be demonstrated in epithelial cells in the ovine nasopharyngeal tract, although it may be possible that the anti-vimentin clone used was not fully cross-reactive with sheep vimentin. Similarly, cytokeratin 18 has been shown to mark porcine PP M cells (Gebert *et al.*, 1994) and cytokeratin 8 has been shown to mark rat PP M cells (Rautenberg *et al.*, 1996), but none of the anti-cytokeratin peptides used in this study were specific for ovine nasal M cells. There appears to be site and species specificity in the expression of these cytoskeletal intermediate filaments, since there are no reports of M cell labelling in NALT using the expression of intermediate filaments.

Another means by which M cells may be identified is by demonstration of their functional activity in the uptake of particulate material. Particle uptake into ovine nasal epithelium could be demonstrated both *in vitro* and *in vivo*, and this uptake appeared to be specific to areas of FAE overlying MALT. Particles not only bound specifically to the surface of the epithelium but were also observed within epithelial cells and in the dome area of the underlying MALT. Thus cells with the functional activity of M cells were present in the epithelium overlying areas of MALT in the ovine nasopharyngeal tract. However, in the absence of an M-cell specific marker for ovine NALT, it was not possible to identify individual cells responsible for particle uptake or prove that the cells had the morphological characteristics of M cells. Levels of uptake of microparticles of different sizes were comparable, although it was not possible to quantify particle uptake. It was clear that particles in the nanoparticle size range of 0.1 – 1.0μm diameter were efficiently transported across the epithelium.

The *in vitro* application of microparticles on tissue explants was developed to provide a useful model to optimise particle uptake. Many particles were taken up into the FAE of these explants but quantification of this uptake was difficult. Particle uptake was facilitated by the large numbers of particles placed on the surface of very small areas of tissue. Relatively large numbers of microparticles could be seen within the epithelium as little as 30 min after particles were applied, but they did not seem to be translocated into the underlying lymphoid tissue within culture periods of up to 4 hours. This may have been due to the conditions of the tissue in the culture system, although little or no degenerative changes were observed during this period of incubation. Such *in vitro* work is often hampered by inadequate maintenance or tissue viability, which leads to limited and non-selective particle uptake (Pietzonka *et al.*, 2002), but this did not appear to be relevant for the short culture times required to allow the uptake of particles in this system.

The quantities of particles that could be demonstrated within the epithelium from the *in vivo* experiments were considerably lower. This may have been because few particles from the intra-nasal spray had contact with areas of FAE, or due to the dilution effect of applying a small volume of particles to such a large target area. In addition some particles may have been swallowed or removed by clearance mechanisms operating in the upper respiratory tract. However, it was interesting to note that particle uptake could be observed in the epithelium overlying both the nasal lymphoid nodules and the pharyngeal tonsil following application of particles with this intra-nasal spray. It was also encouraging to note that particle uptake appeared to occur only in areas of FAE, and that particles had managed to penetrate the entire depth of the epithelium within 45 min, and could occasionally be observed in contact with lymphocytes in the dome epithelium. Similar sized particles have been shown to be taken up into the nasal mucosa in the rat and mouse and have reached NALT, draining cervical lymph nodes and the bloodstream in as little as 15 min (Alpar *et al.*, 1994; Brooking *et al.*, 2001; Eyles *et al.*, 2001). In the gut *in vivo* studies of particle uptake using ligated gut loops have been employed to compare uptake into the epithelium overlying PP in different conditions, and such gut loops have been used successfully in sheep to model mucosal immune responses (Gerdts *et al.*, 2001), but are not applicable in the nasopharyngeal region.

In conclusion, the present study has shown that particle uptake in the ovine nasal epithelium occurs specifically in areas overlying MALT. The following chapter describes the development of a microparticle delivery system to target and optimise antigen uptake by ovine nasal M cells.

CHAPTER 3

Development of a Biodegradable Microsphere Delivery System for Mucosal Vaccination

3.1 INTRODUCTION

The aim of the research described in this chapter was to develop an appropriate delivery system to target M cells in the mucosal epithelium overlying NALT in the sheep. Microparticles or nanoparticles prepared from the biodegradable and biocompatible poly(D,L-lactide-co-glycolide) (PLG) polymer have been extensively studied as delivery vehicles for protein and polypeptide antigens in mucosal vaccination (reviewed in Brayden & Baird, 2001). The nasal absorption of PLG particles has not been studied in the sheep, but the use of other bioadhesive microsphere delivery systems has been shown to enhance nasal absorption and bioavailability of a number of pharmaceutical compounds or drugs, such as biosynthetic human growth hormone or desmopressin (Illum *et al.*, 1990; Critchley *et al.*, 1994).

Poly (L-lactide) is a crystalline polymer, whereas the glycolide monomer forms an amorphous structure. Therefore the copolymer PLG contains a mixture of both crystalline and amorphous features dependent on the ratio of lactide: coglycolide. The chemical structure of PLG is shown in Figure 3.1. PLG degrades by random hydrolysis of the ester linkages, initially to smaller polymeric fragments and ultimately to the constituent monomeric acids.

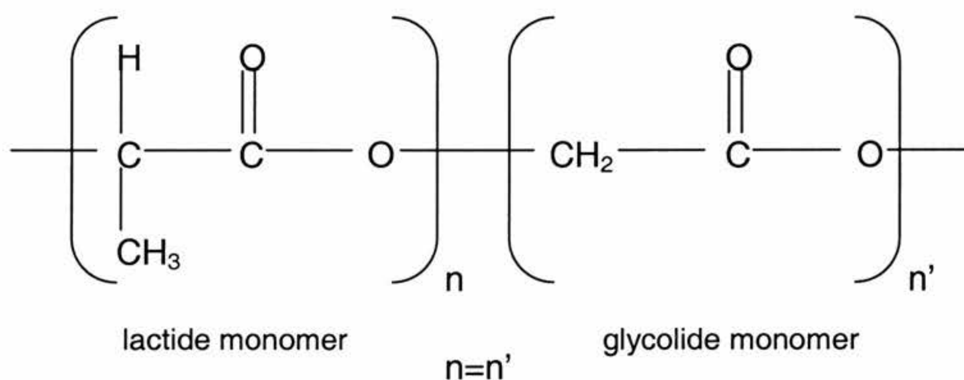


Figure 3.1 *Chemical structure of PLG*

There are a number of techniques available for the preparation of PLG microparticles. For the purpose of this study the water/oil/water (w/o/w) double emulsion, solvent evaporation technique was used, demonstrated in Figure 3.2. This is a phase separation process, and the most widely used technique for preparing microparticles containing water soluble drugs and proteins (Rafati *et al.*, 1997).

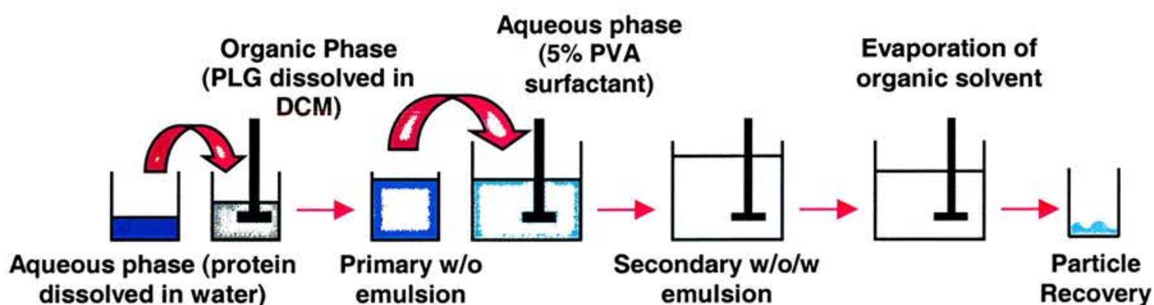


Figure 3.2 *Diagrammatic representation of w/o/w double emulsion solvent evaporation technique of particle production*

Firstly a primary emulsion is produced by homogenising an aqueous phase containing protein with an organic solvent phase consisting of PLG polymer dissolved in dichloromethane (DCM). This primary emulsion is then homogenised with a large volume of surfactant, polyvinyl alcohol (PVA), to produce a secondary emulsion. PVA acts as an emulsion stabiliser by decreasing the interfacial tension between the suspended organic polymer droplets and the continuous phase, thereby preventing aggregation and reducing the coalescence of the droplets. Excess DCM then partitions into the continuous phase and evaporates at the air-water interface for several hours. As the solvent evaporates from the organic droplets the PLG precipitates and hardens, forming microspheres and entrapping the protein. Finally the particles are washed several times to remove excess PVA, and lyophilised. Figure 3.3 demonstrates the smooth spherical particles that are typically produced by the w/o/w double emulsion solvent evaporation technique.

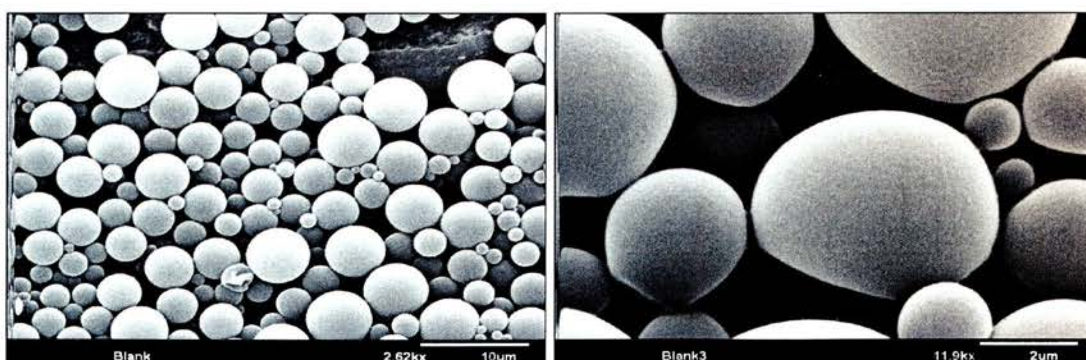


Figure 3.3 *SEM images of typical PLG microspheres demonstrating their smooth surface and spherical shape*

The formulation conditions of microencapsulation in the water/oil/water double emulsion, solvent evaporation technique are critical to the size and size distribution of PLG particles, the amount and distribution of protein associated with particles, and the protein release characteristics of particles (Jeffery *et al.*, 1993; Cohen *et al.*, 1991; Yan *et al.*, 1994; Hora *et al.*, 1990). The properties of particles produced are influenced by a range of key factors such as the concentration and type of polymer, protein and stabiliser (Alonso *et al.*, 1993; Arshady, 1990; Jeffrey *et al.*, 1993), shear conditions (Sah *et al.*, 1994; Yan *et al.*, 1994), and the stability of the primary w/o emulsion (Nihant *et al.*, 1994). Microspheres can be produced over a wide size range, from less than 200nm to several hundred microns. The principle factors that control particle size are the speed, equipment and technique used for mixing the two phases and the concentration of polymer. Particle size tends to decrease exponentially with increasing mixing speed, accompanied by a narrowing of the particle size distribution, and tends to increase with polymer concentration (Watts *et al.*, 1990). The effect of a number of variables on protein encapsulation and particle size are investigated in this study, with the aim of optimising formulation conditions for the preparation of microparticles suitable for use as an intranasal delivery system in sheep.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Two 50:50 PLG copolymers of different molecular weights (MW) were used. The low MW polymer (Resomer RG502H, Boehringer Ingelheim, Germany) and the high MW polymer (P2191, Sigma, Dorset, UK) M_r 8,400-8,600 (Callacombe *et al.*, 1997; Park *et al.*, 1998) and M_r 40,000-70,000 respectively. PVA (M_r 30,000-70,000), bovine serum albumin (BSA) (M_r 67,000), BSA conjugated to fluorescein isothiocyanate (FITC-BSA) and ovalbumin (OVA) (M_r 43,000) were obtained from Sigma (Dorset, UK). Dichloromethane was obtained from Fisher Scientific (Leicestershire, UK).

3.2.2 Development of Microparticles in the Appropriate Size Range

A reproducible method was required to produce microspheres in the size range optimal for M cell uptake, whilst minimising damage to the encapsulated protein. The desired size range was between 0.1 – 3.0μm in diameter, but ideally less than 1μm in diameter. The speed and length of time of homogenisation for both primary and secondary emulsions were varied in an attempt to produce particles of the desired size. No protein was used at this stage, instead the particles were made with deionised water.

3.2.2.1 Particle Preparation

A primary emulsion was produced by homogenising 1ml of water with 5ml of 5% PLG (Resomer RG502H lactide:glycolide 50:50, Boehringer Ingelheim, Germany) in DCM at speeds ranging from 16,000rpm to 19,000rpm for 2 min. A secondary emulsion was then produced by homogenising the primary emulsion with 50ml of 5% PVA at speeds of 19,000rpm, 21,000rpm or 24, 000rpm for either 2 min or 3 min. The homogeniser used was a T25 Basic Ultra-Turrax homogeniser (IKA-Werke, Staufen, Germany). Secondary emulsions were maintained overnight under magnetic stirring to facilitate the evaporation of excess DCM. Particles were then centrifuged in a Beckman J2-21 centrifuge using a JA20.1 rotor (Beckman Coulter, High Wycombe, UK) at 12,000rpm for 10 min. The supernatant was discarded and particles were resuspended in 20ml distilled water, and centrifuged at 8,000rpm for 3 min. Particles were washed again by repeating this step, resuspended in approximately 2ml distilled water, and lyophilised (Edwards Modulyo 4K freeze-drier, Fred Baker Scientific, Cheshire, UK) for at least 48 hours.

3.2.2.2 Analysis of Particle Size

A small sample of the particles was suspended in water, and microparticle size distribution was determined by laser diffractometry in a Coulter LS 230 particle size analyser (Beckman Coulter, High Wycombe, UK). The average particle size was expressed as the volume mean diameter.

3.2.2.3 SEM

Particle size and surface morphology were examined using SEM. A small sample of microparticles was suspended in a small volume of distilled water, placed on aluminium stubs and allowed to air dry. The stubs were then sputter coated with gold (SCD030 Balzers Union Ltd., Liechtenstein) and examined in a Phillips 505 scanning electron microscope (Phillips, Eidhoven, Netherlands). Size was evaluated and demonstrated on the micrographs produced.

3.2.3 Optimising Protein Encapsulation into Microparticles

Once methods had been established to produce particles within the appropriate size range, the effect of a number of variables on protein encapsulation was investigated. Variables included the protein used, protein loading, the molecular weight of the PLG polymer, and the length and time of homogenisation. The parameters measured were the total amount of protein associated with the particles and the ratio of surface-bound: encapsulated protein. BSA was used as a model protein unless otherwise specified.

3.2.3.1 General Particle Preparation

The appropriate amount of protein was weighed out and hydrated in a volume of either 100µl or 200µl of distilled water (as detailed below). A primary emulsion was made by homogenising this aqueous protein solution with 2ml of 5% PLG in DCM in a glass test-tube at the speed and for the length of time specified below. The low MW PLG polymer was used (Resomer RG502H, Boehringer Ingelheim, Germany) unless otherwise specified. The primary emulsion was then homogenised with 20ml of 5% PVA at the speed and for the length of time specified below to create the secondary emulsion. Secondary emulsions were maintained overnight under magnetic stirring to facilitate the evaporation of excess DCM, and then centrifuged in a Beckman J2-21 centrifuge using a JA20.1 rotor (Beckman Coulter, High Wycombe, UK) at 12,000rpm for 10 min. The microparticles were washed twice to remove excess PVA by redispersion in 20ml of distilled water and centrifugation at 8,000rpm for 3 min. Particles were then rehydrated in

approximately 2ml of distilled water and lyophilised for at least 48 h. Variations in this method are detailed in sections 3.2.4.2 – 3.2.4.6 below.

3.2.3.2 Effect of Homogenisation Speed

Both primary and secondary emulsions were homogenised for a constant time of 2 min. The primary emulsion for each batch was made with 100μl of a 20mg/ml solution of BSA and a 5% PLG solution in DCM. The homogenisation speed was varied for both the primary and secondary emulsions as detailed in Table 3.1 below.

Batch	Primary Emulsion Speed (rpm)	Secondary Emulsion Speed (rpm)
1	6,000	14,000
2	6,000	19,000
3	11,000	19,000
4	16,000	11,000
5	16,000	14,000
6	16,000	16,000
7	16,000	19,000
8	19,000	14,000
9	19,000	19,000

Table 3.1 *Variations in primary and secondary emulsion homogenisation speed*

3.2.3.3 Effect of Length of Homogenisation

The length of time of the secondary emulsion homogenisation was investigated for effect on protein encapsulation into the microparticles. The primary emulsion homogenisation time was not increased further due to the localised heating and high shear produced in the small volume of the primary emulsion which is likely to lead to denaturisation of the protein. The primary emulsion for each batch was made with 100μl of a 20mg/ml solution of BSA and a 5% PLG solution in DCM. The primary emulsion homogenisation was kept constant at 16,000rpm for 2 min, and the secondary emulsion homogenisation speed and time were varied as detailed in Table 3.2 below.

Primary Emulsion		Secondary Emulsion	
Speed (rpm)	Time (min)	Speed (rpm)	Time (min)
16,000	2	11,000	2 or 3
16,000	2	14,000	2 or 3
16,000	2	16,000	2 or 3
16,000	2	19,000	2 or 3

Table 3.2 *Variation in secondary emulsion homogenisation speed and time*

3.2.3.4 Effect of Molecular Weight of PLG Polymer Used

Different molecular weight PLG polymers have differences in polymer chain length and hydrophobicity, and protein encapsulation into particles made from a higher MW PLG polymer was investigated. Both polymers were 50:50 lactide/glycolide PLG copolymers. The low MW PLG polymer M_r 8,400-8,600 (Callacombe *et al.*, 1997; Park *et al.*, 1998) had an estimated half-life of 1 week. The high MW PLG polymer M_r 40,000-70,000 had an estimated half-life of 1 month. Each primary emulsion was made with 2mg of BSA in 100μl. Standard homogenisations of 16,000rpm for 2 min in the primary emulsion, and 19,000rpm for 2 min in the secondary emulsion were used. 2 batches of particles were made with each polymer.

3.2.3.5 Effect of Protein Loading

BSA loading was varied by altering the concentration and volume of protein solution added to the primary emulsion. Standard homogenisations of 16,000rpm for 2 min in the primary emulsion, and 19,000rpm for 2 min in the secondary emulsion were used. The comparisons of protein loading were as follows:

a) 100μl of a 20mg/ml BSA solution (~2mg)
b) 200μl of a 20mg/ml BSA solution (~4mg)
c) 100μl of a 40mg/ml BSA solution (~4mg)

3.2.3.6 Comparison of Different Proteins

It has been documented that encapsulation efficiency varies for different proteins (Blanco & Alonso, 1998; Takahata *et al.*, 1998), and the encapsulation of BSA was thus compared with OVA. All primary emulsions were made at 16,000rpm for 2 min, and all secondary emulsions were made at 19,000rpm for 2 min. The lower molecular weight PLG polymer was used. The amounts of protein used (BSA versus OVA) were as detailed for BSA in section 3.2.4.5, with an additional 200μl of a 40mg/ml OVA solution (~8mg).

3.2.3.7 Quantification of the Ratio of Surface-Associated: Encapsulated Protein

Surface-adsorbed protein is known to be displaced by sodium dodecyl sulfate (SDS) which binds to hydrophobic regions of protein molecules causing them to unfold and freeing them from association with other molecules. 10mg microparticles were dispersed in 0.5ml of 0.01M PBS, pH 7.4 containing 2% w/v SDS. The resulting suspension was rotated at room temperature for 1 h, and then centrifuged at 14,000rpm for 5 min. The supernatant was removed and analysed for protein content to give a measurement of the amount of protein associated with the surface of the microparticles. The microparticle pellet was then resuspended in 0.5ml of 0.5M NaOH containing 5% w/v SDS and this suspension was rotated gently for 3-4 h at room temperature until particles had completely dissolved. The increase in pH due to the NaOH considerably enhances lactic/glycolic acid polymer degradation. This suspension was then assayed for protein content to give a measurement of the amount of protein encapsulated within the particles.

3.2.3.8 Quantification of Total Protein Content

5mg microparticles were dispersed in 0.5ml of 0.2M NaOH containing 5% w/v SDS and gently mixed for 3-4 hours until the particles had completely dissolved. This solution was then assayed for total protein content. Data is presented as the encapsulation efficiency, which indicates the percentage of protein associated with the particles compared to the original amount of protein used to make the primary emulsion.

3.2.3.9 Pierce BCA Assay

Protein assays were performed with the bicinchoninic acid (BCA) enhanced protein assay (Pierce Chemical Company, Rockford, Illinois, USA) using albumin fraction V (Pierce Chemical Company, Rockford, Illinois, USA) as a standard as described by the manufacturer's instructions. This assay is based on the reduction of

copper (II) to copper (I) by protein under alkaline conditions. A colorimetric reaction is produced with the BCA reagent that can be detected by reading the absorbance at 562nm.

3.2.4 Confirmation of Presence of Protein in Particles

A batch of microparticles was made using BSA conjugated to fluorescein isothiocyanate (FITC-BSA) following the methods detailed in section 3.2.3.1. A higher protein loading of 5% was used (5mg BSA-FITC in 100mg PLG). The primary emulsion was homogenised at 16,000rpm for 2 min, and the secondary emulsion was homogenised at 19,000rpm for 2 min. Following lyophilisation approximately 10mg of microparticles was rehydrated in approximately 50µl water, and a 5-10µl of this suspension were allowed to dry on a microscope slide. The slide was then mounted in Citifluor mountant (Citifluor Ltd., London, UK) and examined using an Olympus BX50 microscope (Olympus, London, UK) with a Natural Blue filter at 470-490nm, and a Leica TCS NT/SO series confocal system (Leica, Germany). An argon-krypton ion gas laser with excitation lines at 491nm was used to induce fluorescence.

3.2.5 Rate of Protein Release

A total of eight batches of microparticles were made to determine the rate of protein release, four with the low MW PLG polymer and four with the high MW PLG polymer. For each batch, the primary emulsion was made by homogenising 2mg of BSA in 100µl of water with 100mg PLG in 2ml DCM at 16,000rpm for 2 min. The secondary emulsion was made by homogenising the primary emulsion with 20ml of 5% PVA at 19,000rpm for 2 min. After the particles had been washed and lyophilised, 50mg of each batch were suspended in 250µl of PBS and mixed gently at 37°C for 15 min. The particles were then centrifuged at 8,000rpm for 3 min, and each supernatant was collected for protein analysis. The remaining particles were resuspended in 250µl of PBS and mixed gently at 37°C for a further 45 min before the particles were centrifuged and the supernatant collected as before. Supernatant samples were collected at the following time points after the particles had been suspended: 15 min, 1 hr, 4 hr, 1 day, 2 days, 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, and 10 weeks. Two 100µl aliquots from each supernatant sample were

analysed for protein content by the Pierce BCA assay as detailed in Section 3.2.3.9 and an average of the two samples was taken.

3.2.6 Particles Made with Secreted Proteins from *Listeria monocytogenes*

Further to the encapsulation of model proteins, BSA and OVA, the encapsulation of a mixture of biologically active and functional proteins was investigated. The secreted proteins from *L. monocytogenes* provided a useful model for this purpose.

3.2.6.1 *Listeria monocytogenes*

Supernatant fluid was obtained from a dialysis sac culture containing approximately 2×10^{10} colony forming units per ml of *Listeria monocytogenes* serovar 4b (strain L1059) originally isolated from a sheep with listeric encephalitis at the Moredun Research Institute, Edinburgh, UK (Low *et al.*, 1992). This supernatant contained a mixture of proteins secreted by *L.monocytogenes*, including listeriolysin O (LLO), a secreted soluble protein against which there is a specific polyclonal rabbit antibody (kindly gifted by W. Donachie, Moredun Research Institute, Edinburgh, UK). LLO is a thiol-activated cytolysin which lyses red blood cells at a pH of approximately 6.0 (Geoffroy *et al.*, 1987) The functional activity of this protein provides a useful means of assessing the functionality of encapsulated proteins, using a simple haemolysin assay.

3.2.6.2 Particle Preparation

The secreted proteins from *L. monocytogenes* were concentrated to approximately 10mg/ml using Centriprep® YM-10 concentrators (Amicon, Stonehouse, UK) at 3,000 x *g*. Particles were prepared using 200μl of this protein solution with both high and low MW PLG polymers using the methods detailed in Section 3.2.3.1. The primary emulsion was prepared at 16,000rpm for 2 min, and the secondary emulsion at 19,000rpm for 2 min. The protein content of these particles was measured as detailed in Sections 3.2.3.7 – 3.2.3.9.

3.2.6.3 Demonstration of LLO Within Particles

A small quantity of each batch of microparticles made in Section 3.2.6.2 was rehydrated in distilled water, and 5μl or 10μl amounts of the suspensions were dotted onto electrostatically charged microscope slides (SuperFrost® Plus, Menzel-Glaser®, Germany). The particles were left for approximately 1 h to dry onto the slides. The slides were then washed thoroughly with PBS containing 1% w/v BSA and 0.05% v/v Tween®20 (Sigma, Dorset, UK), and incubated in a 1:1,000 dilution of the polyclonal rabbit anti-LLO antibody for 1 h at room temperature (RT). The slides were washed again in PBS and incubated in a 1:400 dilution of a goat anti-rabbit antibody conjugated to biotin (Dako, Ely, UK) for 1 h at RT. The slides were washed again and incubated in a 1:100 dilution of streptavidin conjugated to FITC (Dako, Ely, UK) for 1 h at RT. Slides were then washed in PBS and mounted in Citifluor® (CitiFluor Ltd., London, UK). Slides were examined using an Olympus BX50 microscope (Olympus, London, UK) with a ×40 objective and a natural blue (NB) filter at a wavelength of 470-490nm.

3.2.6.4 Analysis of Proteins Within the Particles

Samples of surface–associated and encapsulated proteins were obtained from high and low MW PLG microparticles containing proteins from *L. monocytogenes* as detailed in Section 3.2.3.7, and then concentrated to a volume of approximately 50μl using Centriprep® YM-10 concentrators (Amicon, Stonehouse, UK) at 3,000 x *g*. For comparison, samples of the original material were also used, diluted 1:10 in PBS. Proteins were analysed initiall by SDS-PAGE and Western blots, and then by the haemolysis of red blood cells.

3.2.6.4.1 Sodium Dodecylsulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated by gel electrophoresis as outlined by Laemmli (1970) using the Mini-Protean II gel system (Bio-Rad, Hemel Hempstead, UK). 15μl of each sample were mixed with an equal volume of reducing sample buffer (62.5mM Tris-HCl pH 6.8, 2% w/v SDS, 10% w/v glycerol, 0.002% w/v bromophenol blue, 5% v/v β-mercaptoethanol) prior to loading into the wells of discontinuous SDS-

PAGE minigels. 10% acrylamide gels that had been made in separating gel buffer (0.375M Tris-HCl pH8.8, 1% w/v SDS, 0.1% v/v TEMED, 0.1% w/v ammonium persulfate (APS)) were used for protein separation. The separating gel was overlaid with a 4% acrylamide stacking gel made in stacking gel buffer (0.15M TrisHCl pH 6.8, 0.5% w/v SDS, 0.2% v/v TEMED, 0.2% w/v APS). Proteins were separated at 200V for 45-60mins in tank buffer (25mM Tris, 200mM glycine, 5mM SDS pH8.3) until the dye front had reached the bottom of the gel. SeeBlue™ Plus2 Pre-Stained Standards (Novex, Invitrogen, Paisley, UK) molecular weight markers were included on all gels, consisting of phosphorylase B (M_r 148,000), BSA (M_r 98,000), glutamic dehydrogenase (M_r 64,000), alcohol dehydrogenase (M_r 50,000), carbonic anhydrase (M_r 36,000), myoglobin red (M_r 22,000) and lysozyme (M_r 16,000).

3.2.6.4.2 Protein Detection on SDS-PAGE Gels

Separated proteins were visualised by silver staining (Morrissey, 1981). Firstly the proteins in the gels were fixed in 50% (v/v) methanol, 10% (v/v) acetic acid in distilled water for 20 min, then twice in 5% (v/v) methanol, 7% (v/v) acetic acid in distilled water for 10 min. After two 5 min washes in distilled water, proteins were reduced in 5μg/ml dithiothreitol (DTT) in distilled water for 15 min, washed again, and stained in 0.1% silver nitrate in distilled water for 20 min. Gels were then washed briefly and finally developed for stained protein by immersion in 3% sodium carbonate containing 0.05% formaldehyde. Development was terminated by the addition of citric acid to 1% w/v.

3.2.6.4.3 Western Blot Analysis

Immuno-blot analysis was performed on proteins separated by SDS-PAGE and electrotransferred to Protran BA 83 nitrocellulose membranes (Schleicher and Schull, Anderman, Kingston upon Thames, UK) by semi-dry Western blot. The gel and membrane were sandwiched between several sheets of 3mm filter paper soaked in transfer buffer (24mM Tris, 192mM glycine, 20% v/v methanol, pH8.3) and placed in the Western blot apparatus. Transfer took place at 70mA/gel (2mA/cm^2 of gel) for 1 h. The membranes were then blocked in PBS containing 5% non-fat milk powder (Marvel, Chivers, Dublin, Ireland) for 30 min at RT and washed thoroughly

in wash buffer (PBS containing 0.5M NaCl and 0.5% v/v Tween®80). The membranes were then probed with a polyclonal rabbit anti-LLO antibody diluted to 1:500 in blot wash buffer for 1h at RT. Negative controls were incubated in wash buffer alone. After extensive washing of the blot, bound antibody was visualised by a further 1 h incubation with a 1:2,000 dilution of goat anti-rabbit antibody conjugated to biotin (Dako, Ely, UK) in wash buffer, followed by further washing and a 1 h incubation in a 1:5,000 dilution of streptavidin conjugated to horseradish peroxidase (HRP) (Dako, Ely, UK). After a final wash HRP was detected by treating blots with the enhanced chemiluminescence (ECL) reagent (Amersham International, Little Chalfont, UK) according to the manufacturer's instructions, and exposure to Hyperfilm ECL (Amersham International, Little Chalfont, UK) for approximately 5 sec before development.

3.2.6.5 Functional Activity of Encapsulated LLO Protein

The surface-associated and encapsulated proteins from 15mg samples of both high and low MW particles were obtained using the methods detailed in section 3.2.3.7. Following particle dissolution in NaOH the resulting suspensions were dialysed in PBS, pH 6.0. All samples were concentrated to a volume of approximately 250μl using Centriprep® YM-10 concentrators (Amicon, Stonehouse, UK) at 3,000 x *g*. Haemolytic activity in *L. monocytogenes* broth culture supernatant fluid (diluted 1:10) and the samples extracted from the particles were determined in a haemolysis assay according to the method of Kreft *et al.* (1989). Briefly 200μl of test samples were double diluted in 100μl PBS, pH 6.0, in U-bottomed 96-well microtitre plates and 10μl of 0.1M dithioerythritol (DTE, Sigma, Dorset, UK) was added to all wells. Plates were covered and incubated on an orbital shaker for 10 min at 37°C before 10μl of a 10% suspension of washed sheep erythrocytes in PBS, pH 6.0, was added to all wells and mixed thoroughly. Plates were covered and incubated for 30 min at 37°C then cells were allowed to settle overnight at 4°C. Positive control wells consisted of 10μl 10% washed sheep erythrocytes in 100μl distilled water with 10μl DTE. Negative control wells contained 10μl of washed sheep erythrocytes in 100μl PBS, pH 6.0, with 10μl 0.1M DTE.

3.3 RESULTS

3.3.1 Size Range of Microspheres Produced

The Coulter LS 230 particle size analyser revealed that the size distribution of every batch of microparticles prepared in Section 3.2.2.1 was within the desired range of 0.1μm – 3μm regardless of the length of homogenisation or the homogenisation speed used. Not only were most particles within the desired size range, but the majority of particles were approximately 1.0μm in diameter or less.

The average size of particles ranged between 0.5μm and 1.5μm, and a typical bimodal distribution of sizes was produced. Figure 3.4 shows a typical particle size distribution plot for microspheres prepared. A bimodal distribution is observed with the main peak at approximately 0.5μm, and a secondary peak at 1.5μm diameter. In this particular batch the average size of microparticle was approximately 0.7μm diameter.

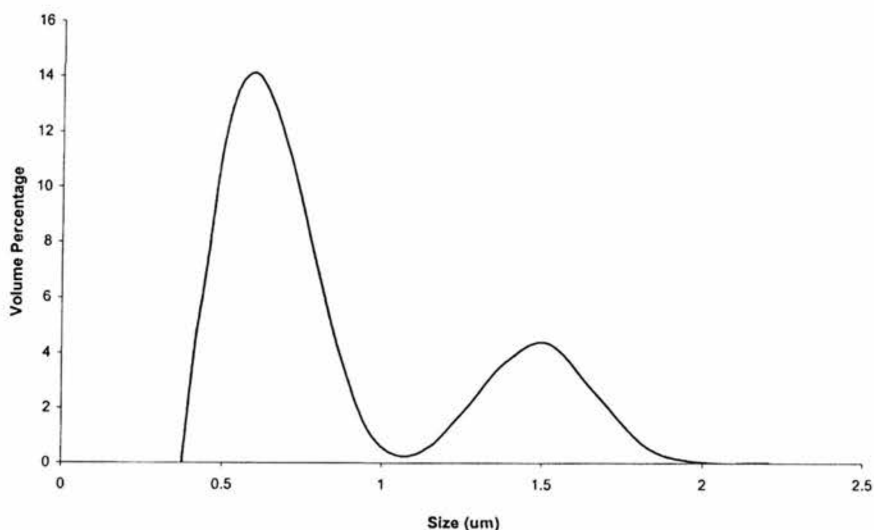


Figure 3.4 *Size distribution plot of microspheres produced by the Coulter LS 230 particle size analyser*

Particles prepared with higher secondary emulsion homogenisation speeds tended on average to be slightly smaller, as did those that were homogenised for 3 min rather than 2 min. However, even the particles prepared using the slowest homogenisation speed for the shortest time were within the desired size range, with a considerable proportion being less than 1μm in diameter. Some background variation in size range existed between different batches of particles prepared using the same emulsion times and speeds. Variation in primary emulsion homogenisation speed did not appear to have any significant effect on the size of particles produced, above the background levels previously mentioned.

SEM confirmed the size distribution of the microspheres as measured by the particle size analyser. Figure 3.5 shows a typical micrograph of particles made using the standard homogenisation of 16,000rpm for 2 min for the primary emulsion and 19,000rpm for 2 min for the secondary emulsion. Measurements were performed on the particles under the electron microscope and are demonstrated on the micrograph, and particles between 0.2μm and 1μm diameter can be observed. All microspheres were spherical, but it was difficult to determine any surface details due to the poor resolution achieved at high magnification. The SEM shows the typical spherical form and smooth surfaces of the protein-loaded PLG microspheres.

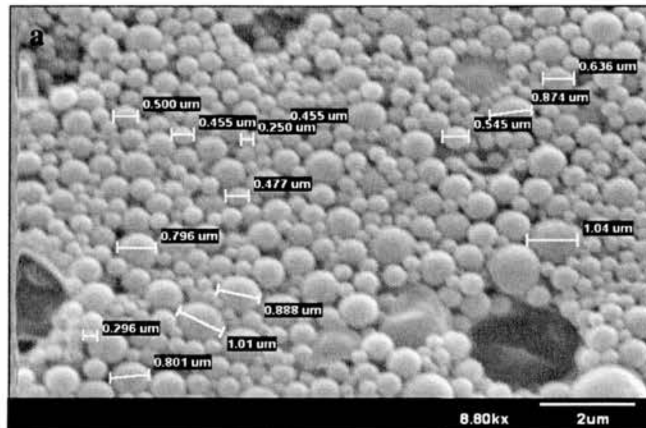


Figure 3.5 *Scanning electron micrograph demonstrating the morphology and size range of PLG particles*

3.3.2.1 Location of Protein Within the Particles

Particles incorporating FITC-BSA were clearly visible as small fluorescent green spheres when examined under a NB filter at 470-490nm demonstrated in Figure 3.6. This demonstrated that the FITC-BSA protein had been successfully incorporated into the microparticles. The confocal images in Figure 3.7 also confirmed the presence of protein as green fluorescence within the particles. The exact location of protein within the microspheres could not be determined due to their small size, but under this higher magnification the protein appeared to be evenly distributed both inside the particles and on their surface.

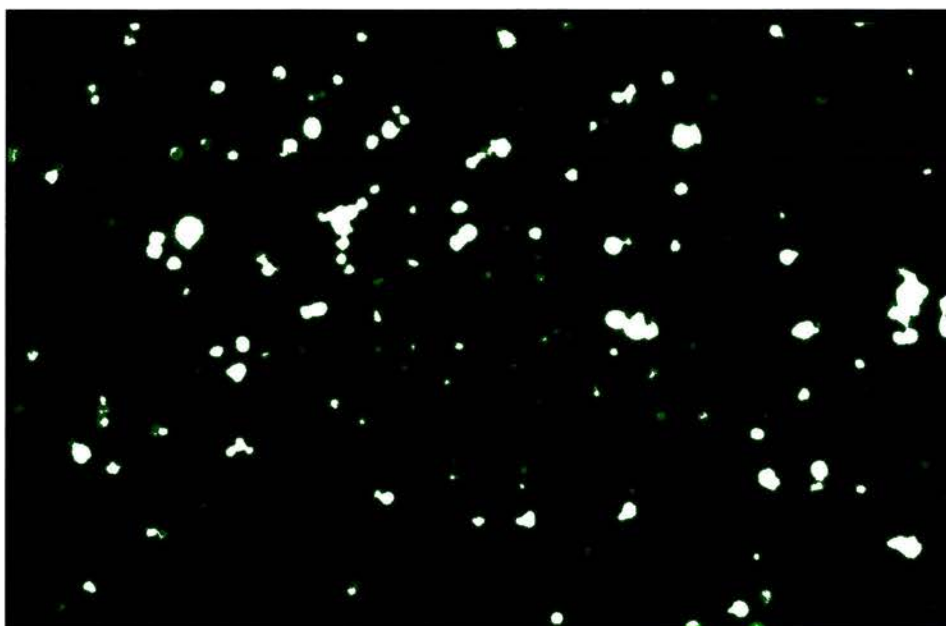


Figure 3.6 *PLG microspheres loaded with 5% w/w BSA-FITC x500 magnification*

Figure 3.7 *Confocal images of PLG microspheres prepared with 5% w/w BSA-FITC ×350 and ×2700 magnification*

their small size, but under this higher magnification the protein appeared to be evenly distributed both inside the particles and on their surface.

3.3.2.2 Effect of Homogenisation Speed

The ratio of surface-bound to encapsulated protein and the total protein encapsulation efficiency for each preparation of microparticles are detailed in Table 3.3 and demonstrated in Figure 3.8.

Primary Emulsion (rpm)	Secondary Emulsion (rpm)	Surface: Encapsulated Protein (%)	Total Protein (μg/mg particles)	Encapsulation Efficiency (%)
6,000	14,000	49:51	15.49	77.45
6,000	19,000	37:63	11.33	56.65
11,000	19,000	65:35	12.37	61.85
16,000	11,000	39:61	14.82	74.10
16,000	14,000	59:41	13.81	69.05
16,000	16,000	67:33	14.54	72.70
16,000	19,000	40:60	17.39	86.95
19,000	14,000	60:40	16.76	83.80
19,000	19,000	66:34	19.25	96.25

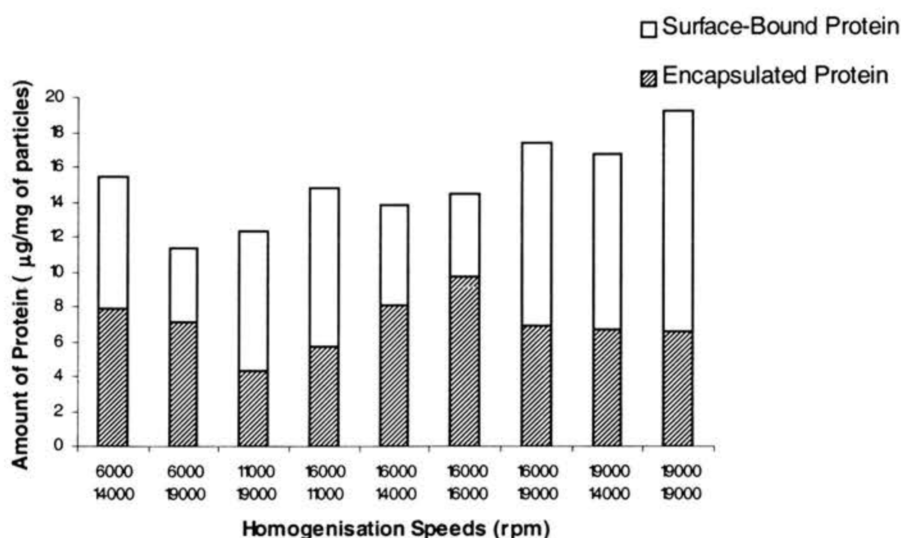


Figure 3.8 *Comparison of protein encapsulation of microparticles prepared using different homogenisation speeds for both primary and secondary emulsions*

The variation of homogenisation speed for the preparation of both primary and secondary emulsions had no obvious effect on the encapsulation efficiency, i.e. the total amount of protein that became associated with the microparticles. The results were variable but in general, the trend was that the higher the homogenisation speed for the preparation of either primary or secondary emulsion, the greater the encapsulation efficiency of the BSA protein. Encapsulation efficiencies ranged between 56 - 96%, and the three batches containing the greatest amount of protein

Table 3.3 *Effect of homogenisation speed on encapsulation efficiency and ratio of surface-bound to encapsulated protein*

are highlighted in red in Table 3.3.

Variation in homogenisation speed appeared to have a more marked effect on the outcome of the ratio of protein loosely associated with the particle surface compared to the protein encapsulated within the particles. In general it seemed that the higher the homogenisation speed for the preparation of either the primary or secondary emulsion, the higher the proportion of protein that remained surface-bound rather than becoming encapsulated. This effect appeared to be more marked on the increase of the secondary emulsion homogenisation speed. The three batches with the maximum proportion of encapsulated protein are highlighted in blue in Table 3.3. It would appear that homogenisation at 16,000rpm for 2 min for the primary emulsion, followed by 19,000rpm for 2 min for the secondary emulsion resulted in both maximum encapsulation of the protein into the particles rather than loose association with the particle surface, and high total protein encapsulation efficiency.

3.3.2.3 Effect of Homogenisation Time

The effect of the duration of homogenisation on the ratio of surface-bound to encapsulated protein and the total protein encapsulation efficiency for each preparation of microparticles are detailed in Table 3.4 and demonstrated in Figure 3.9. Increasing the length of time for the preparation of the secondary emulsion did not appear to have a marked effect on the encapsulation efficiency of BSA into the

microparticles. There were variations in the total amount of protein encapsulated into the particles, but the encapsulation efficiency did not necessarily increase with homogenisation duration. However, increasing the length of the secondary emulsion tended to increase the ratio of protein that remained bound to the surface of the microparticles.

Secondary Emulsion		Surface: Encapsulated Protein (%)	Total Protein (μg/mg)	Encapsulation Efficiency (%)
Speed (rpm)	Time (min)			
11,000	2	39:61	14.82	74.10
11,000	3	62:38	15.21	76.05
14,000	2	59:41	13.81	69.05
14,000	3	73:27	19.73	98.65
16,000	2	67:33	14.54	72.70
16,000	3	68:32	12.87	64.35
19,000	2	40:60	17.39	86.95
19,000	3	61:39	12.13	60.65

Table 3.4 *Effect of homogenisation speed on encapsulation efficiency and ratio of surface-bound to encapsulated protein*

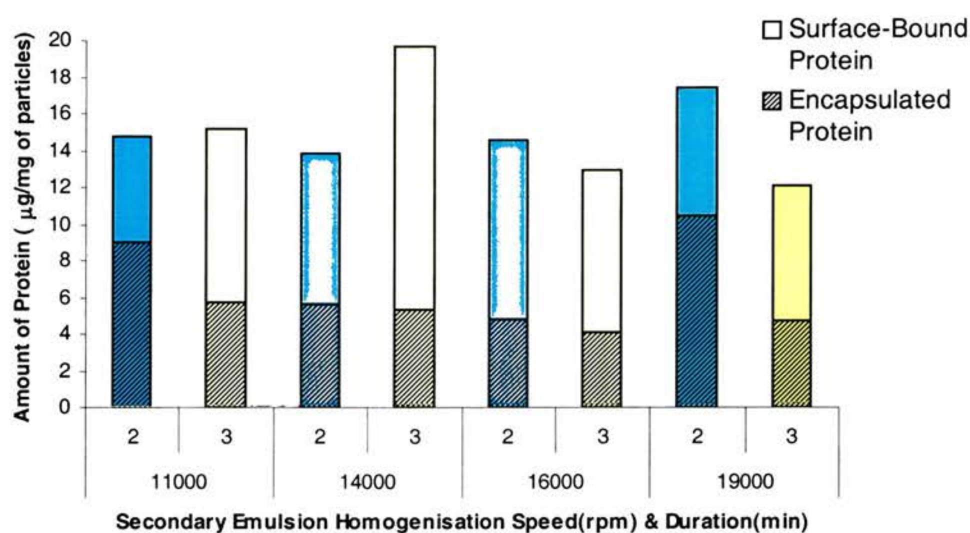


Figure 3.9 *Comparison of protein encapsulation of microparticles prepared using different lengths of time of homogenisation for the preparation of the secondary emulsion*

When the results from the variation of both homogenisation speed and time were taken as a whole, the formulation in which the primary and secondary

emulsions were prepared by homogenisation at 16,000rpm and 19,000rpm respectively, for 2 min appeared to be the most efficient at protein encapsulation, and were used for all further batches of particles. Not only was the total protein encapsulation efficiency high for this formulation, but the majority of the protein was encapsulated into the particles.

3.3.2.4 Effect of PLG Polymer MW

The ratio of surface-bound to encapsulated protein and the total protein encapsulation efficiency for microparticles prepared with different MW PLG polymers are detailed in Table 3.5 below and demonstrated in Figure 3.10 overleaf. The encapsulation efficiency of BSA was slightly higher in particles made from the lower MW PLG polymer (>80%) than the higher MW PLG polymer (approximately 70%). However when the higher MW PLG polymer was used, proportionally less protein remained only loosely associated with the surface and more protein was actually encapsulated into the particles.

Polymer (MW)	Surface: Encapsulated Protein (%)	Total Protein (μg/mg)	Encapsulation Efficiency (%)
Low 1	40:60	17.39	86.95
Low 2	52:48	16.50	82.50
High 1	30:70	13.73	68.65
High 2	40:60	14.25	71.25

Table 3.5 *Comparison of encapsulation efficiency and the ratio of surface-associated to encapsulated protein in particles made from low and high MW PLG polymers*

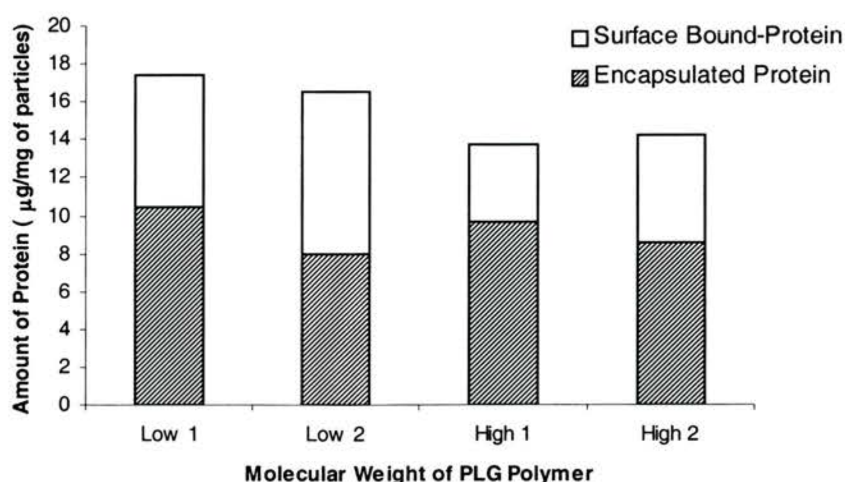


Figure 3.10 *Comparison of protein encapsulation of microparticles prepared using different molecular weight PLG polymers (2 batches for each polymer)*

3.3.2.5 Effect of Protein Concentration and Volume

The effect of the amount of protein included in the primary emulsion on the ratio of surface-bound to encapsulated protein and the total protein encapsulation efficiency of microparticles are detailed in Table 3.6 below and demonstrated in Figure 3.11 overleaf. Protein loading of particles depended on the volume and concentration of protein in the primary emulsion. However, above a certain level of protein the encapsulation efficiency declined, such that proportionally less protein became associated with the particles. The encapsulation efficiency was highest when the standard amount of 2mg of BSA in 100μl of solution was used. The proportion of surface-bound to encapsulated protein remained approximately the same when the protein amount was varied. When examined under SEM, more split and deformed microspheres were observed following higher protein loading, perhaps due to the higher volume of aqueous phase in the primary emulsion.

Amount of Protein	Surface: Encapsulated Protein (%)	Total Protein (μg/mg)	Encapsulation Efficiency (%)
2mg in 100μl	48:52	14.97	74.85
4mg in 200μl	55:45	26.46	66.15
4mg in 100μl	52:48	23.21	58.03

Table 3.6 *Effect of protein loading on encapsulation efficiency and the ratio of surface-associated to encapsulated protein*

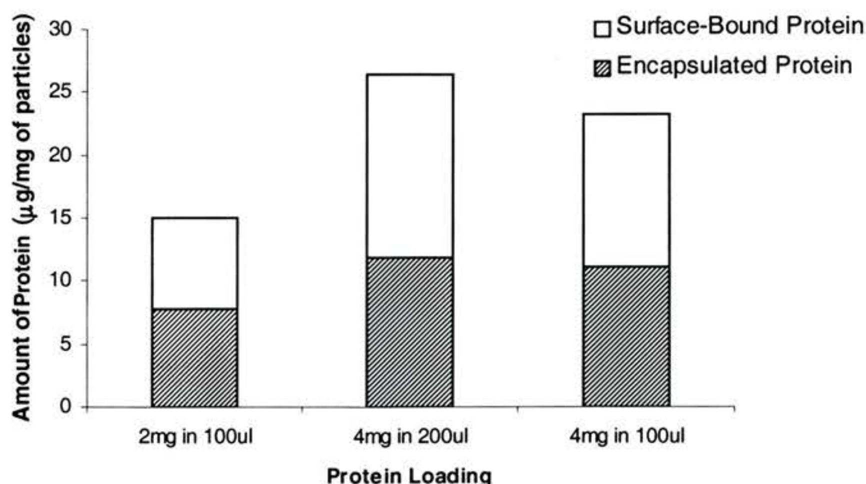


Figure 3.11 *Effect of protein loading on encapsulation efficiency and the ratio of surface-associated to encapsulated protein*

3.3.2.6 Encapsulation Efficiency of Different Proteins

The ratio of surface-bound to encapsulated protein and the total protein encapsulation efficiency for microparticles prepared with BSA and OVA are detailed in Table 3.7 and demonstrated in Figure 3.12 overleaf. The encapsulation efficiency was much lower for OVA than for BSA for all the different amounts, volumes and concentrations of protein used to make the primary emulsion. Less than half the amount of OVA became associated with the particles than BSA. In addition, proportionally more of the OVA became encapsulated than the BSA (60-78%). With BSA, approximately half the protein became encapsulated, whilst the other half remained surface-bound. The encapsulation efficiency of secreted proteins from *L. monocytogenes* was approximately 54%, a level in between that of BSA and OVA. Like OVA, most of the listeria protein became encapsulated, leaving only approximately 20% surface-bound. These results are based on a mixture of proteins, because specific *L. monocytogenes* proteins could not be quantified.

Protein	Amount	Surface: Encapsulated Protein (%)	Total Protein Content (μg/mg)	Encapsulation Efficiency (%)
BSA	2mg in 100μl	48:52	14.97	74.85
OVA	2mg in 100μl	35:65	7.23	36.15
BSA	4mg in 200μl	55:45	26.46	66.15
OVA	4mg in 200μl	22:78	10.85	27.13
BSA	4mg in 100μl	52:48	23.21	58.03
OVA	4mg in 100μl	36:64	9.59	23.98
OVA	8mg in 200μl	40:60	23.67	29.59

Table 3.7 *Comparison of the encapsulation efficiency and the ratio of surface-associated to encapsulated protein for BSA and OVA*

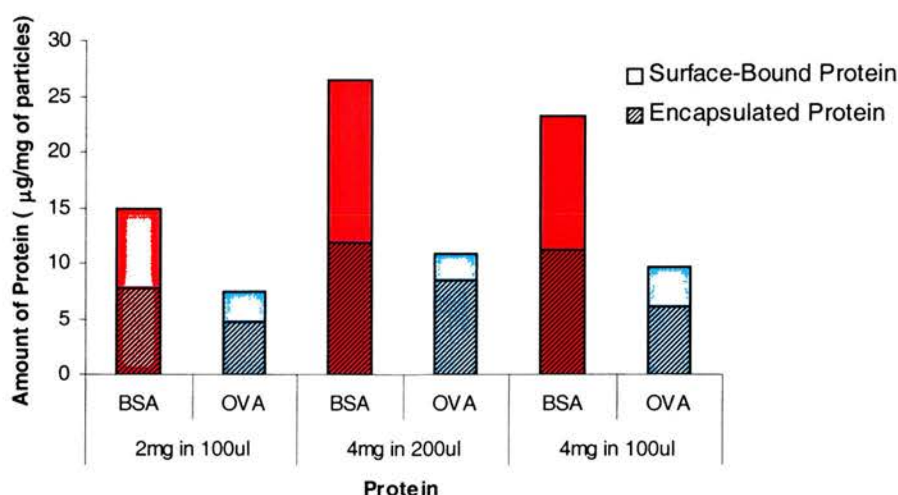


Figure 3.12 *Comparison of protein encapsulation of microparticles prepared with ovalbumin and BSA at different protein volume and/or concentration*

3.3.3 Release of Protein from PLG Particles

The encapsulation efficiency of BSA and the ratio of surface-bound to encapsulated protein for the 4 batches of microparticles prepared with the high MW PLG polymer and the 4 batches prepared with the low MW PLG polymer are shown in Table 3.8 overleaf. The method used resulted in encapsulation efficiencies for BSA ranging from 65% to 85%. On average particles prepared with the higher MW PLG polymer had better encapsulation efficiency of BSA (approximately 80%) than those prepared with the lower MW PLG polymer (approximately 70%). Particles prepared with the higher MW polymer had less protein bound to their surface and more protein encapsulated within the particles, on average the ratio was 30%:70%, compared to 50%:50% for the lower MW PLG polymer.

Batch	Surface Protein	Encapsulated Protein	Ratio S:E	Total Protein Content	Encapsulation Efficiency
	μg/mg particles		%	μg/mg	%
H1	5.99	11.07	**35:65**	17.06	**85**
H2	2.74	11.05	**20:80**	13.79	**69**
H3	5.40	11.49	**32:68**	16.89	**85**
H4	7.42	9.60	**44:56**	17.02	**85**
L1	7.99	5.10	**61:39**	13.09	**65**
L2	7.98	8.71	**48:52**	16.69	**83**
L3	5.57	7.91	**41:59**	13.48	**67**
L4	7.19	6.34	**53:47**	13.53	**68**

Table 3.8 *Details of the different batches of particles prepared with BSA for experiments examining the rate of protein release*

The average rate of release of BSA protein from microparticles made from the two different molecular weight polymers are shown in Figure 3.13a, and Figure 3.13b demonstrates the amount of protein released at each time point. For both PLG polymers, there was an initial burst phase of protein release from the particles within 15 min to 1 h of being suspended in PBS, after which protein release continued at a relatively slow rate for several weeks. The burst release was likely to be due to surface-bound protein. More protein was released from the lower MW PLG particles within this first hour, and by the end of the 10 week period these particles had released more protein than the higher MW PLG microparticles. At the end of 10 weeks no further protein release appeared to occur from the lower MW particles.

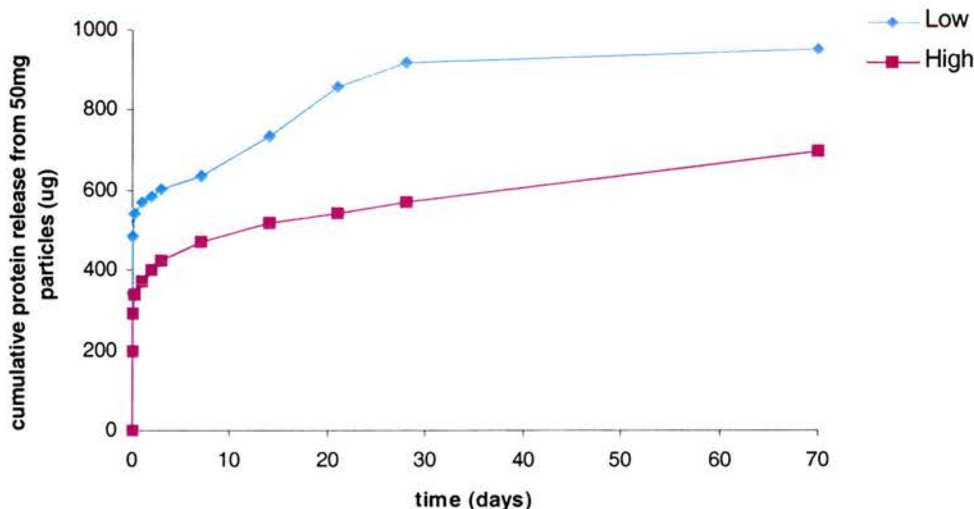


Figure 3.13a *Cumulative protein release from high and low MW PLG particles over 10 weeks at 37°C*

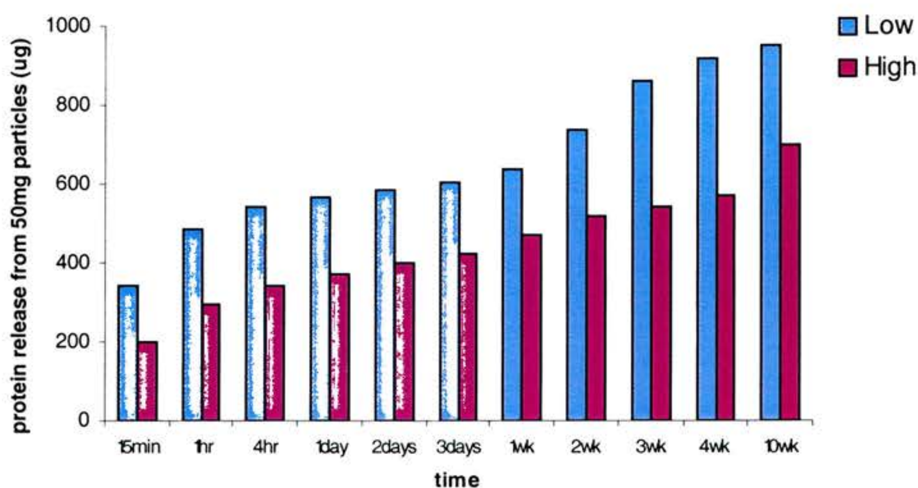


Figure 3.13b *Cumulative protein release from high and low MW PLG particles over 10 weeks at 37°C*

Table 3.9 demonstrates that when the higher proportion of surface-bound protein in the lower MW PLG particles was accounted for (protein released within 1 h), the amount of encapsulated protein released from particles made from the 2 different MW polymers over the 10 week period of the experiment was very similar.

PLG Polymer	μg protein released from 50mg particles		
	Surface (1h)	**Total (10 wk)**	**Encapsulated (10wk – 1h)**
Low	0.48	0.95	**0.47**
High	0.29	0.7	**0.41**

Table 3.9 *Comparison of encapsulated protein release from high and low molecular weight PLG particles*

3.3.4 Particles Prepared With Proteins Secreted from *L. monocytogenes*

Particles were successfully prepared using the secreted supernatant proteins from *L. monocytogenes*. The encapsulation efficiency was approximately 50% of the total protein loaded. Of the protein associated with the particles, on average 23% was surface-bound and 77% was encapsulated within the particles. The encapsulation efficiencies are shown in Table 3.10 below. Particles were smooth and spherical when examined by SEM and many particles were less than 1μm in diameter, as demonstrated in Figure 3.14.

Batch	Surface Protein	Encapsulated Protein	Ratio S:E	Total 1	Total 2	Average Total	Encapsulation Efficiency
	μg/mg particles		%	μg/mg particles			%
LmH1	1.02	5.26	**16:84**	6.28	6.06	6.17	**44**
LmH2	1.71	6.08	**22:78**	7.79	10.13	8.96	**64**
LmL1	2.48	4.99	**33:67**	7.47	5.29	6.38	**58**
LmL2	2.26	4.37	**34:66**	6.63	5.50	6.07	**46**

Table 3.10 *Protein encapsulation of particles incorporating secreted proteins from* L. monocytogenes

When the particles were incubated with a polyclonal antibody against LLO and observed with an FITC label under a 470-490nm NB filter, the majority of particles fluoresced strongly indicating the incorporation of LLO (Figure 3.15). No differences could be observed in the labelling for LLO between the particles made with the low and high MW PLG polymers.

Figure 3.16a shows proteins present in the original sample of secreted proteins from *L. monocytogenes* separated on a 10% gel, and Figure 3.16b shows a Western blot of these proteins probed with a monoclonal antibody against LLO. There are a number of protein bands visible on the gel, and the anti-LLO antibody reacted with two major bands on the blot at molecular weights of approximately

58kDa and 45kDa, which probably represented LLO and a breakdown product of LLO respectively. Both of these proteins were present on the surface and encapsulated within particles prepared with the lower MW PLG, whereas these proteins were encapsulated into but absent on the surface of particles prepared with the high MW PLG. The Western blot of the surface-bound and encapsulated proteins from both the high and low MW PLG particles, probed with the antibody against LLO is demonstrated in Figure 3.16c.

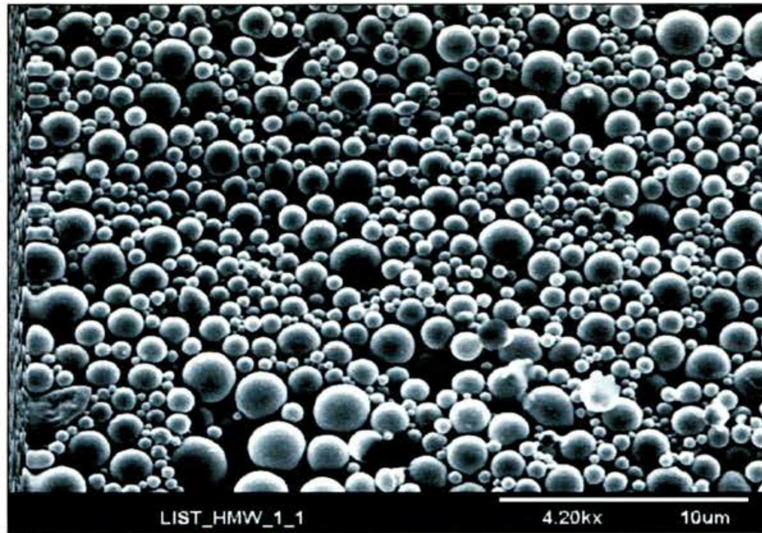


Figure 3.14 *High MW PLG microparticles incorporating proteins from* L. monocytogenes

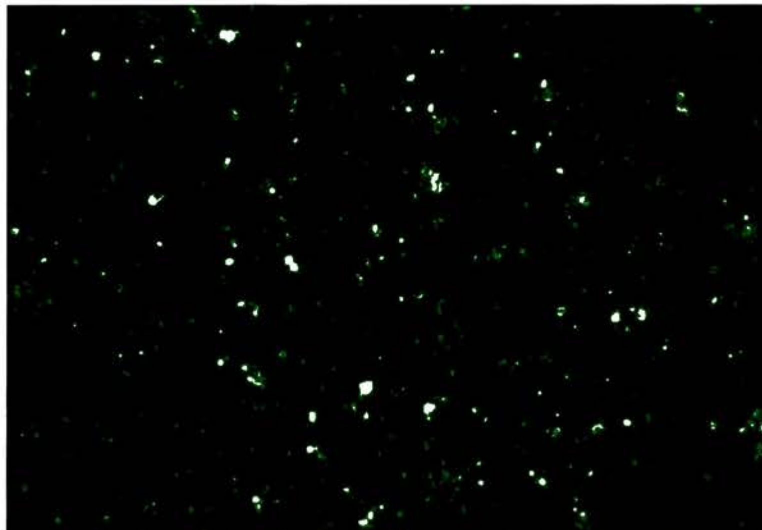


Figure 3.15 *Particles labelled with polyclonal anti-LLO antibody, ×272 magnification*

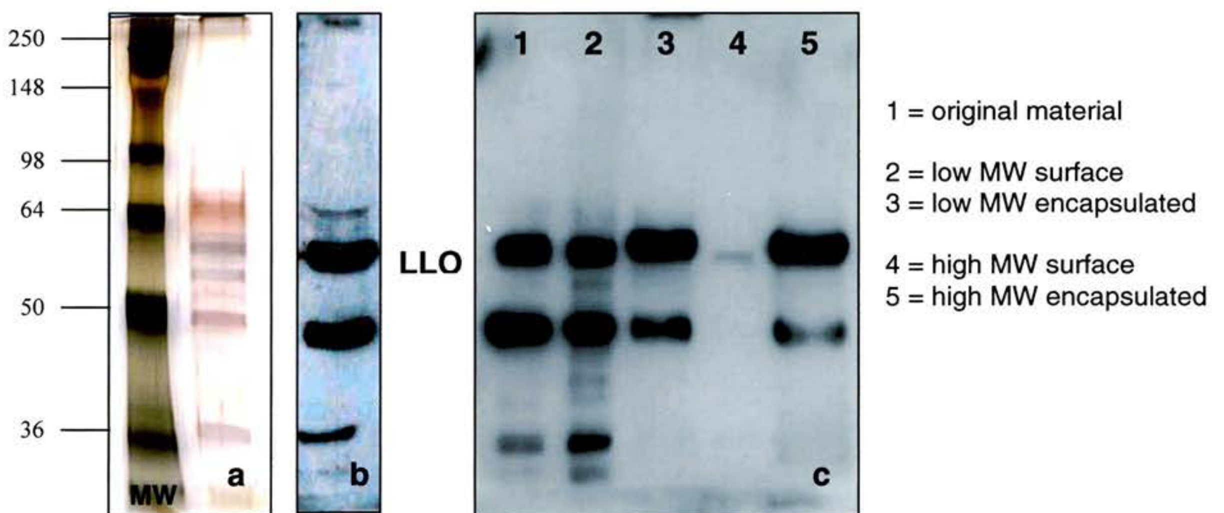


Figure 3.16 *Molecular weight standards and secretory proteins from a culture of L. monocytogenes a) silver stained, and b) Western blot probed with antibody against LLO. c) Western blot of surface and encapsulated proteins from high and low MW PLG particles probed with antibody against LLO*

3.3.5 Functional Activity of Encapsulated LLO Protein

The microtitre plate from the haemolysis assay is shown in Figure 3.17 overleaf. The seventh column of wells contained the original culture supernatant fluid, diluted 1:10 in PBS, pH 6.0, and double diluted down the plate. At all dilutions, the red blood cells in all of the wells were completely lysed, demonstrating that LLO was present and functionally active in sufficient quantities to lyse the quantity of cells present.

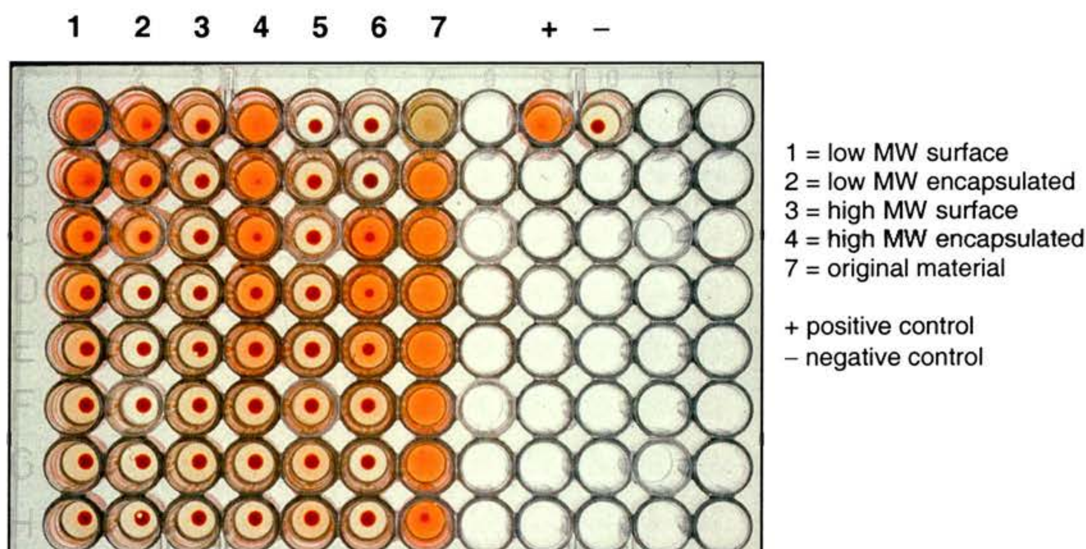


Figure 3.17 *Haemolysin assay demonstrating functional activity of surface-bound and encapsulated LLO in both low and high MW PLG microparticles*

Columns 1 and 2 show the proteins extracted from the low MW PLG microparticles, double diluted down the plate. Red blood cells have been lysed in the first three wells in column 1 and the first two wells in column 2, indicating that the LLO protein extracted from the low MW PLG microparticles remained functionally active. Columns 3 and 4 contain surface-bound and encapsulated proteins respectively from the high MW PLG microparticles. The lack of lysis in column 3 supports the evidence from the Western blots, indicating that LLO was absent on the surface of the high MW particles. Red blood cells were lysed in the sample of encapsulated protein from the high MW PLG microparticles, showing that LLO was present inside the particles and had retained its functional activity. The amounts of LLO detected were considerably lower than in the original material because a relatively small aliquot of particles (15mg) was used.

3.4 DISCUSSION

The results presented in this study demonstrate the generation of PLG particles in the nanoparticle size range with maximum protein encapsulation and long-term release properties for future mucosal immunisation studies. The range of emulsification rates and times used in this study consistently resulted in the production of microparticles within the optimal size range for M cell uptake (<2μm in diameter) (Brayden & Baird, 2001). The different speeds and duration of homogenisation were clearly appropriate for the development of sub-micron particles. After a certain point increasing the homogenisation time or speed had no further effect on particle size and the droplet size remained stable. The bimodal distribution of particle size is typically associated with the use of relatively high concentrations of PLG polymer and low concentrations of PVA in the double emulsion technique (Rafati *et al.*, 1997). Polymer droplets in the primary emulsion contained variable amounts of the aqueous protein phase, resulting in non-uniform droplet size reduction on solvent evaporation, non-uniform shrinkage of particles and a heterogeneous particle size distribution. Microparticles of different dimensions within a distinct defined size range may be beneficial as a mucosal delivery system and should ensure that a proportion are taken up by M cells, as there is still debate concerning the most appropriate size of particle to optimise M cell uptake.

The encapsulation efficiency for BSA was consistently relatively high for all particle formulations, with on average a 50:50 ratio of surface:encapsulated protein. This high proportion of surface-associated protein is associated with small microspheres that have a large relative surface area (Watts *et al.*, 1990; Rafati *et al.*, 1997). The concentrations of PVA and PLG used in these studies remained constant, and were chosen with the aim of optimising protein encapsulation within a small particle size range. A relatively high concentration of polymer was used, and is thought to restrict migration of the inner aqueous/protein phase in emulsion droplets to the external water phase to improve protein entrapment efficiency (Ogawa *et al.*, 1988; Yan *et al.*, 1994). At this concentration of PLG, variation in PVA concentration is thought to have minimal effect on protein loading (Rafati *et al.*, 1997).

Variation in the speed and duration of the primary or secondary emulsion homogenisation did not appear to have marked effects on protein encapsulation efficiency, which may be a consequence of the considerable batch variation in protein encapsulation that is commonly associated with the double emulsion solvent evaporation technique. Increasing the homogenisation speed may tend to increase protein encapsulation, by forcing more protein to become associated with the PLG. However, it would appear that subtle differences in emulsification rate and time within the range examined in this study have little bearing on the final amount of protein that becomes associated with the PLG polymer. Further replications of each batch are required to make more definitive conclusions, but the range of protein encapsulation produced was relatively high (75% on average) and sufficient for the encapsulation of protein antigen for immunisation studies. At lower concentrations of PVA decreasing homogenisation rate in the secondary emulsion has been shown to improve protein loading (Rafati *et al.*, 1997), but this effect could not demonstrated in this study using 5% PVA.

Protein loading of particles (in μg/mg particles) could be increased by the addition of an increased amount of protein to the PLG polymer in the primary emulsion, either by increasing the volume or protein concentration. However, increasing the amount of protein decreased the encapsulation efficiency. Whilst particles contained an increased amount of protein, the proportion of the original protein that became associated with the polymer was significantly reduced, suggesting that there may be a saturation level of protein that can be incorporated into the polymer. This confirms previous findings that increasing the volume fraction of the internal aqueous phase in the primary w/o emulsion lowers encapsulation efficiency (Herrman & Bodmeier, 1995). In addition in the present study SEM revealed more split and deformed microspheres at higher protein loading. Split particles are likely to be due to the higher volume of aqueous phase in the primary emulsion that leads to increased formation of aqueous channels and increased porosity, particularly in the more hydrophilic low MW PLG.

In general there was a high proportion of surface-associated protein on particles produced in this study. Considerable surface association of albumin with sub-5μm PLG microparticles has previously been documented using the double

emulsion solvent evaporation technique (Coombes *et al.*, 1996; Rafati *et al.*, 1997). Increasing the homogenisation speed and time tended to further increase the proportion of surface-associated protein, suggesting smaller particles may have been produced under these conditions with a larger relative surface area that facilitated further surface association of protein. The role of the distribution of surface:core protein in immunisation is unclear, but it may exert a profound effect on the immune responses produced. A higher proportion of encapsulated protein is desirable, as this protein is more protected from the harsh mucosal environment and is retained within the PLG particle until M cell uptake occurs.

BSA has been widely used as a model antigen for this type of study (e.g. Rafati *et al.*, 1997; Ho *et al.*, 1998). BSA and PLG have been shown to interact favourably and instantaneously by co-precipitation, forming an interfacial film that has a stabilising effect on primary water/oil emulsions (Nihant *et al.*, 1994, 1995). However, other proteins may interact with the polymer differently, and hence the encapsulation of BSA was compared with OVA, a protein related to BSA. Both proteins are hydrophilic and should be structurally similar. However, the encapsulation efficiency was much lower for OVA than for BSA (less than half), and proportionally more BSA remained surface-bound than OVA. Similarly, in particles made from a 75:25 PLG polymer BSA loading was more than doubled compared to OVA (Takahata *et al.*, 1998). However, a higher proportion of BSA than OVA was encapsulated in the particles made from this polymer, possibly due its higher hydrophobicity. The reasons for the differences in the association or the affinity of the two proteins with PLG are unclear, but may be related to the structure of the proteins, their relative hydrophilicity or molecular weight. These results demonstrate that the encapsulation of individual proteins into the PLG microparticle delivery system must be determined, and that the results from one protein may not necessarily be translated to another protein.

The chemical composition of the polymer and its affinity for protein are also thought to have an effect on protein encapsulation (Blanco & Alonso, 1997). Low MW PLG is more hydrophilic than high MW PLG because it consists of shorter polymer chains with more hydrophilic end carboxyl groups. Increased hydrophilicity leads to increased influx of the aqueous phase containing protein into the dispersed

polymer droplets during primary emulsification, and therefore better protein encapsulation, as was demonstrated in the present study. In addition, as the lower MW PLG is more water-soluble it precipitates more slowly out of the secondary emulsion, so it takes longer for the protein to diffuse out of the microparticles into the continuous aqueous phase, due to the osmotic pressure difference. The other main difference between the two polymers was that particles prepared with the higher MW PLG polymer had a lower proportion of surface-bound protein than the particles prepared with the lower MW PLG. The more hydrophobic high MW PLG polymer precipitates more quickly which leads to improved encapsulation of the protein and slower release. Some of the high proportion of surface-associated protein on the lower MW PLG particles may have been due to protein that had diffused out of the particles as discussed above. In addition, higher MW PLG particles tend to be slightly larger than lower MW PLG particles, because the high MW polymer solution is more viscous and therefore more difficult to disperse (Gasper *et al.*, 1998). Therefore high MW PLG particles may have a smaller surface area for protein to become associated with.

An initial burst phase of protein release occurred from both high and low MW PLG particles when incubated at 37°C. This occurred within 24 hours, but the majority was released within one hour. Burst release of protein from PLG particles is a common phenomenon considered to be due to the release of protein loosely associated with the surface of the particles (Wang *et al.*, 1991). More protein was released from particles made from the lower MW PLG polymer, reflecting the higher proportion of surface-bound protein associated with these particles. Within the first 24 hours, 40% of protein had been released from particles made from the low MW PLG polymer whereas 23% had been released from particles made from the high MW PLG polymer. In concurrence with these figures, previous analysis had demonstrated that 50% of protein was surface-associated with the low MW particles, and 30% with the high MW particles.

Following this burst phase of release, encapsulated protein is released more slowly from the particle core. Protein release initially occurs by diffusion through fluid filled pores (McGinity & O'Donnell, 1997). The first stage in the biodegradation process is a decrease in polymer MW, caused by random hydrolytic

cleavage of the ester linkages. The second stage is onset of polymer mass loss (Pitt *et al.*, 1981). As degradation occurs protein is released through the polymer matrix. Protein release depends on a number of factors, such as the degradation rate of the PLG copolymer matrix, the denaturation and aggregation of protein molecules, and the level of polymer-protein binding. The 50:50 lactide/glycolide copolymers are thought to have the fastest degradation rate, and complete degradation occurs in about 50-90 days *in vivo.*

Most protein release from the lower MW PLG particles occurred within the first 4-5 weeks, after which time approximately 92% of the BSA associated with the particles had been released. Particles made from the higher MW PLG polymer released encapsulated protein more slowly. Protein release continued for the duration of the experiment, by which stage approximately 69% of protein had been released. This suggested that degradation of the higher MW PLG polymer matrix occurred more slowly, perhaps due to increased polymer chain length and increased hydrophobicity. Water ingress into more hydrophobic PLG is reduced, hence it takes longer for the polymer to be broken down into smaller water-soluble molecules by hydrolysis to the critical level at which protein release can occur. Incomplete protein release from the low MW microparticles may be due to protein aggregation and non-specific adsorption (Lu & Park, 1995a,b). However, despite differences in the rate of protein release between the two polymers, by the end of the experiment similar amounts of encapsulated protein had been released from both types of particle. Prolonged release of protein from particles is desirable for vaccination and may result in prolonged stimulation of the immune system, minimising the need for booster immunisations.

These results offer a reflection of the release characteristics of the PLG particles following uptake into the MALT by M cells, but the different physiological conditions in the body may alter the release properties. Recently, the rate of degradation of PLG has been confirmed to be faster *in vivo* than *in vitro* (Gupta *et al.*, 1996). Historically, several investigators have reported that enzymes play a significant role in the breakdown of the polymer and that the rate of degradation is dependent on cell uptake (Herrman *et al.*, 1970; Williams & Mort, 1977). Plasma proteins have also been demonstrated to accelerate the degradation rate (Makino *et*

al., 1987). The rate of degradation may also have an effect on the immune response produced. Following oral vaccination with OVA in mice, a more rapidly degrading polymer was shown to be effective for the induction of salivary IgA while a more slowly degrading polymer was more effective for the induction of serum IgG (O'Hagan *et al.*, 1994).

A mixture of secreted proteins from *L. monocytogenes* was also successfully encapsulated into sub-micron PLG particles, showing that the methods developed in this chapter could be used to encapsulate a biologically relevant protein or mixture of proteins with potential for use as antigens for mucosal immunisation. Most data concerning protein encapsulation, stability and functionality focus on a single commercially available protein, and there is little literature available concerning the encapsulation of a mixture of proteins. Approximately 50% of the total secreted proteins from *L. monocytogenes* added to the primary emulsion became associated with both high and low MW PLG microparticles, an encapsulation efficiency between OVA and BSA. As with BSA, more protein became associated with the surface of particles made with the low MW PLG. Of particular interest was LLO, clearly shown to be encapsulated in the particles by labelling with fluorescent antibody. Western blot analysis and haemolysis assay revealed an interesting discrepancy between the low and high MW PLG polymers. LLO was only present in the core of particles made from the high MW particles but was present in the core and on the surface of particles made from the low MW polymer.

A major challenge in the microencapsulation of proteins in PLG microspheres is to retain their functional activity and tertiary structure (Gombotz & Pettit, 1995). A number of structural modifications can occur due to denaturation, aggregation, conformational changes, chemical degradation and adsorption onto polymer surfaces. Such changes may be induced by solvent interactions or mechanical processing. In addition, an acidic environment is created within the PLG microparticles as they degrade due to the cleavage of ester groups during degradation of the polymer matrix, which can denature proteins and impede their release (Park *et al.*, 1995). Despite these factors some evidence suggests that PLG microspheres can protect enzyme from activity loss. Unencapsulated HRP can lose 80% of its activity in solution at 37°C in a few days, whereas encapsulated enzyme can retain more than

55% of its activity after 21 days incubation at 37°C (Cohen *et al.*, 1991). LLO appears to retain its stability and antigenicity and is clearly visible as a single band on an SDS-PAGE gel following encapsulation. Furthermore, the haemolysis assay demonstrated that LLO retained its functional activity following encapsulation, despite the factors described above. Further investigation of the retention of functional activity following particle degradation and protein release is now required.

These studies show that there is potential for proteins to retain antigenicity and functionality following encapsulation into PLG microparticles. A range of particle sizes can be produced, including particles below 1μm in diameter. This range of sizes should ensure that particles of adequate size for M cell uptake are present in the vaccine formulation. The high MW polymer was chosen to produce particles for immunisation studies. Particles made from this polymer contained more core protein, and the controlled release of protein from high MW PLG particles continued for longer periods of time. In addition this polymer is more hydrophobic, and previous work has demonstrated that increasing the hydrophobicity of antigen-associated microspheres may improve the immune response (Alpar & Almeida, 1994). The following chapter describes the use of the methods developed in this study to encapsulate antigen from *Toxoplasma gondii* together with an adjuvant into PLG nanoparticles. These particles shall be used to target M cells overlying ovine NALT in intranasal immunisation studies.

CHAPTER 4

Preparation of Antigen from *Toxoplasma gondii* and Encapsulation into the Microsphere Delivery System

4.1 INTRODUCTION

The optimal formulation conditions developed in Chapter 3 shall now be applied to the encapsulation of protein antigen for intranasal immunisation studies in sheep. The model disease system to be used in these studies is *Toxoplasma gondii*. The purification and encapsulation of antigen from the tachyzoite stage of the parasite life cycle into PLG nanoparticles shall be investigated. Immunity against this initial fast-replicating stage of the life-cycle is thought to be central to the prevention of infection. Although there may be antigenic differences between tachyzoites, bradyzoites and sporozoites (Lunde & Jacobs, 1983; Kasper *et al.*, 1984; Suzuki *et al.*, 1988; Woodison & Smith, 1990), tachyzoite-derived antigens given subcutaneously have been shown to stimulate substantial protective immunity against an oral oocyst challenge in sheep (Buxton *et al.*, 1991).

The main tachyzoite antigen of interest is the 30kDa major surface antigen (SAG1), which is thought to be involved in the process of invasion (Mineo & Kasper, 1994). SAG1 is the most abundant protein in the tachyzoite, constituting 5% of the total protein (Kasper *et al.*, 1983). It is also thought to be one of the immunodominant proteins recognised by serum IgG and sIgA antibodies (Chardes *et al.*, 1990). SAG1 is the principle vaccine candidate antigen of *T. gondii* and several studies in mice have shown the value of SAG1 as a protective antigen following parenteral and more recently mucosal immunisation (Bulow & Boothroyd, 1991; Darcy *et al.*, 1992; Khan *et al.*, 1991; Debard *et al.*, 1996; Velge-Roussel *et al.*, 2,000; Bonenfant *et al.*, 2001). Vaccination with purified natural SAG1 (Debard *et al.*, 1996), with recombinant SAG1 (Petersen *et al.*, 1998), or with SAG1 derived peptides (Darcy *et al.*, 1992; Velge-Roussel *et al.*, 1997) have all demonstrated the development of significant protection against lethal challenge in animal models.

The potential to use cholera toxin (CT) as a mucosal adjuvant for intranasal immunisation in sheep will also be investigated. Vaccines consisting of homogenates or soluble extracts of killed toxoplasma parasites are often less immunogenic than live ones, and may not be able to induce a satisfactory level of protection (Bout *et al.*, 2002). Cholera toxin has been shown to be a potent mucosal adjuvant for both total antigen of *T. gondii* and SAG1 using the oral or intranasal immunisation route in mice (Bourguin *et al.*, 1993; Debard *et al.*, 1996). CT is thought to enhance both the

anti-*T. gondii* IgA response and mucosal cellular immunity with increased IFNγ secretion .

CT can either be co-entrapped with the toxoplasma antigen in the PLG particles or it can be delivered with the particles. However co-entrapment of the adjuvant with the antigen is likely to be superior for immunisation because the two agents are maintained in close proximity at the time of release from particles to antigen-presenting cells, and encapsulation protects the adjuvant from the mucosal environment. CT present on the surface of particles may also directly interact with the epithelial surface to enhance particle uptake. In mice a vaccine dose of 20μg of SAG1 and 1μg of CT is effective for the induction of protective immunity (Debard *et al.*, 1996). This 20:1 ratio will be used to prepare PLG particles containing both toxoplasma antigen and CT for intranasal delivery to sheep.

To the best of the author's knowledge the encapsulation of SAG1 or other proteins from *T. gondii* into PLG microparticles has not been previously investigated. It is hoped that the results presented in Chapter 3 will maximise protein encapsulation into particles of the appropriate size for M cell uptake with long-term controlled release properties.

4.2 MATERIALS AND METHODS

4.2.1 Mice

110 adult female Swiss White (outbred) and Porton mice (minimally inbred) were used to passage and maintain *T. gondii* tachyzoites of the RH strain. Mice were group housed and supplied with proprietary food and water ad lib.

4.2.2 *Toxoplasma gondii*

4.2.2.1 RH strain

T. gondii incomplete strain RH was originally isolated in Cincinnati (USA) from mice inoculated with a tissue sample from the CNS of a 6 year old boy with a fatal acute nonsuppurative encephalitis (Sabin, 1941).

4.2.2.2 Maintenance of Tachyzoites

T. gondii tachyzoites of the RH strain were maintained by passage in female mice. Five adult female Swiss White mice were each inoculated intraperitoneally with c.2×10^7 *T. gondii* tachyzoites (0.2ml inoculum) taken directly from storage in liquid nitrogen, and examined daily for symptoms of disease, including fever, staring coats and panting. Mice that appeared dull and lethargic were culled immediately to avoid further distress. After 5 days mice were killed by carbon dioxide inhalation and tachyzoites were harvested by repeatedly irrigating the peritoneal cavity with PBS using a 26 gauge (26G) needle and syringe until the washes became clear. Tachyzoites were then counted in an improved Neubauer haemocytometer using an Olympus BX50 microscope with a ×40 objective (Olympus, London, UK), and an inoculum containing 10^7 tachyzoites/ml was prepared in 50% PBS and 50% tissue culture strength penicillin/streptomycin. A further 5 adult female Swiss White mice were then inoculated with 10^6 tachyzoites per mouse from the above inoculum (0.1ml inoculum per mouse), left for 3 days, and checked daily for signs of disease. The mice were then killed by carbon dioxide inhalation and tachyzoites harvested as described above. Sufficient inoculum was prepared from these tachyzoites for a batch of 100 adult female Swiss White or Porton mice, which were then inoculated as described above. Mice were left for 3 days, culled and parasites harvested as before. Peritoneal washings were now white in appearance and any that were excessively contaminated by blood were discarded.

4.2.2.3 Extraction of Proteins from Toxoplasma Tachyzoites

The peritoneal washings collected from infected mice were given up to 10 alternate washes in PBS and Hank's Balanced Salt Solution (HBSS) pH7.4 (Gibco, Invitrogen, Paisley, UK), by centrifugation at 500×*g* for 5 min and resuspension in 10ml of buffer, to minimise host cell contamination. After the final wash the pellet was resuspended in 10ml of PBS, and a 100μl aliquot was taken and diluted in 2% glutaraldehyde either 1:10 to count cellular contamination or 1:1,000 to count tachyzoites. The counts were performed in an improved Neubauer haemocytometer using an Olympus BX50 microscope with a ×40 objective (Olympus, London, UK). The tachyzoites were then pelleted at 500×*g* for 5 min, stored at -20°C until required

and freeze-thawed three times before use. A crude extract of proteins was prepared by lysing 2×10^8 RH tachyzoites per ml of extraction buffer (50mM TrisHCl pH8.3, 0.5% Nonidet P-40, 150mM NaCl, 2mM EDTA, 1mM PMSF, 0.02% (w/v) aprotinin) on ice (adapted from Debard *et al.*, 1996). The lysates were centrifuged at 40,000×*g* for 1 h at 4°C and the supernatants were collected.

4.2.3 Protein Analysis

4.2.3.1 SDS-PAGE

Proteins were separated by gel electrophoresis as outlined by Laemmli (1970) using the Mini-Protean II gel system (Bio-Rad, Hemel Hempstead, UK). 15μl of each sample were mixed with an equal volume of non-reducing sample buffer (62.5mM Tris-HCl pH 6.8, 2% w/v SDS, 10% w/v glycerol, 0.002% w/v bromophenol blue) prior to loading into the wells of discontinuous SDS-PAGE minigels. 12% acrylamide gels that had been made in separating gel buffer (0.375M Tris-HCl pH8.8, 1% w/v SDS, 0.1% v/v TEMED, 0.1% w/v ammonium persulfate (APS)) were used for protein separation. The separating gel was overlaid with a 4% acrylamide stacking gel made in stacking gel buffer (0.15M TrisHCl pH 6.8, 0.5% w/v SDS, 0.2% v/v TEMED, 0.2% w/v APS). Proteins were separated at 200V for 45-60mins in tank buffer (25mM Tris, 200mM glycine, 5mM SDS pH8.3) until the dye front had reached the bottom of the gel. Molecular weight markers were included in all gels, either Mark12™ Unstained Standards (Novex, Invitrogen, Paisley, UK) or SeeBlue™ Plus2 Pre-Stained Standards (Novex, Invitrogen, Paisley, UK).

4.2.3.2 Protein Detection on SDS-PAGE Gels

Separated proteins were visualised by silver staining (Morrissey, 1981). Firstly the proteins in the gels were fixed in 50% (v/v) methanol, 10% (v/v) acetic acid in distilled water for 20 min, then twice in 5% (v/v) methanol, 7% (v/v) acetic acid in distilled water for 10 min. After two 5 min washes in distilled water, proteins were reduced in 5μg/ml dithiothreitol (DTT) in distilled water for 15 min, washed again, and stained in 0.1% silver nitrate in distilled water for 20 min. Gels were then washed briefly and finally developed for stained protein by immersion in 3% sodium

carbonate containing 0.05% formaldehyde. Development was terminated by the addition of citric acid to 1% w/v.

4.2.3.3 Western Blot Analysis

Immuno-blot analysis was performed on proteins separated by SDS-PAGE and electrotransferred to Protran BA 83 nitrocellulose membranes (Schleicher and Schull, Anderman, Kingston upon Thames, UK) by semi-dry Western blot. The gel and membrane were sandwiched between several sheets of 3mm filter paper soaked in transfer buffer (24mM Tris, 192mM glycine, 20% v/v methanol) and placed in the Western blot apparatus. Transfer took place at 70mA/gel (2mA/cm^2 of gel) for 1 hr. The membranes were then blocked in PBS containing 5% non-fat milk powder (Marvel, Chivers, Dublin, Ireland) for 30min at RT and washed thoroughly in wash buffer (PBS containing 0.5M NaCl and 0.5% (v/v) Tween®80). The membranes were then probed with a mouse IgG anti-SAG1 monoclonal antibody (Couvreur *et al.*, 1988) diluted to 1:5,000 in blot wash buffer for 1h at RT (Mab 1E5, kindly gifted by J.F. Dubremetz, U42 INSERM, Villeneuve d'Ascq, France). Negative controls were provided by omitting the primary antibody. Following extensive washing of the blot, bound antibody was visualised by a further 1 h incubation with a 1:2,000 dilution of a goat anti-mouse antibody conjugated to biotin (Dako, Ely, UK) in wash buffer, followed by further washing and a 1 h incubation in a 1:2500 dilution of streptavidin conjugated to horseradish peroxidase (HRP) (Dako, Ely, UK). After a final wash HRP was detected by treating blots with either DAB (Sigma Fast™ DAB Tablet, Sigma, Dorset, UK) or the enhanced chemiluminescence (ECL) reagent (Amersham International, Little Chalfont, UK) according to the manufacturer's instructions, and exposure to Hyperfilm ECL (Amersham International, Little Chalfont, UK) for approximately 5 sec before development.

4.2.3.4 Protein Assays

Protein assays were performed with the bicinchoninic acid (BCA) enhanced protein assay (Pierce Chemical Company, Rockford, Illinois, USA) using either the microplate or the test-tube method as described by the manufacturer's protocol.

4.2.3.5 Concentration of Proteins

Proteins were concentrated at least 10-fold using centrifugal filter units. For volumes up to 2ml, proteins were concentrated in Centricon® YM-10 (molecular weight cut-off 10,000 kDa) microconcentrator cells (Amicon, Stonehouse, UK) at 5,000 x *g*. For volumes up to 15ml, proteins were concentrated in Centriprep® YM-10 concentrators (Amicon, Stonehouse, UK) at 3,000 x *g*.

4.2.4 Purification of SAG1

4.2.4.1 Gel Filtration Chromatography

Chromatography was carried out using a High Performance Liquid Chromatography (HPLC) system (Waters, Milford, USA). Proteins were separated on the basis of size by gel filtration chromatography. It has been established that SAG1 has a molecular weight of approximately 30kDa (Couvreur *et al.*, 1988). Superose 12 (Amersham Pharmacia Biotech, Little Chalfont, UK), which separates proteins between 1- 300 kDa, was chosen as the optimal chromatographic column for separation of this protein.

200μl volumes of the toxoplasma protein extract were loaded onto a 10mm × 300mm Superose 12 HR column (Amersham Pharmacia Biotech, Little Chalfont, UK) equilibrated with running buffer (50mM Tris-HCl pH8.3, 0.5% Triton-X100 reduced, 150mM NaCl, 2mM EDTA). Column flow rate was kept constant at 0.2ml/min and 0.4ml fractions were collected every two minutes. OD at 280nm was monitored using an absorbance detector and fractions relating to peaks were retained for further analysis.

4.2.4.2 Analysis of Fractions

2μl of each fraction collected and retained from the column were dotted on small squares of Protran BA 83 nitrocellulose membrane (Schleicher & Schull, Dassel, Germany) and allowed to dry completely for dotblot analysis. A positive control of original material was also included. The nitrocellulose was then blocked in PBS containing 5% non-fat milk powder (Marvel, Chivers, Dublin, Ireland) and probed with the monoclonal anti-SAG1 antibody as detailed in Section 4.2.3.3.

Fractions which tested positive for SAG1 were pooled, concentrated 10-fold (Section 4.2.3.5) and analysed further by SDS-PAGE (Section 4.2.3.1) and Western blot probed with the monoclonal anti-SAG1 antibody (Section 4.2.3.3).

4.2.5 Preparation of PLG Microparticles for Immunisation Studies

4.2.5.1 Microparticles Incorporating Proteins from *T. gondii* Tachyzoites

Toxoplasma antigen was prepared according to the methods described in Section 4.2.2.3 and concentrated to approximately 20mg/ml as detailed in Section 4.2.3.5. A primary emulsion was made by homogenising 100μl of this protein solution with 2ml of a 5% PLG solution in DCM (50:50 PLG copolymer, P2191, M_r 40,000-70,000, Sigma, Dorset, UK) in a glass test-tube at 16,000rpm for 2 min. The primary emulsion was then homogenised with 20ml of a 5% solution of PVA at 19,000rpm for 2 min to create the secondary emulsion. The secondary emulsion was maintained overnight under magnetic stirring to facilitate the evaporation of excess DCM. Particles were then centrifuged in a Beckman J2-21 centrifuge using a JA20.1 rotor (Beckman Coulter, High Wycombe, UK) at 12,000rpm for 10 min. The microparticles were washed twice to remove excess PVA by redispersion in 20ml of distilled water and centrifugation at 8,000rpm for 3 min. Particles were then rehydrated in approximately 2ml of distilled water and lyophilised for at least 48 h. Three batches of particles were prepared using this method.

4.2.5.2 Microparticles Incorporating Proteins from *T. gondii* Tachyzoites and Cholera Toxin

Cholera holotoxin (Sigma, Dorset, UK) was reconstituted to a concentration of 10mg/ml. The primary emulsion for the microparticles was prepared using the methods detailed in Section 4.2.5.1 incorporating 100μl of the toxoplasma antigen and 10μl of the cholera toxin solution into the PLG solution in the primary emulsion to give a 20:1 ratio of antigen:adjuvant in the particles. Three batches of particles were prepared using this method.

4.2.5.3 Negative Control Microparticles

Particles were made according to Section 4.2.5.1, using 100μl of distilled water to make the primary emulsion. Three batches of blank microparticles were prepared using this method.

4.2.6 Analysis of Particles

4.2.6.1 Analysis of Protein Content of Particles

10mg microparticles were dispersed in 0.5ml of 0.01M PBS, pH 7.4 containing 2% w/v SDS. The resulting suspension was rotated at room temperature for 1 h, and then centrifuged at 14,000rpm for 5 min. The supernatant was removed and analysed to give a measurement of the amount of protein associated with the surface of the microparticles. The microparticle pellet was then resuspended in 0.5ml of 0.5M NaOH containing 5% w/v SDS and this suspension was rotated gently for 3-4 h at room temperature until particles had completely dissolved. This suspension was then assayed to give a measurement of the amount of protein encapsulated within the particles. A further 5mg microparticles were dispersed in 0.5ml of 0.2M NaOH containing 5% w/v SDS and gently mixed for 3-4 hours until the particles had completely dissolved. This solution was then assayed to give a measure of protein encapsulation efficiency. Protein contents were measured by the test tube method using the Pierce BCA assay detailed in Section 4.2.3.4

4.2.6.2 Analysis of Particle Size and Morphology

Particle size and surface morphology were examined using SEM. A small sample of microparticles was suspended in a small volume of distilled water, placed on aluminium stubs and allowed to air dry. The stubs were then sputter coated with gold (SCD030 Balzers Union Ltd., Liechtenstein) and examined in a Phillips 505 scanning electron microscope (Phillips, Eidhoven, Netherlands). The range of diameter of the particles was assessed on the micrographs produced.

4.2.6.3 Comparison of Surface and Encapsulated Proteins

Surface-associated and encapsulated protein samples were obtained from microparticles as detailed in Section 4.2.6.1 and concentrated approximately 10-fold as detailed in Section 4.2.3.5. Proteins were separated on 12% non-reducing gels as detailed in Section 4.2.3.1 and visualised using silver stain as detailed in Section 4.2.3.2. Further gels were blotted and probed for SAG1 using the monoclonal anti-SAG1 antibody as detailed in Section 4.2.3.3 or a 1:5,000 dilution of a rabbit anti-cholera toxin antibody (C3062, Sigma, Dorset, UK).

4.2.6.4 Dual Staining of Particles with Antibodies Against SAG1 and CT

A small quantity of toxoplasma + cholera toxin microparticles was rehydrated in distilled water, and 5μl or 10μl amounts of the suspension were dotted onto electrostatically charged microscope slides (SuperFrost® Plus, Menzel-Glaser®, Germany). The particles were then left for approximately 1 h to dry completely onto the slides. The slides were washed thoroughly with PBS containing 1% BSA and 0.05% Tween®20 (Sigma, Dorset, UK), and then incubated in a 1:1,000 dilution of a polyclonal rabbit anti-cholera toxin antibody (C3062, Sigma, Dorset, UK) for 1 h at RT. The slides were washed thoroughly in PBS and incubated in a 1:50 dilution of a goat anti-rabbit IgG antibody conjugated to tetramethyl rhodamine isothiocyanate (TRITC) for 1 h at RT (T6778, Sigma, Dorset, UK). The slides were washed again and incubated in a 1:1,000 dilution of the monoclonal mouse anti-SAG1 antibody for 1 h at RT. The slides were washed in PBS and incubated in a 1:400 dilution of a goat anti-mouse IgG antibody conjugated to biotin (Dako, Ely, UK) for 1 h at RT. The slides were washed again and incubated in a 1:100 dilution of streptavidin conjugated to fluorescein isothiocyanate (FITC) (Dako, Ely, UK) for 1 h at RT, before being washed thoroughly in PBS and mounted in Citifluor® (CitiFluor Ltd., London, UK). Negative controls were produced by staining blank particles with the same method, or by staining toxoplasma + cholera toxin particles with unrelated isotype-matched monoclonal antibodies raised in both rabbit and mouse. Slides were examined under an Olympus BX50 microscope microscope (Olympus, London, UK) using a ×40 objective. TRITC was visualised under a green (NG) filter at 530-550nm, and FITC was visualised under a blue (NB) filter at 470-490nm.

4.3 RESULTS

4.3.1 Extraction of Proteins from *T. gondii* Tachyzoites

In total 5.4×10^9 tachyzoites were harvested from the peritoneal cavities of the mice used in this study, and were divided into 10 aliquots, each of 6×10^8 tachyzoites. Each aliquot of tachyzoites was lysed in 3ml of extraction buffer as described in Section 4.2.2.3. The protein concentration of the extraction buffer from each aliquot is shown in Table 4.1 below. Comparable amounts of protein were obtained from each aliquot and in total approximately 38mg protein were obtained from the toxoplasma tachyzoites harvested in this study. The average protein concentration of each extraction was 1.25mg/ml, equating to approximately 3.75mg protein from each aliquot of 6×10^8 tachyzoites. The extracts were pooled and a small aliquot was kept aside for later assays.

Aliquot	Volume (ml)	Protein Concentration (mg/ml)	Total Protein (mg)
1	3	1.032	3.096
2	3	1.34	4.02
3	3	1.421	4.263
4	3	1.409	4.227
5	3	1.381	4.143
6	3	1.147	3.441
7	3	1.447	4.341
8	3	0.952	2.856
9	3	1.153	3.459
10	3	1.245	3.735

Table 4.1 *Protein concentration in extraction from each aliquot of tachyzoites*

The extracted tachyzoite proteins are demonstrated in Figure 4.1. The silver-stained gel in Figure 4.1a shows that there were a large number of proteins present in the tachyzoite extract, in a wide range of molecular weights. There was a large, darkly stained band visible at the approximate location of the carbonic anhydrase marker (31kDa). This protein was the most likely candidate for SAG1, which has a published molecular weight between 30kDa – 32kDa (Couvreur *et al.*, 1988). The intensity of the staining of the band shows that this protein was present in large quantities and was one of the most abundant proteins in the extract.

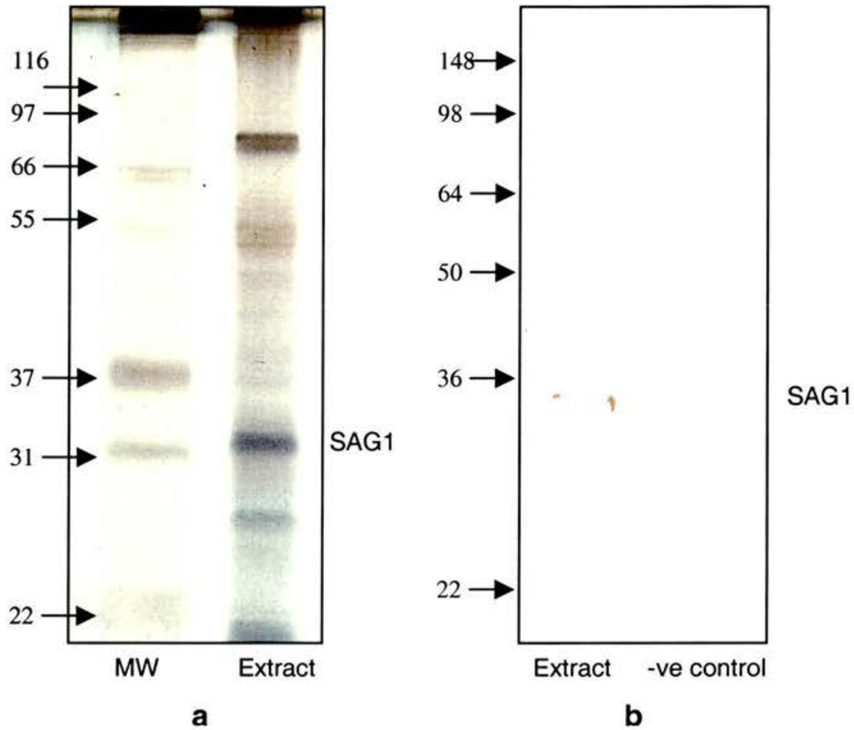


Figure 4.1 *Extract of proteins from toxoplasma tachyzoites separated on 12% non-reducing gels. Figure 4.1a shows a silver-stained gel while 4.1b shows a Western blot probed with the anti-SAG1 monoclonal antibody, visualised using DAB*

When a Western blot of the extract was probed with the monoclonal antibody, SAG1 was clearly visible as a large, darkly stained band at a molecular weight of approximately 30kDa, shown in Figure 4.1b. The position of SAG1 corresponded to the location of the major band discussed on the silver stained gel, confirming that this protein was SAG1. Some other proteins in the extract with higher molecular weights appeared to show a small degree of cross-reactivity with the antibody but this was negligible compared to the strong staining visible for SAG1. No non-specific staining was visible on the negative control.

4.3.2 Partial Purification of SAG1 using Gel Filtration Chromatography

The chromatogram produced by gel filtration of the tachyzoite extract using Superose 12 is demonstrated in Figure 4.2. A small peak of protein was eluted from the column at a retention time of 40 min (fraction 20), and a larger peak at a retention time of approximately 56 min (fraction 28). The bulk of protein was eluted from the column directly after this peak in one very large peak that had a shoulder (retention time 65 min, fraction 32), suggesting that good separation of proteins may not have been achieved. A very small amount of protein was also eluted from the peak at a retention time of 84-90 min.

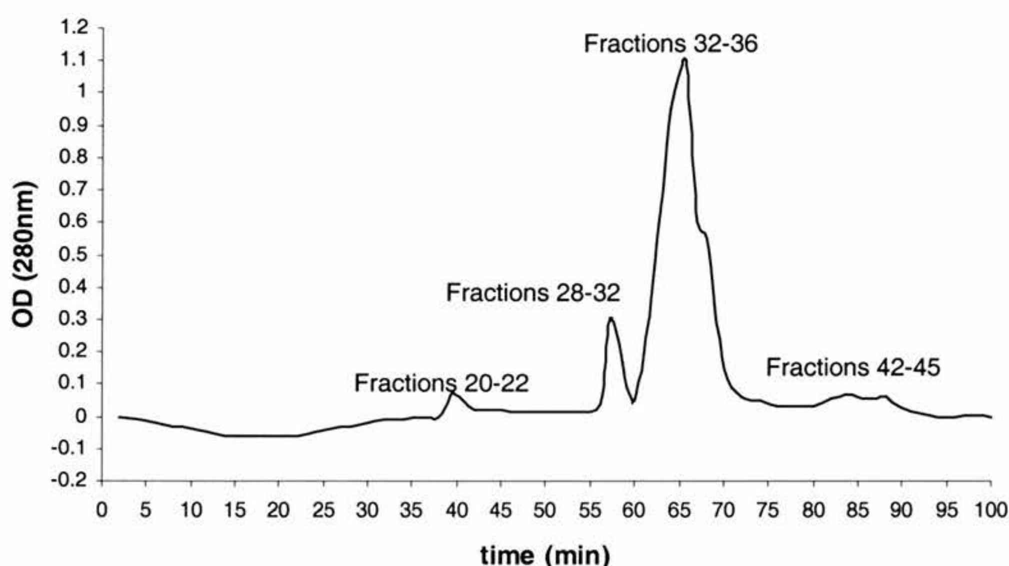


Figure 4.2 *Gel filtration chromatogram of toxoplasma tachyzoite proteins*

Individual fractions were analysed by dotblot using the monoclonal anti-SAG1 antibody and a positive result for the SAG1 protein was shown within the first two peaks (represented by fractions 20-22 and 28-32), but not in the major peak. The fractions within each of these two peaks were then pooled, concentrated and proteins were separated on 12% non-reducing gels. The silver stained-gel shown in Figure 4.3 demonstrates the presence of an intensely stained band at approximately 30kDa, likely to be SAG1, in the peak represented by fractions 28-32, as well as a number of other proteins in a range of molecular weights. In comparison relatively few bands were present in the peak represented by fractions 20-22 suggesting few proteins were present. SAG1 appeared to be absent from fractions 20-22.

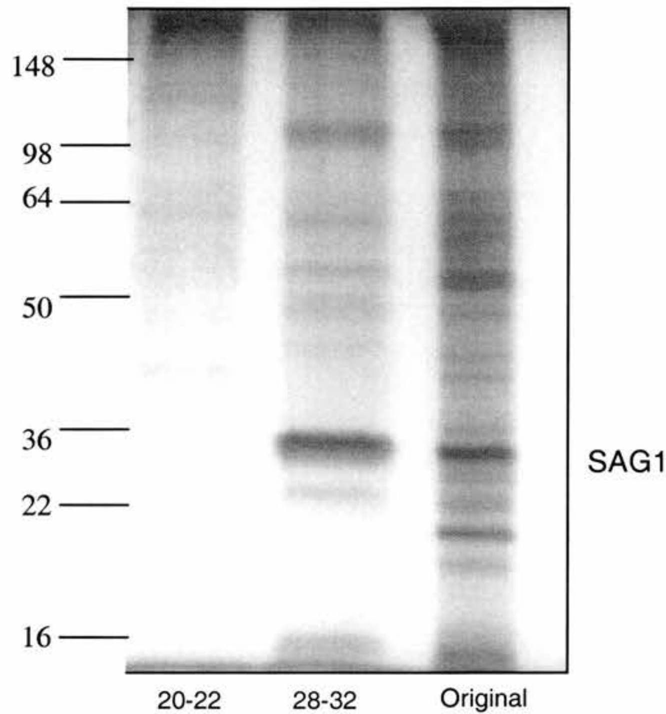


Figure 4.3 *Silver-stained gel of original toxoplasma tachyzoite extract, and fractions 20-22 and 28-32 from gel filtration chromatography*

The Western blot probed with the anti-SAG1 monoclonal antibody shown in Figure 4.4 overleaf confirmed that SAG1 was present in the peak represented by fractions 28-32 but not in the pool of fractions 20-22. An intensely stained band at approximately 30kDa was observed in fractions 28-32 and the original extract, but was absent in fractions 20-22. Visualisation of the SAG1 using the ECL reagent resulted in the demonstration of more intense bands than with DAB, and the higher molecular weight proteins that the antibody cross-reacted with were more clearly visible. It seems likely that the positive result on the dotblot for fractions 20-22 was caused by cross-reactivity of the antibody with some of these other proteins. A small amount of non-specific background was observed in the negative control on the Western blot.

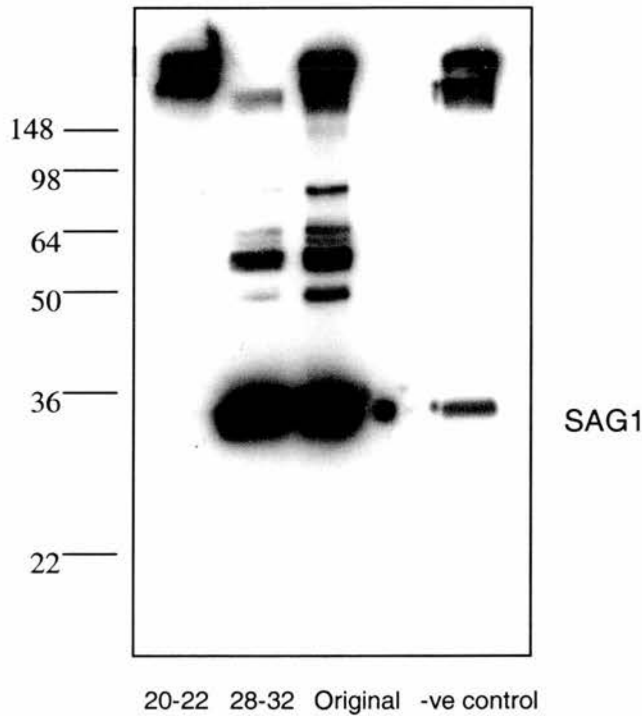


Figure 4.4 *Western blot probed with anti-SAG1 antibody demonstrating presence of SAG1 within fractions 28-32 from gel filtration chromatography*

Therefore the peak containing fractions 28-32 represented good partial purification and enrichment of SAG1. However a number of other contaminating proteins were also present within this fraction, and further purification steps were required. Slowing the flow rate to 0.1ml/min did not result in better separation of the protein. A Pierce BCA protein assay revealed that each column run of 200µl of tachyzoite extract returned approximately 25µg of protein in the pool of fractions 28-32, representing approximately 10% of the total protein originally applied to the column. Up to 50% of this protein may have been SAG1. However, further purification of SAG1 was not attempted due to expected further losses of SAG1. When the quantity of protein obtained from the partial purification was scaled up for the total amount of protein obtained from the tachyzoite extraction insufficient protein was available for the proposed immunisation studies. This resulted in the decision to encapsulate the crude extract of toxoplasma tachyzoite proteins into the PLG microsphere delivery system.

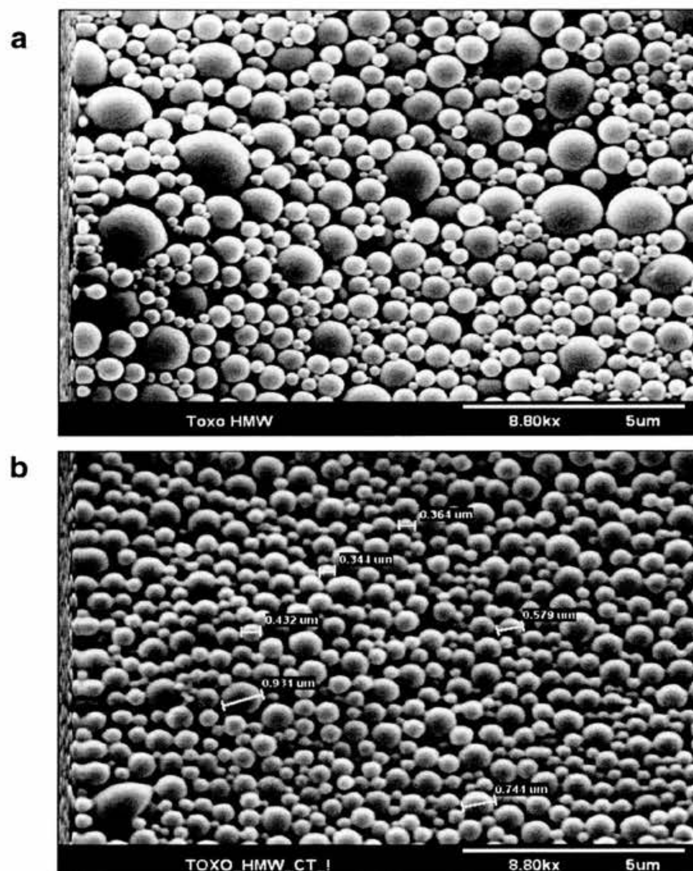
4.3.3 Toxoplasma-Loaded Microspheres for Use in Vaccination Studies

4.3.3.1 Preparation of Antigen

The remaining toxoplasma tachyzoite extract, which contained approximately 33mg of protein, was concentrated to a volume of 1.7ml. This provided an antigen with a protein concentration of approximately 20mg/ml for microsphere development. Proteins in the concentrate and the original extract were separated and compared on a 12% gel, demonstrating that concentration of the material had no effect on the constituent proteins.

4.3.3.2 Morphology and Size Range

SEM revealed that all particles containing toxoplasma tachyzoite antigen (with and without CT) were smooth and roughly spherical. A scanning electron micrograph of particles incorporating toxoplasma antigen alone is demonstrated in Figure 4.5a and a micrograph of particles incorporating toxoplasma antigen and CT is demonstrated in Figure 4.5b. The size of particles is highlighted on Figure 4.5b but particle size can also be compared to the scale bars on the micrographs.



Figures 4.5a and b *Scanning clcctron micrographs demonstrating morphology and size range of microparticles incorporating a) proteins from toxoplasma tachyzoites and b) proteins from toxoplasma tachyzoites and cholera toxin*

Particles were of comparable size to those produced in Chapter 3, maximum 2μm in diameter with the majority below 1μm. Particles demonstrated in Figure 4.5a are slightly larger than those in Figure 4.5b. A bimodal size distribution is apparent in Figure 4.5a, but particles in Figure 4.5b have a more uniform size distribution. These differences are likely to be due to the considerable batch variation associated with the double emulsion solvent evaporation technique.

4.3.3.3 Protein Content

The protein contents of the different batches of microparticles were generally relatively high, demonstrated in Table 4.2. No protein could be detected in the negative control particles. The two methods used to determine the total protein content of each batch of microparticles produced similar protein concentrations in μg protein/mg particles. Encapsulation efficiency for toxoplasma tachyzoite antigen was relatively high, with an overall average encapsulation efficiency of 80%. The majority of protein in every batch of toxoplasma-loaded microparticles was encapsulated within the core of the particles. On average 80% protein was encapsulated whilst 20% protein remained surface bound.

Batch	Surface Protein	Enapsulated Protein	Ratio S:E	Total 1	Total 2	Average Total	Encapsulation Efficiency
	μg/mg particles		%	μg/mg particles			%
Toxo1	3.42	12.90	**21:79**	16.32	12.64	14.48	**60**
Toxo2	3.01	16.06	**16:84**	19.07	17.86	18.47	**77**
Toxo3	3.41	16.68	**17:83**	20.09	22.02	21.01	**88**
T+CT1	3.01	18.48	**14:86**	21.49	21.94	21.72	**90**
T+CT2	3.08	13.76	**18:82**	16.84	19.64	18.24	**76**
T+CT3	4.35	16.26	**21:79**	20.61	22.91	21.76	**91**

Table 4.2 *Protein content of toxoplasma-loaded PLG microspheres*

4.3.3.4 Comparison of Surface-Bound and Encapsulated Proteins

A silver-stained 12% non-reducing SDS-PAGE gel comparing the proteins located on the surface and encapsulated within toxoplasma-loaded particles with and without CT is shown in Figure 4.6. The first track of the gel shows the total mixture of proteins present within the original tachyzoite extract for comparison, and the position of SAG1at approximately 30kDa is highlighted. Tracks 2 and 3 are the surface-bound and encapsulated proteins respectively from particles incorporating toxoplasma tachyzoite antigen alone. Tracks 4 and 5 are surface-bound and encapsulated proteins respectively from particles incorporating toxoplasma tachyzoite antigen and cholera toxin.

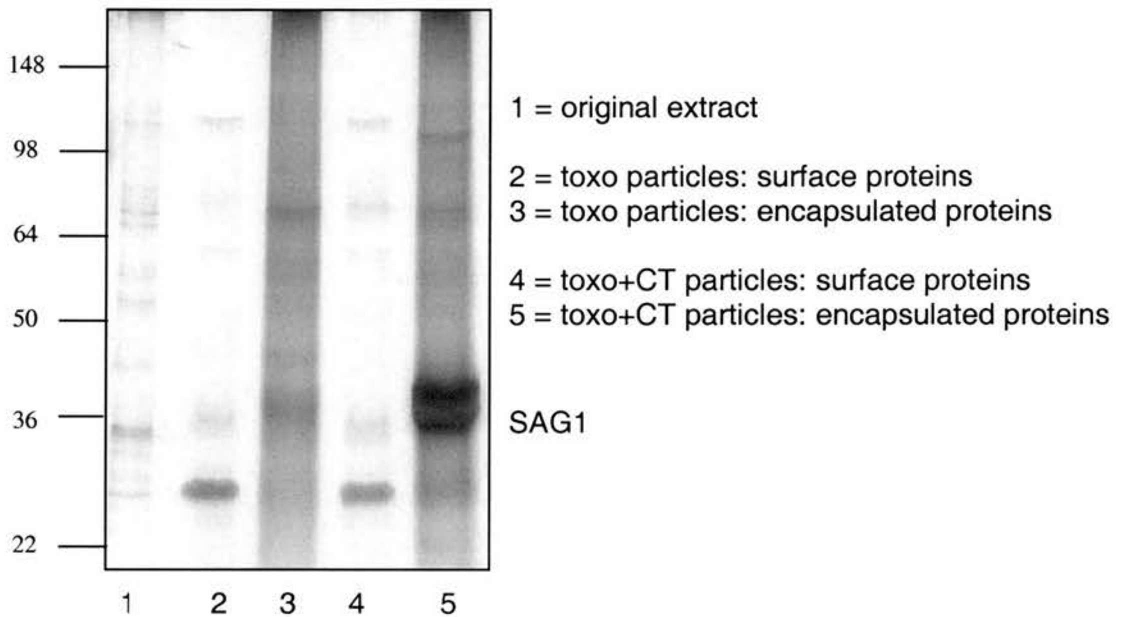


Figure 4.6 *Silver-stained 12% non-reducing gel showing surface bound and encapsulated proteins from microparticles incorporating toxoplasma tachyzoite antigen with and without CT*

The encapsulated proteins from the different particles were not clearly visible, perhaps due to the effects of the dissolved polymer on the separation of the proteins within the gel. However, a number of bands were visible in the samples of surface-associated proteins. Proteins on the surface of both types of particle appeared to be identical, representing a range of proteins from the original extract in a wide range of sizes. This suggested that no preferential encapsulation of proteins of certain molecular weights had occurred. A faint band was visible at 30kDa in the surface-

associated samples, suggesting that some SAG1 was located on the surface of both types of particle. The band was much more weakly stained than in the original extract, suggesting that only a small proportion of the total SAG1 remained surface-bound. It was unfortunate that comparisons with encapsulated proteins could not be performed, although it seems likely that proteins with a high affinity for the PLG polymer are located both on the surface and inside the particles.

A Western blot of these samples probed with the monoclonal anti-SAG1 antibody is shown in Figure 4.7. Tracks 1 and 2 represent surface-bound and encapsulated proteins respectively from particles incorporating toxoplasma protein alone, and tracks 3 and 4 are from particles incorporating toxoplasma protein and cholera toxin.

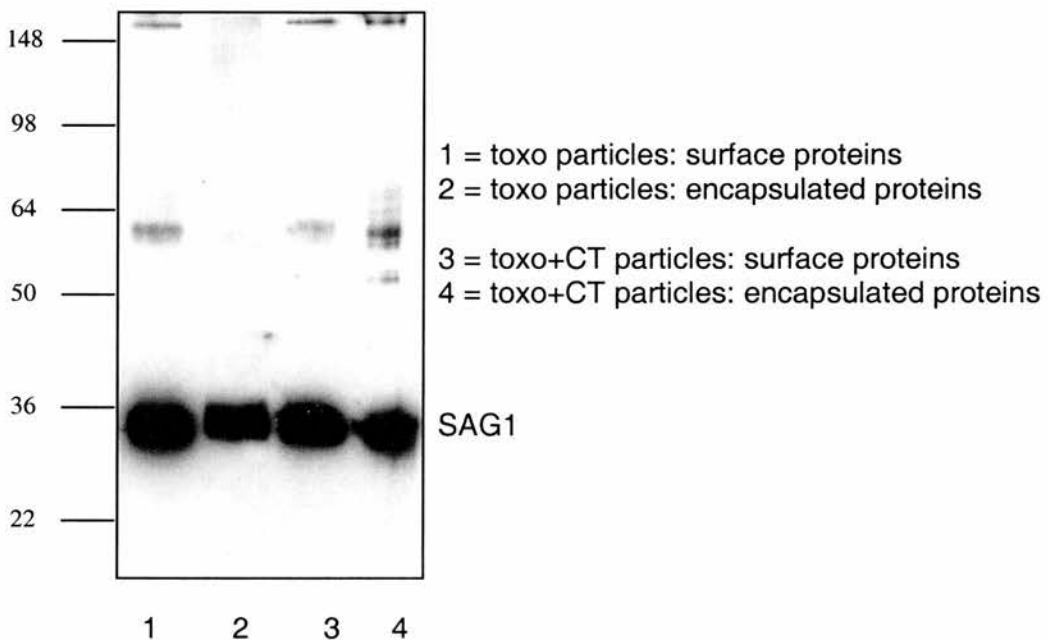


Figure 4.7 *Western blot probed with monoclonal anti-SAG1 antibody, visualised using chemiluminescence*

SAG1, represented by the strongly stained band at 30kDa, was present on the surface and encapsulated within both types of particles in significant detectable quantities. Despite the fact that the dissolved polymer masked the proteins present in the silver-stained gel, the antibody was still able to detect the protein on the blot. No cholera toxin could be observed in identical Western blots probed with antibody

against cholera toxin, probably due to dilution factors and the extremely small amounts of CT present in the particles.

4.3.3.5 Detection of Cholera Toxin and SAG1 within the Particles

Both antibodies used in the dual staining procedure showed immunoreactivity, and staining could be visualised simultaneously for the two different antibodies using different coloured filters on the microscope. Positive labelling clearly corresponded to the location of the microparticles, and no labelling was observed on negative control slides. At this magnification the microparticles were clearly visible as numerous small round spherical objects, which occurred either individually or in small clusters. Cholera toxin, visualised using TRITC, was clearly associated with the particles, shown in Figure 4.8b. Cholera toxin appeared to be present both within the particles and bound to their surface. The perimeter of individual microparticles appeared to be as intensely stained as the centre, suggesting that a significant proportion of the cholera toxin was associated with the surface of the microparticles.

SAG1 was also clearly associated with the microparticles and showed a very similar distribution to the cholera toxin. SAG1, visualised using FITC, is shown in Figure 4.8a. Again intense staining could be observed around the perimeter of the particles suggesting a significant proportion of the protein was surface-bound as well as internalised. The dual exposure photograph shown in Figure 4.8c demonstrated that the same particles were stained for both cholera toxin and SAG1. There did not appear to be any particles that were only labelled for one of the proteins. Thus the particles contained both cholera toxin and SAG1 from the toxoplasma tachyzoites, both encapsulated and on the surface. However, it was not possible to compare the amounts of the two different proteins by their staining intensities.

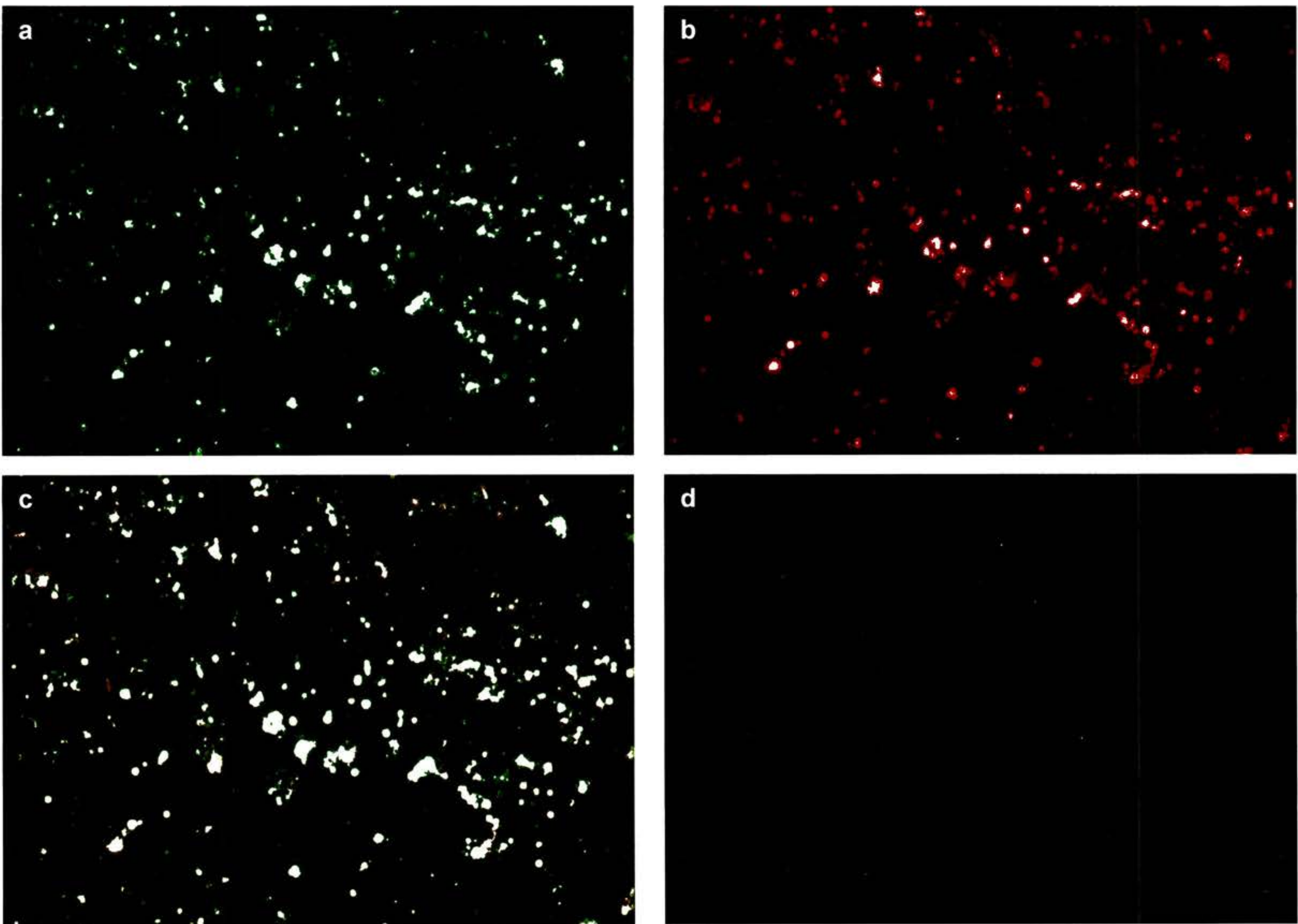


Figure 4.8 *Dual staining of microparticles with antibodies for SAG1 and cholera toxin. a) anti- SAG1 (FITC), b) anti-cholera toxin (TRITC), c) TRITC and FITC together, and d) negative control ×272 magnification*

4.4 DISCUSSION

Lysis of the toxoplasma tachyzoites resulted in the successful extraction of a substantial amount of a number of proteins, including SAG1. However, insufficient protein was available to provide enough purified SAG1-encapsulated microparticles for the proposed immunisation studies, and it was therefore decided to encapsulate the crude tachyzoite extract. This is the first demonstration that soluble toxoplasma tachyzoite extract can be encapsulated with high efficiency into PLG nanoparticles, both alone and with cholera toxin. Such whole extracts of soluble tachyzoite proteins have been successfully used as an antigen in previous mucosal immunisation studies in mice with CT as an adjuvant (Bourguin *et al.*, 1991, 1993; Chardes *et al.*, 1993).

The monoclonal antibody permitted confirmation that the major band in the tachyzoite extract was SAG1 at approximately 30kDa. The molecular weight of SAG1 has been published between 27kDa (Handman *et al.*, 1980) and 32kDa (Couvreur *et al.*, 1988). The intensity of staining on SDS-PAGE gels revealed that SAG1 was a major component of the tachyzoite extract and a good candidate protein for purification because it was one of the most dominant proteins, and present in relatively large quantities. Detergent was an essential component of the lysis buffer to maintain the solubility of membrane-bound proteins such as SAG1. Non-reducing conditions were used to separate proteins in the tachyzoite extract on SDS-PAGE because under reducing conditions the SAG1 protein was not detected by the monoclonal antibody on Western blot, suggesting that the tertiary structure of the epitope may be important. Most of the surface proteins are thought to electrophorese more slowly under reduced gel conditions (Tomavo, 1996). Other authors have suggested that SAG1 is recognised following separation in reducing conditions (Couvreur *et al.*, 1988) and that the P35 and P30 (SAG1) proteins co-migrate under non-reduced conditions (Tomavo, 1996).

The antibody appeared to exhibit some cross-reactivity with other higher molecular weight proteins in the tachyzoite extract, which were more apparent on Western blots developed with the ECL reagent. These cross-reactive proteins may have been contaminants in the original inoculum, they could share the same epitope as SAG1, or could be dimerised forms of SAG1. Some cross-reactivity was also exhibited in protein complexes that were too large to enter the gel and remained in

the wells at the top of the gel. It is probable that these protein complexes were complexes of SAG1 with other proteins that were too large to run into the gel under non-reducing conditions. Low levels of background staining could be observed on the negative control with the ECL reagent, likely to be due a low level of cross-reactivity between SAG1 and the secondary antibody. However, this cross-reactivity was negligible when compared to the staining observed for SAG1 when the primary antibody was included.

For the purpose of this study it was not possible to purify SAG1 as a single band for use as an antigen in the proposed immunisation studies. Gel filtration chromatography led to the elution of SAG1 in a small distinct peak visible on the chromatogram that appeared to be distinct and separate from the majority of proteins in the tachyzoite extract. However, SDS-PAGE analysis revealed the presence of several contaminating proteins within the same peak as SAG1. Thus, further purification steps were required to purify SAG1 as a single band, and ion-exchange chromatography and immunoaffinity chromatography were considered. Purification of SAG1 has been previously achieved using immunoaffinity chromatography (Kasper *et al.*, 1983) with the monoclonal anti-SAG1 antibody used in this study. However insufficient quantities of this antibody were available to purify enough of the SAG1 protein.

Protein assays revealed that each column run of 200μl of the tachyzoite extract resulted in a return of approximately one-tenth of the original protein in the peak containing SAG1, representing an enrichment of SAG1 in which 90% of contaminant proteins were removed. However, when this amount was scaled up for the total number of tachyzoites produced for this study, only approximately 3.75mg of protein could be obtained in total by gel filtration purification, and only a proportion of this protein would be SAG1. On past experience by a number of workers at the Moredun Research Institute (Edinburgh, UK) sheep normally generate a good serum antibody response to subcutaneous injections of 100-500μg of protein (J. Huntley, personal communication). However, the dose and frequency of stimulation has not been previously established for the optimal mucosal stimulation of sheep, and it was decided that 3 doses of 200μg per dose would provide a reasonable and practical regime for stimulation. After gel filtration purification, it

was apparent that insufficient SAG1 could be prepared for the proposed immunisation studies and the crude extract, albeit with a major SAG1 component, was encapsulated for immunisation. Other proteins in the extract may also be highly immunogenic, and the mixture of proteins in their native form may be more immunogenic than a single purified protein. SAG1 is one of the major proteins in the tachyzoite extract and has been shown to be the immunodominant protein in toxoplasma tachyzoites (Chardes *et al.*, 1990), and it is therefore likely that the majority of the immune response may be directed towards SAG1.

Microparticles prepared incorporating the toxoplasma tachyzoite extract were smooth and spherical, and the incidence of split or damaged particles was low. The majority of particles were below 1μm in diameter, and within the desired size range to facilitate M cell uptake. The toxoplasma tachyzoite antigen was encapsulated with high efficiency into the PLG microsphere delivery system that was developed in Chapter 3 and these particles are now ready to use in the proposed intranasal immunisation study. High encapsulation efficiencies, ranging between 60-91%, were demonstrated for this mixture of proteins, suggesting there had been high affinity for the polymer. The encapsulation efficiencies demonstrated for toxoplasma tachyzoite antigen were comparable to those published for other bacterial and viral antigens. For example, encapsulation efficiencies for Hepatitis B surface antigen were 80% (Singh *et al.*, 1997), for *Helicobacter pylori* whole lysates were between 62-75% (Kim *et al.*, 1999), for *Vibrio cholerae* were up to 97.8% (Yeh *et al.*, 2002a) and for Japanese encephalitis virus were up to 98% (Yeh *et al.*, 2002b). The encapsulation efficiency in the present study was approximately the same as for BSA, and much higher than OVA (see Chapter 3) and meant that little protein was lost during particle preparation. Most of the protein became encapsulated within the particles, with only approximately 20% remaining surface-bound. This 20% of the protein will be released rapidly from the particles following rehydration, but controlled release of the majority of protein will occur as the particles biodegrade.

Only surface-bound proteins could be visualised following separation by SDS-PAGE, clearly revealing the presence of SAG1 in small quantities on the surface of particles. Samples of encapsulated proteins did not seem to separate into distinct bands, likely to be due to the effects of the dissolved polymer in the sample.

Neutralising the pH did not further improve protein separation in these samples. Therefore it was not possible to determine whether certain proteins in the mixture were preferentially encapsulated or surface-associated. The surface bound proteins were clearly visible, and had a broad range of molecular weights, suggesting that protein size did not have an effect on association with the polymer. The presence of only low quantities of SAG1 on the surface suggested that the majority of SAG1 had been encapsulated into the microparticles; and the presence of cholera toxin did not seem to affect the constituent surface proteins. Western blot analysis confirmed that SAG1 was present both on the surface and encapsulated within the microparticles, and the intensity of antibody binding suggested that approximately equal amounts were surface-associated and encapsulated. The fact that SAG1 was recognised by the monoclonal antibody as a distinct band following encapsulation demonstrated that the conformation of the protein had been retained and suggested that the protein had remained antigenically intact, despite the harsh conditions of encapsulation. There is no method available to determine whether functional activity of encapsulated proteins was retained, but functionality is not necessary as long as proteins retain their antigenicity. The presence of cholera toxin, both on the surface and encapsulated within the particles, was clearly demonstrated with a dual labelling technique. Surface-associated cholera toxin may enhance particle uptake across the epithelium, and it is hoped that encapsulated cholera toxin will act as a mucosal adjuvant when it is presented to the mucosal immune system at the same time as the toxoplasma tachyzoite antigen.

These toxoplasma-containing microparticles will be used to deliver the toxoplasma tachyzoite antigen to the mucosal immune system of the sheep, in the hope of stimulating a protective immune response. The delivery system has been tailored to achieve M cell uptake and mucosal delivery of antigen in the ovine nasopharynx. As proteins are both surface-bound and contained within the particles, there should be an initial burst of protein release immediately after delivery. As the particles are processed and begin to degrade within the body further protein should be released to boost the immune system. The particles may thus have an intrinsic adjuvant effect, which may be enhanced by the presence of cholera toxin.

CHAPTER 5

Intra-nasal Immunisation of Sheep with Soluble and Particulate Forms of *Toxoplasma gondii* Tachyzoite Antigen

5.1 INTRODUCTION

The aim of the pilot immunisation study described in this chapter was to determine whether intranasal immunisation could stimulate antigen-specific mucosal and systemic immune responses. The model system used for this purpose was the zoonotic protozoan parasite *Toxoplasma gondii,* which is an important cause of abortion and neonatal mortality in sheep (Buxton, 1998). In addition, the consumption of poorly cooked infected sheep meat can lead to human infection (Jacobs *et al.*, 1960). *T. gondii* was chosen for this purpose because there are good assays available to monitor toxoplasma-specific immune responses, and a well-defined febrile response occurs following infection that can be used to assess whether these responses are protective (McColgan *et al.*, 1988).

Several studies in mice have shown that immunisation with whole *T. gondii* tachyzoite extracts, specific native antigens or excretory-secretory antigens can confer protection against tissue cyst or tachyzoite challenge as assessed by reduction in the number of brain cysts (reviewed in Jenkins, 2001). Immunity sufficient to reduce foetal mortality caused by *T. gondii* infection in sheep can be induced by a live attenuated vaccine (Buxton *et al.*, 1991), but attempts with killed vaccines have been essentially unsuccessful (Beverley *et al.*, 1971; Wilkins *et al.*, 1987). Most recently, attempts to immunise sheep with *T. gondii* antigens incorporated into ISCOMs have been shown to induce both humoral and cell-mediated responses. However these responses were not protective when sheep were infected with oocysts (Buxton *et al.*, 1989; Lunden, 1995), and an alternative to the live attenuated vaccine that currently exists for sheep remains highly desirable due to the short shelf-life and high production costs associated with the live vaccine. In addition, a successful killed vaccine in sheep would offer significant progress towards a human vaccine, since the use of a live attenuated vaccine in humans is unacceptable. An effective vaccine must stimulate protective immunity, which is thought to be due to a cytotoxic T cell response involving IFNγ (Khan *et al.*, 1994; Subauste & Remington, 1991).

Mucosal immunisation strategies against *T. gondii* have not been previously investigated in sheep, despite the fact that the natural route of infection occurs via the mucosal surfaces, and a vaccine that stimulates mucosal defences to control further infection may be highly effective. A number of studies in mice have shown that there

is potential to stimulate protective immunity via the mucosal route and that mucosal immunisation is a good way to stimulate both IgA and cell-mediated immunity against *T. gondii*. Initial studies demonstrated that oral immunisation with a toxoplasma sonicate combined with cholera toxin as a mucosal adjuvant induced both cell-mediated and humoral responses leading to approximately 50% protection assessed by a reduction in the number of cerebral cysts (Bourguin *et al.*, 1991, 1993; Chardes *et al.*, 1993). Since then the potential of intranasal vaccination has been assessed with promising results. Intranasal immunisation with SAG1 combined with CT protected mice against *T. gondii* (Debard *et al.*, 1996) with good correlation between the level of protection and the immune response in the intestinal mucosa. Protective immunity induced by this route was shown to be associated with a specific cellular response in both the NALT and the GALT compartments (Velge-Roussel *et al.*, 2000). In addition, non-toxic mutant *Escherichia coli* heat-labile enterotoxin (LT) as an adjuvant can induce protective immunity as good as the native toxin or CT when delivered with SAG1 via this route (Bonenfant *et al.*, 2001). All studies in mice have used soluble antigen for intranasal delivery, and it is hoped that by using a particulate antigen the immune response may be further improved.

The success of intranasal immunisation against *T. gondii* in mice indicates that investigation of the potential of this strategy in larger animals would be worthwhile. Sheep are a natural host, and in the future pregnant ewe studies may offer a useful model of the human infection. Toxoplasma tachyzoite antigen, containing a crude mixture of soluble tachyzoite proteins, has been successfully encapsulated into PLG nanoparticles and microparticles. These particles will be used as a delivery system to intranasally immunise sheep against *T. gondii*. Both humoral and cell-mediated responses will be assessed in reponse to immunisation, and compared to responses in sheep immunised with soluble toxoplasma tachyzoite antigen, negative control sheep or sheep infected with oocysts. The efficacy of cholera toxin as a mucosal adjuvant for the tachyzoite antigen will also be assessed. As part of the experiment all sheep will be challenged orally with *T. gondii* oocysts and the resulting febrile response used to determine whether immune responses induced by intranasal immunisation were protective.

5.2 MATERIALS AND METHODS

5.2.1 Animals

5.2.1.1 Sheep

Twenty-four entire male Scottish Blackface hoggs, aged approximately 6 months - 1 year old, were obtained from Moredun's Firth Mains farm (Edinburgh, UK). Sheep were shown to be seronegative by ELISA for IgG antibodies to *T. gondii* and non-responsive to toxoplasma antigen in lymphocyte stimulation assays. Animals were housed under conventional conditions for the duration of the trial and fed on concentrates and hay and water ad lib. Animal in each treatment group were housed in separate pens.

5.2.1.2 Cats

Two male cats, under 6 months of age and seronegative for antibodies to *T. gondii* were obtained from an approved supplier, and used to obtain viable *T. gondii* oocysts of the Moredun M3 isolate. Cats were housed using barrier containment in an SPF facility to minimise contamination of the environment with infective oocysts.

5.2.2 *Toxoplasma gondii*

5.2.2.1 Moredun M3 Isolate

The M3 isolate of *T. gondii* was originally isolated at the Moredun Research Institute (Edinburgh, UK) on 25th April 1986 by the intraperitoneal (i.p.) inoculation of 2 female Swiss White mice with the brain of a stillborn lamb. It was passaged for the first time 3 months later, and maintained by once yearly passage in Porton mice (each passage was an intraperitoneal inoculation of brain homogenate containing 40-100 tissue cysts). The brains were aseptically removed and homogenised in Hank's balanced salt solution (HBSS) (Gibco, Invitrogen, Paisley, UK) by passage through a 16 gauge (G) needle. The M3 isolate was chosen for this project because it is a complete isolate, capable of causing clinical toxoplasmosis in sheep, and M3 tissue cyst bradyzoites can induce oocyst formation in seronegative cats. Non-pregnant

sheep, dosed orally with M3 oocysts, develop a very predictable febrile response to provide a convenient model system for measuring the efficacy of a vaccine. This isolate was used experimentally for the first time by Buxton *et al.* (1991).

5.2.2.2 M3 Tissue Cysts

Brains from four chronically infected Porton mice were removed and homogenised in an equal volume of PBS using a 16G needle and syringe. Five 5μl aliquots of this suspension were spotted onto a microscope slide and tissue cysts were counted in each spot. Suspensions containing an estimated total of 10^3 M3 *T. gondii* tissue cysts were prepared.

5.2.2.3 M3 Oocysts

Each cat was given an estimated 10^3 M3 *T. gondii* tissue cysts mixed in a small amount of food. Thereafter faeces were collected daily and screened for the presence of oocysts. Oocyst shedding normally commences 5 days post-infection, and continues until day 10. Sporulated oocysts were obtained from infected cat faeces using the Saturated Salt Flotation Technique described by Buxton *et al.* (1988). Briefly, infected faeces were diluted 1:10 in water, homogenised thoroughly and the resulting suspension sieved through a 1mm mesh. 50ml aliquots were centrifuged for 10 minutes at 1000×*g*, the supernatant was discarded, and the pellet resuspended in 50ml of saturated sodium chloride. After the mixture was centrifuged at 1000×*g* for 10 minutes, the meniscus was removed (approximately 5ml) and diluted 1:10 with water and centrifuged for a further 10 minutes at 1000×*g*. The pellet was resuspended in a small volume of water and oocysts were counted in an improved Neubauer haemocytometer. Oocysts were allowed to sporulate in 2% sulphuric acid at 22°C for 7 days and then stored at 4°C until required. The final suspension contained 15000 oocysts in 2ml sulphuric acid, 50% of which were viable. Prior to use the oocysts were pelleted at 500×*g* for 5 min and resuspended in PBS.

5.2.2.4 Titration of M3 Oocyst Dose

The 4 sheep to be given an oral infection of oocysts were housed in a separate pen (for experimental design see Section 5.2.5). Two animals were given a dose of 200 oocysts and 2 animals were given a dose of 500 oocysts. For dosing, a gag was used to open the mouth and depress the tongue and a tube was inserted down the oesophagus into the rumen. A 2-way syringe was used to administer a 2ml aliquot of oocysts into the stomach, which was then washed down with 50ml water. This system is demonstrated in Figure 5.1. Rectal temperatures were recorded with a clinical electronic thermometer in the sheep for the 2 days prior to any inoculation and for 14 days following inoculation. Fever was defined as a temperature of 40°C or higher.

These animals were also used as positive control animals for the immunisation study. They were bled prior to dosing and weekly thereafter. Blood collected from these animals was used to establish T cell proliferation assays (including titration of antigen) and isotype-specific ELISAs. Approximately 8 weeks after initial oocyst infection the animals were challenged with oocysts again to provide a measure of secondary response to re-infection with *T. gondii*.

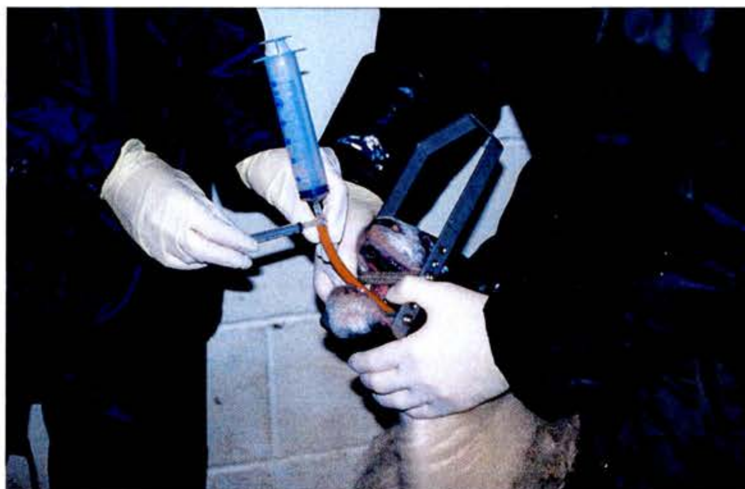


Figure 5.1 *Delivery of an oocyst infection*

5.2.3 Intranasal Vaccine Spray

The intranasal spray used to deliver the vaccine was optimised to deliver a liquid to the appropriate area of the ovine nasopharynx, and is demonstrated in Figure 5.2. An Arnolds® dog catheter tube with female luer mount (2.6mm × 50cm) (SIMS Portex Ltd, UK) was reduced to approximately half the original length. The end of the tube was sealed with a small amount of Araldite® (Bostik Ltd., Leicester, UK) and left to dry overnight. Five small holes were punched on one side of the sealed end of the tube with a 16G needle (Microlance®, Becton Dickinson, Dublin, Ireland).

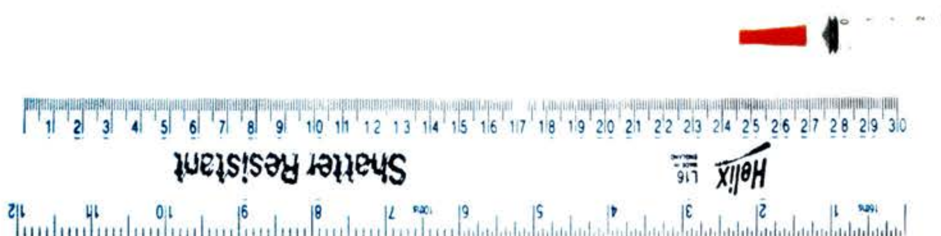


Figure 5.2 *Delivery system for intra-nasal vaccination in sheep*

Distances of 15, 16 and 17cm from the holes were marked on the tube with a permanent marker so that depth of insertion into the nasal cavity could be measured. A 6ml syringe with a male Luer lock (Monoject®, Sherwood Medical, Northern Ireland) was attached to the corresponding female Luer lock on the tube. Before use the spraying capacity of each tube was assessed using water. To apply the vaccine the tubing was inserted to a depth of 16cm into the nasal cavity with the holes facing away from the septum, and 1ml of the appropriate vaccine solution from the syringe was applied to each nostril. Prior to the experiment the delivery system was tested on several detached sheep heads. The spray was inserted to a depth of 16cm into the nasal cavity and 1ml of a violet coloured dye (Serva Violet 17, Invitrogen, Paisley, UK) was sprayed into each nostril using the delivery system. The heads were then sagitally Sectioned and examined to determine the location of the dye in the nasopharyngeal tract. Intra-nasal delivery is demonstrated in Figure 5.3.

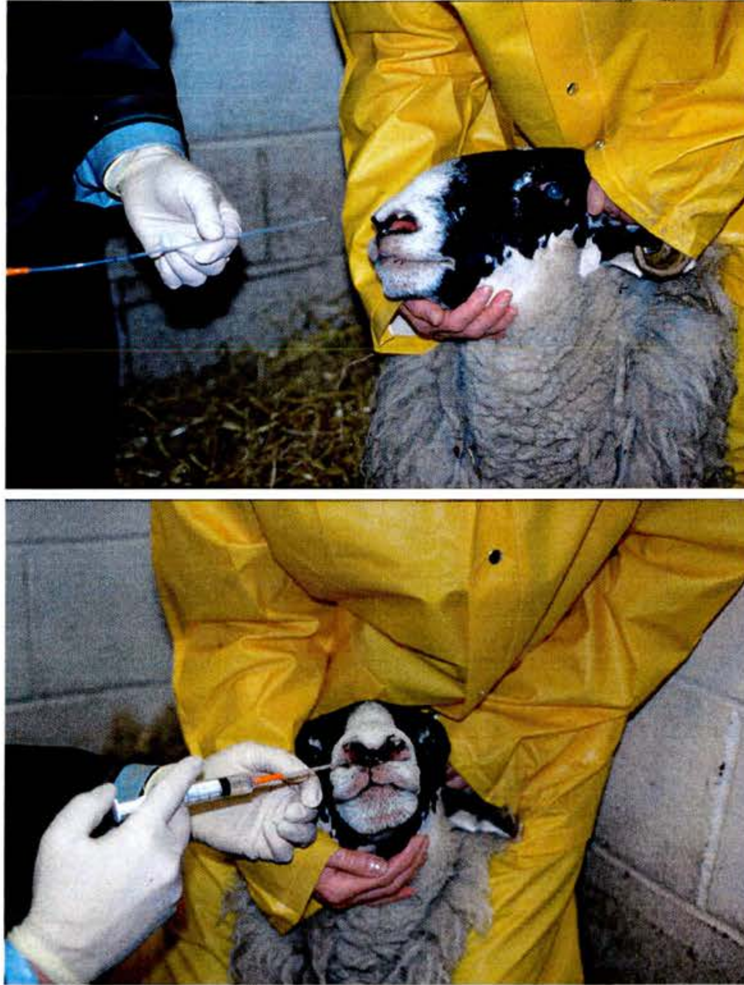


Figure 5.3 *Intranasal vaccine delivery to sheep*

5.2.4 Antigens

Toxoplasma tachyzoites were produced and lysed as described in Section 4.2.2.3 to provide a soluble extract of tachyzoite proteins. The protein concentration of a proportion of this extract was adjusted to 0.2mg/ml for use as soluble antigen. The remaining tachyzoite extract was concentrated and encapsulated into PLG microparticles as described in Section 4.2.5.1. A 20:1 ratio of cholera toxin was also included with the tachyzoite extract in half of these particles as described in Section 4.2.5.2. Finally PLG particles containing no protein were prepared as detailed in Section 4.2.5.3 for delivery to negative control sheep.

5.2.5 Experimental Design

The first treatment group, consisting of 4 sheep, were given an oral challenge of oocysts (known as group 5) (see Section 5.2.2.4). The remaining 20 sheep were randomly assigned into the groups detailed below. The individual animals in each group and the dates of vaccination and challenge are shown in Table 5.1 overleaf.

1. Four sheep were given control inoculum. For each immunisation each animal received 5mg of blank particles suspended in 1 ml of water into each nostril.
2. Eight sheep were dosed intranasally with soluble toxoplasma tachyzoite antigen. For each immunisation, each animal received 100μg of toxoplasma antigen in a volume of 1ml into each nostril.

3. & 4. Eight sheep were dosed intranasally with toxoplasma tachyzoite antigen encapsulated into the microparticulate delivery system, the preparation of which is detailed in Chapter 4. For each immunisation, each animal received particles containing 100μg of protein suspended in 1ml of water into each nostril. The total protein content of the microparticles (in μg/mg particles) was used to calculate the weight of each batch of particles that would contain a dose of 200μg of protein, as detailed in Table 5.2. The particles were suspended in 2ml water immediately prior to delivery to the animals. This group was sub-divided as follows:

3. Four sheep given microparticles incorporating toxoplasma antigen

4. Four sheep given microparticles incorporating toxoplasma antigen and CT

Batch	Particle Weight		Batch	Particle Weight
Toxo 1	13.8mg		Toxo + CT 1	9.2mg
Toxo 2	10.8mg		Toxo + CT 2	11.0mg
Toxo 3	9.5mg		Toxo + CT 3	9.2mg

Table 5.2 *Weight of each batch of PLG microparticles that contained 200μg of toxoplasma tachyzoite protein antigen*

Animals were immunised 3 times two-weeks apart. Each vaccine dose contained 200μg antigen (100μg delivered into each nostril). All animals were monitored for a further 4 weeks after the final vaccine dose and were then each dosed orally with 200 oocysts. Animals that were initially infected with oocysts received a second dose at week 8 post initial infection. Immune responses were monitored in all animals for a further 2 weeks post-challenge and then euthanised.

Group	Animals	n	Treatment	1st dose	2nd dose	3rd dose	Oocyst ⊗ challenge	Post-Mortem Examination
1. Negative Control	913N	4	3 x i.n. doses of 10mg blank PLG microparticles	12APR02	26APR02	10MAY02	11JUN02	25JUN02
	915N							25JUN02
	950N							24JUN02
	1048N							24JUN02
2. Soluble toxoplasma antigen	638N	8	3 x i.n. doses of 200μg of soluble toxoplasma antigen	12APR02	26APR02	10MAY02	11JUN02	26JUN02
	748N							25JUN02
	919N							24JUN02
	923N							26JUN02
	993N							25JUN02
	1031N							24JUN02
	1034N							26JUN02
	1117N							26JUN02
3. Particulate toxoplasma antigen	715N	4	3 x i.n. doses of particulate toxoplasma antigen	12APR02	26APR02	10MAY02	13JUN02	27JUN02
	739N							28JUN02
	764N							28JUN02
	1067N							27JUN02
4. Particulate toxoplasma antigen + cholera toxin	770N	4	3 x i.n. doses of particulate toxoplasma antigen + CT	12APR02	26APR02	10MAY02	13JUN02	28JUN02
	989N							27JUN02
	1030N							27JUN02
	1116N							28JUN02
5. Oocyst challenge	927N	4	1 x dose of toxoplasma oocysts *	21MAR02	-	-	16MAY02	31MAY02
	976N							31MAY02
	1032N							31MAY02
	1084N							31MAY02

Table 5.1 *Details of animals used in immunisation studies and timings of vaccine doses, oocyst infections and post-mortems*

* Either 200 (to two) or 500 (to two) sporulated oocysts orally ⊗ 200 sporulated oocysts orally

5.2.6 Collection of Samples

Blood samples and nasal secretions were collected from each animal prior to immunisation and weekly thereafter.

5.2.6.1 Serum

Approximately 7ml of blood was collected from the jugular vein of each sheep into preservative-free evacuated blood collection tubes, (Vacutainer, Becton Dickinson, Oxford, UK) and allowed to clot. Following retraction of the clot, serum was obtained after centrifugation at 3000rpm (2060 × *g*) for 30 min and stored at –20°C until required.

5.2.6.2 Peripheral Blood Mononuclear Cells (PBMCs) from Whole Blood

Approximately 10-14 ml of blood was collected from the jugular vein of each sheep into preservative-free heparinised evacuated blood collection tubes (Vacutainer, Becton-Dickinson, Oxford, UK) and mixed well.

5.2.6.3 Nasal Secretions

Nasal secretions were collected using non-applicator tampons (Lil-lets, Accantia, Birmingham, UK). Regular size tampons were cut into 4 equal sized pieces, and one of these pieces was inserted into the nostril of each sheep for approximately 15min, before it was removed and the secretions squeezed out using a 5ml syringe. Nasal secretions were stored at -20°C.

5.2.6.4 Tissue Collection at Post-Mortem Examination

Retropharyngeal (RP LN), mediastinal (Med LN), pre-femoral (P-F LN) and mesenteric lymph nodes (MLN), pharyngeal tonsil (PT) and a piece of spleen were collected using aseptic precautions immediately after euthanasia and placed in wash medium, consisting of HBSS supplemented with 2% heat inactivated foetal bovine serum (FBS) (Labtech International, Sussex, UK), 100 U/ml penicillin and 100μg/ml streptomycin (Gibco, Invitrogen, Paisley, UK). The areas these lymph nodes drain are detailed in Table 5.3.

Lymph Node	Draining From
Retropharyngeal	NALT
Pharyngeal Tonsil	-
Mediastinal	Lungs
Mesenteric	Gut
Pre-femoral	Unrelated area
Spleen	Systemic IS

Table 5.3 *Lymph nodes collected and regions they drain*

Small areas (approximately 1cm^2) of the following tissues were also collected from each animal: NALT, pharyngeal tonsil, trachea, bronchus, lung, abomasal fold, duodenum, jejunum, ileum, Peyer's patch, large intestine, rectum, spleen, mesenteric lymph node. The mucosa was scraped off the following tissues: duodenum, jejunum, ileum, large intestine, rectum. Epithelium was dissected away from the cartilage of the trachea. All tissues were stored separately at -20°C until required.

5.2.6.5 Tissue Homogenisation

Tissues were thawed at room temperature for 1 h, weighed and suspended in 1:5 ratio (w/v) of PBS containing 0.5M NaCl and 0.5% Tween 80® (Sigma, Dorset, UK). They were then homogenised at 10,000rpm (Cat Homogenizer X1030D, M. Zipperer GmbH, Staufen, Germany) for approximately 30 sec until fully disrupted. This suspension was then centrifuged at 3,000rpm (2060 × *g*) for 15 min to remove all remaining tissue. The supernatant was collected and stored at -20°C until use.

5.2.7 Humoral Immune Response

Antibody levels were measured using the enzyme-linked immunosorbent assay (ELISA). Prior to the experiment the checkerboard technique was used to optimise the concentrations of antigen, serum and antibodies in each isotype-specific ELISA, using a *T. gondii* specific positive serum sample.

5.2.7.1 ELISA for Antigen-Specific IgG Antibody

96 well ELISA plates (M129B, Greiner Laborotechnik, Dursley, UK) were coated with 50μl per well toxoplasma tachyzoite antigen at a concentration of 1μg/ml in 0.1M carbonate buffer pH 9.6 at 4°C overnight. Plates were washed twice with wash buffer, consisting of PBS containing 0.05% Tween 20® (Sigma, Dorset, UK), then non-specific binding sites were blocked for 30 min at RT with 50μl per well PBS containing 0.5M NaCl and 0.5% Tween 80® (Sigma, Dorset, UK) and washed again. Each serum sample was diluted serially to 1:10, 1:50, 1:100, 1:500, 1:1000, 1:4000, 1:16000 and 1:64000. Nasal secretions were diluted 1:2 and tissue homogenates were diluted 1:100. Dilutions were prepared in PBS containing 0.5M NaCl and 0.5% Tween 80® and 50μl/well of samples, controls or buffer alone were added to the plate in duplicate. The plates were incubated for 1 h at RT, washed 3 times in wash buffer, then incubated for a further 1 h at RT with 50μl per well primary antibody diluted to 1:4000. The monoclonal antibody was VPM6 (Bird *et al.*, 1995) specific for the heavy chain of IgG. Plates were washed 3 times in wash buffer, and incubated with 50μl per well of a 1:2000 dilution of a biotinylated goat anti-mouse secondary antibody (Dako, Ely, UK). Plates were washed 3 times in wash buffer, and incubated for 1 h at RT with 50μl per well of a 1:5000 dilution of streptavidin conjugated to HRP (Dako, Ely, UK). All dilutions were prepared in PBS containing 0.5M NaCl and 0.5% Tween 80®. The plates were washed as before and the colour reaction was developed with 50μl per well of orthophenylene diamine (OPD) substrate solution (Sigma, Dorset, UK) following the manufacturer's recommendations. Colour was allowed to develop for approximately 10 min, before being terminated by the addition of 25μl per well 2.5M H_2SO_4. The optical density (OD) of the reaction mixture was measured using a MRX Microplate reader (Dynex Technologies, Billingshurst, UK) equipped with a 492nm filter. Samples were unified to the positive control to control for between plate variation using the following equation:

$$\frac{\text{Mean OD sample}}{\text{Mean OD positive control}}$$

The results were either expressed as OD@492nm for serum samples at a dilution of 1:100, or the titre at which the 50% maximum/minimum OD was produced.

5.2.7.2 ELISA for Antigen-Specific IgG1 and IgG2 Antibody

Comparison of IgG1 and IgG2 antibody levels was performed on serum and nasal samples at week 2 post-infection, when high IgG antibody titres could be measured. The method used was as detailed in Section 5.2.7.1. The primary antibody used was a 1:25 dilution of a rat anti-ovine IgG1 or IgG2 (1RSh1 and 2RSh2, kindly gifted by J. Hopkins, University of Edinburgh).

5.2.7.3 ELISA for Antigen-Specific IgA Antibody

Alternate rows of 96 well ELISA plates (M129B, Greiner Laborotechnik, Dursley, UK) were coated with 50µl per well toxoplasma tachyzoite antigen at a concentration of 1µg/ml in 0.1M carbonate buffer pH 9.6 at 4°C overnight. Control rows were coated with carbonate buffer alone to control for non-specific background binding. Plates were washed twice with PBS containing 0.05% Tween 20® (wash buffer), then blocked for 30 min at RT with 50µl per well PBS containing 0.5M NaCl and 0.5% Tween 80®. Each serum sample was diluted serially to 1:2, 1:5, 1:10, 1:20, 1:50, 1:100 and 1:1000. Nasal secretions were diluted 1:50 and tissue homogenates were diluted 1:10 in PBS containing 0.5M NaCl and 0.5% Tween 80®. Plates were washed twice with wash buffer and 50µl/well of each sample were added to the plate in quadruplicate (2 in wells that had been coated with antigen and 2 in wells without antigen). A positive control sample was also included. The ELISA was then carried out as described in Section 5.2.7.1, using a 1:500 dilution of monoclonal anti-ovine/bovine IgA (K84.2F9, Serotec, Oxford, UK) as a primary antibody. The OD value for wells that contained no antigen was subtracted from the OD value for antigen specific binding in wells that contained antigen. Samples were unified to the positive control.

5.2.7.4 ELISA for Antigen-Specific IgE Antibody

The method was adapted from the IgG ELISA method described in Section 5.2.7.1. Sera were diluted 1:10 and nasal secretions were diluted 1:2. The primary antibody was a 1:500 dilution of the mouse anti-ovine IgE (2F1, Winden *et al.*, Veterinary Immunology & Immunopathology, in press).

5.2.8 Immunoblotting to Determine Antibody Specificity

Samples containing 20µg of the original tachyzoite extract were separated in 12% non-reducing gels and blotted as detailed in Section 4.2.3.1 and 4.2.3.3. Western blots were probed with serum from animals as detailed in Tables 5.4 and 5.5. These sera were chosen on the basis of a strong IgA or IgG antibody response by ELISA to tachyzoite antigen, and include day 0 samples as negative controls.

Animal	Serum	Dilution (1 in)	Response
770N	week 0	100	-ve control
Group 4	week 5	100	post-immunisation
(toxo+CT particle)	week 11	1000	post-infection
739N	week 0	100	-ve control
Group 3	week 4	100	post-immunisation
(toxo particle)	week 11	1000	post-infection
1032N	week 0	1000	-ve control
Group5 (oocyst)	week 4	1000	post-infection

Table 5.4 *Serum used to probe blots for antigen-specific IgG*

Animal	Serum	Dilution (1 in)	Response
770N	week 0	100	-ve control
Group 4	week 3	100	post-immunisation
(toxo+CT particle)	week 11	100	post-infection
715N	week 0	100	-ve control
Group 3	week 3	100	post-immunisation
(toxo particle)	week 11	100	post-infection
1084N	week 0	100	-ve control
Group 5 (oocyst)	week 2	100	post-infection

Table 5.5 *Serum used to probe blots for antigen-specific IgA*

Following extensive washing of the blot, bound antibody was visualised by a further 1 h incubation in either a 1:500 dilution of the monoclonal mouse anti-IgA antibody or a 1:2000 dilution of the monoclonal mouse anti-IgG antibody in wash buffer. The blots were then washed thoroughly and incubated with a 1:2000 dilution of a biotinylated goat anti-mouse antibody (Dako, Ely, UK) followed by further washing and a 1 h incubation in a 1:2500 dilution of streptavidin conjugated to HRP (Dako, Ely, UK). After a final wash HRP was detected by treating blots with the enhanced chemiluminescence (ECL) reagent (Amersham International, Little Chalfont, UK) according to the manufacturer's instructions, and exposure to Hyperfilm ECL (Amersham International, Little Chalfont, UK) for approximately 5 sec before development.

5.2.9 Cell-Mediated Immune Response

5.2.9.1 Preparation of PBMCs

Blood samples were diluted 1:2 in approximately 20ml of cold sterile PBS and centrifuged for 20 min at 450×*g* in a CR422 Jouan centrifuge. The interface layer (buffy coat) was removed using a sterile glass pipette and diluted in 2ml of wash medium, consisting of HBSS (Gibco, Invitrogen, Paisley, UK) supplemented with 2% heat inactivated foetal bovine serum (FBS) (Labtech International, Sussex, UK), 100 U/ml penicillin and 100μg/ml streptomycin (Gibco, Invitrogen, Paisley, UK). The cell suspension was gently layered over Lymphoprep (Nycomed, Solihull, UK) in a 15ml conical centrifuge tube and centrifuged at 550×*g* for 30 min. PBMCs were collected from the interface, washed three times in wash medium and resuspended in culture medium, consisting of Iscove's modified Dulbecco's medium (IMDM) (Gibco, Invitrogen, Paisley, UK) supplemented with 10% FBS (Labtech International, Sussex, UK), 100U/ml penicillin and 100μg/ml streptomycin. A small aliquot of cells was stained with 0.1% nigrosin and counted in a haemocytometer. The cell concentration was adjusted to 2×10^6cells/ml for the assay.

5.2.9.2 Preparation of Cells from Lymph Nodes

The lymph nodes collected post mortem were transferred into 5ml of wash medium in a 60mm petri dish, then trimmed and finely chopped with a scalpel blade. This suspension was transferred to a stomacher bag and processed in a Stomacher 80 (Camlab Ltd., Cambridge, UK) for approximately 10 sec. The disrupted tissue was filtered to remove any large lumps. Cells in this suspension were counted as described above and the cell concentration was adjusted to 2×10^6cells/ml for the assay.

5.2.9.3 Cell Proliferation Assay

Cells were cultured with toxoplasma tachyzoite antigen at a concentration of 1μg/ml and 2μg/ml, based on preliminary titration experiments. Positive control cells were cultured with Concanavalin A (Con A) (ICN Biochemicals, Cleveland, Ohio, USA) at a final concentration of 5μg/ml. Con A, the lectin of the jack bean

(*Canavalia ensiformis*), was used as a positive control for the test, as it has been described to selectively stimulate T lymphocytes (Janossy & Greaves, 1971; Rouse & Babiuk, 1974). In contrast negative control cells were cultured with either culture medium alone or Vero African green monkey (*Cercopithecus aethiops*) kidney cells at a concentration of 1μg/ml. Equal volumes of cells and antigen (100μl of each) were set up in quadruplicate in 96 well round-bottomed tissue culture plates (Nunc, Roskilde, Denmark) at 37°C in a humidified 5% CO_2 incubator for 5 days. Cells were pulsed for the final 18h with 18.5 kBq [^{3}H]-thymidine (Amersham Bioscience, Bucks, UK) per well before harvesting onto fibreglass filters (Canberra Packard, Meriden, Connecticut, USA). Cell-associated radioactivity was quantified in a gas proportional counter (beta scintillation counter) (Canberra Packard, Meriden, Connecticut, USA).

5.2.9.4 Determination of Stimulation Index

The results reported as counts per minute (cpm) are the median value of quadruplicate cultures. The differential incorporation of [^{3}H]-thymidine between treated and untreated cultures was used as a measure of proliferation expressed as stimulation index (SI):

$$SI = \frac{\text{cpm of test culture}}{\text{cpm of medium control culture}}$$

5.2.9.5 Interferon Gamma (IFNγ)

Cultures were set up as described above for cell proliferation assays and cell-free supernatants were collected after 5 days of stimulation. The supernatants were stored at -20°C prior to testing for the presence of IFNγ using an ELISA kit (CSL Veterinary, Parkville, Australia), a sandwich enzyme immunoassay capable of detecting less than 50pg/ml of recombinant IFNγ. ELISAs were performed as described by the manufacturer's protocol. Briefly, samples were incubated on microtitre plates coated with antibody to IFNγ for 60 min at RT, and unbound material was removed by extensive washing. Plates were incubated in HRP labelled anti-bovine IFNγ for 60 min at RT and washed as above. Plates were then incubated with enzyme substrate containing H_2O_2 for 30 min at RT. The reaction was stopped

with 0.5M H_2SO_4 and the amount of colour development was estimated spectrophotometrically at 450nm.The ELISA was calibrated using standards of recombinant bovine IFNγ in concentrations of 2000pg/ml to 10pg/ml. A regression line was calculated and pg/ml of IFNγ present in each test sample was determined from the standard curve.

5.2.10 Statistics

If necessary a small constant value was added to each data point for a measured parameter to make all values positive. Data were transformed logarithmically to ensure that observations within each group had an approximately normal distribution with a common variance. Data were analysed firstly for weeks 0-9 to compare the responses produced to immunisation with those produced to oocyst infection, and secondly for the 2 weeks post-infection with oocysts.

A mixed model was fitted to the data using treatment group and week of experiment (time) as fixed factors and individual animals within each group as a random factor. The observations for individual animals over time may have been correlated because the data are of the repeated measures type, hence the correlation structure was modelled by fitting an auto-regressive correlation of order 1. The model was fitted using restricted maximum likelihood (REML) with a statistics package (Genstat for Windows, 6th ed.). The significance of fixed effects (group, time and the interaction between group and time) from the analysis of variance (ANOVA) was assessed using the Wald statistic, and a P value of less than 0.05 was regarded as significant. For variables measured at one time period only (e.g. tissues collected at post-mortem), one-way ANOVAs were performed to examine between group differences, and these were further analysed using the Tukey's test statistic (Minitab version 13). Finally, sheep were classified as responders or non-responders to each treatment on the basis of measurements over the first nine weeks for each parameter. The proportion of animals responding and not responding to the treatment in each group was compared using the Fisher's Exact Test.

5.3 RESULTS

5.3.1 Preliminary Results

5.3.1.1 Titration of Oocyst Dose

The mean rectal temperatures for animals that received infective doses of either 200 or 500 toxoplasma oocysts from the day prior to infection to day 14 post-infection are demonstrated in Figure 5.4. Values for individual animals are shown in Appendix 5.1. There was no difference in the febrile response produced to infection with 200 or 500 oocysts (Wald=7.43, d.f.=15, P=0.945). All animals produced the classic temperature profile to infection with *T. gondii*, with a fever lasting from day 5 to day 9 post-infection (McColgan *et al.*, 1988; Buxton *et al.*, 1991), at levels significantly higher than baseline values (Wald=229.92, d.f.=15, P<0.001). The lower oocyst dose was used for all future challenge infections. There were no differences between animals infected with 200 and 500 oocysts for any other parameters measured.

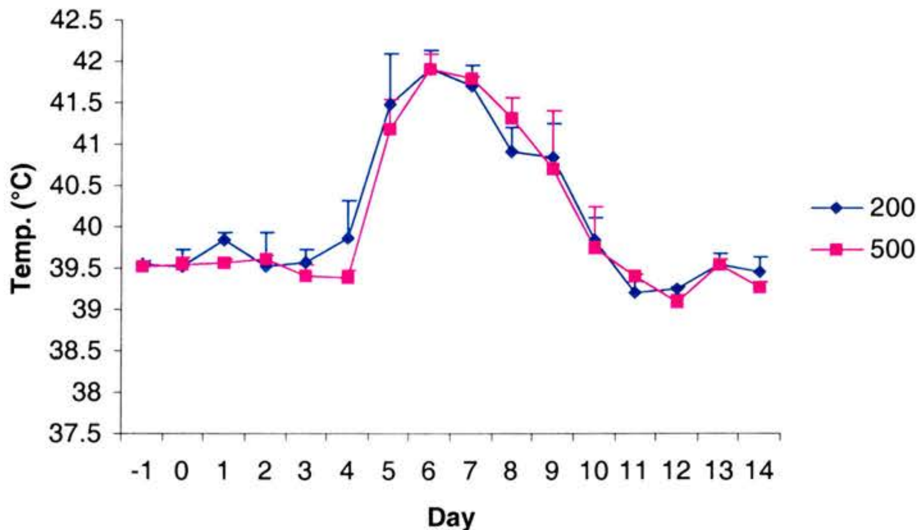


Figure 5.4 *Mean rectal temperatures (±sem) of animals given an infective dose of either 200 or 500 toxoplasma oocysts from the day prior to dosing to day 14*

5.3.1.2 Area Targeted by Intra-Nasal Spray

The intra-nasal spray was effective at targeting the area of the nasopharynx that contained NALT and the pharyngeal tonsil when inserted to a depth of approximately 16cm into the nasal cavity, as demonstrated in Figure 5.5.

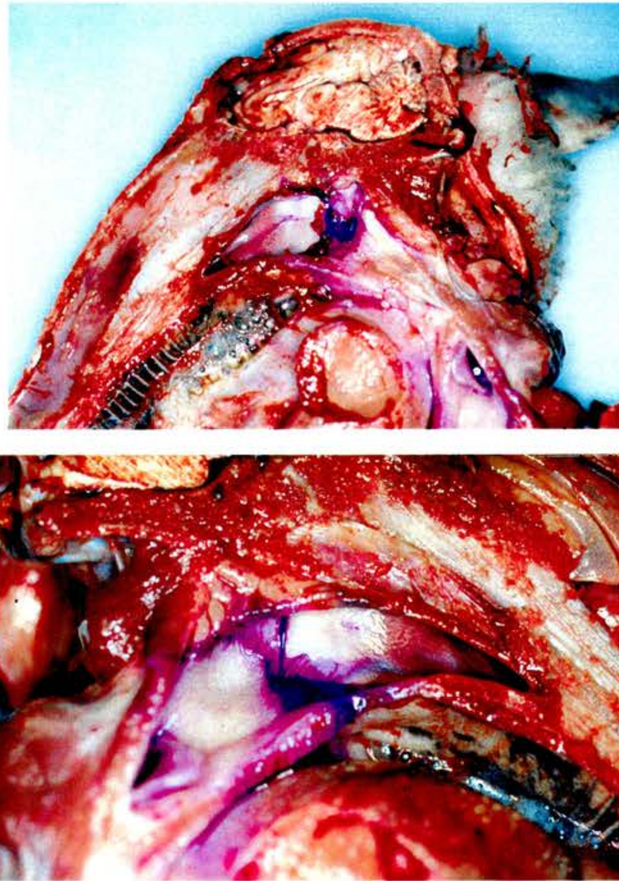


Figure 5.5 *Location of Serva Violet 17 dye after 1ml was delivered at a depth of 16cm in the nasopharyngeal tract of 2 sheep using the intranasal spray*

5.3.2 Clinical Response to Oocyst Infection

5.3.2.1 Clinical Observations

The first clinical signs occurred in the first week post-infection, when all animals displayed an increase in respiratory rate, mild anorexia and a febrile response. Animals in each treatment group fully recovered from the infection, apart from animal 1048N in the control group (immunised with particles containing no

protein) which developed the clinical symptoms of acute toxoplasmosis at day 10 post-infection. This animal had a febrile response that was maintained at or around 41°C for the remainder of the experiment. This animal appeared lethargic, weak, maintained a raised respiratory rate, and had a slight noticeable tremor and slowed reflexes in its hind legs. At post-mortem examination ascitic fluid was noted in the peritoneal cavity, and the brain and spinal cord were collected into formol saline for histological examination. The pathology report showed that the animal had a widespread non-suppurative meningoencephalitis. The inflammation consisted of perivascular lymphoid cuffs in the cerebrum with associated focal meningitis and small scattered foci of microgliosis. These findings are consistent with those seen in protozoal infections such as toxoplasmosis.

5.3.2.2 Temperature Response

Rectal temperature data were analysed using the mean temperature values for each group prior to infection, as baseline values. All five groups of animals showed rectal temperatures below 40°C prior to infection. In naïve animals the mean febrile response lasted for an average of 4-5 days with values above 41°C. Daily temperatures of individual animals are displayed in Appendix 5.2. The mean rectal temperatures recorded from each treatment group for the 14-day period following oral infection are demonstrated in Figure 5.6.

Statistical analysis of the temperature response revealed that there were highly significant differences in the febrile responses observed between treatment groups over time (Wald=306.40, d.f.=60, $P<0.001$). Naïve animals that were orally infected with oocysts produced the characteristic febrile response that has previously been described in sheep (McColgan *et al.*, 1988; Buxton *et al.*, 1991). Fever began at day 5 and lasted until day 9 post-infection, when levels began to slowly drop back down to baseline levels. Following re-infection an increase in temperature to just over 40°C was measured in animals on day 3 post-infection. Temperatures dropped back to baseline values the next day where they remained for the rest of the experiment. After day 3 temperatures were significantly lower than in all the other treatment groups until day 11 post-infection.

A similar febrile response to primary infection was measured in animals in the control group and animals immunised with soluble toxoplasma tachyzoite antigen. A fever was observed from day 5 to day 9 post-infection, after which time the mean temperature returned progressively to baseline values by day 11 or 12 post-infection. In both groups immunised with particulate toxoplasma antigen fever occurred earlier and temperature returned to normal more quickly than in the negative control group. Animals immunised with particulate toxoplasma antigen alone had a fever at day 4 to day 8 post-infection, and temperature returned to baseline at day 11. Animals in the group immunised with particulate antigen and cholera toxin produced a temperature response to infection that contained elements of the response observed in both naïve and immune animals. A slight increase in the mean temperature to just over 40°C was observed on day 2 post-infection in three out of the four animals in the group significantly higher than the baseline values. Temperatures returned to baseline the following day and then increased markedly on day 4. A fever was measured from day 5 to day 8 post-infection. The response in the animal in this group that did not show an early rise in temperature was very similar to the negative control animals, while the temperatures of the other three animals were not as high as those recorded in the negative control and the soluble-antigen group, and they also returned to baseline values sooner.

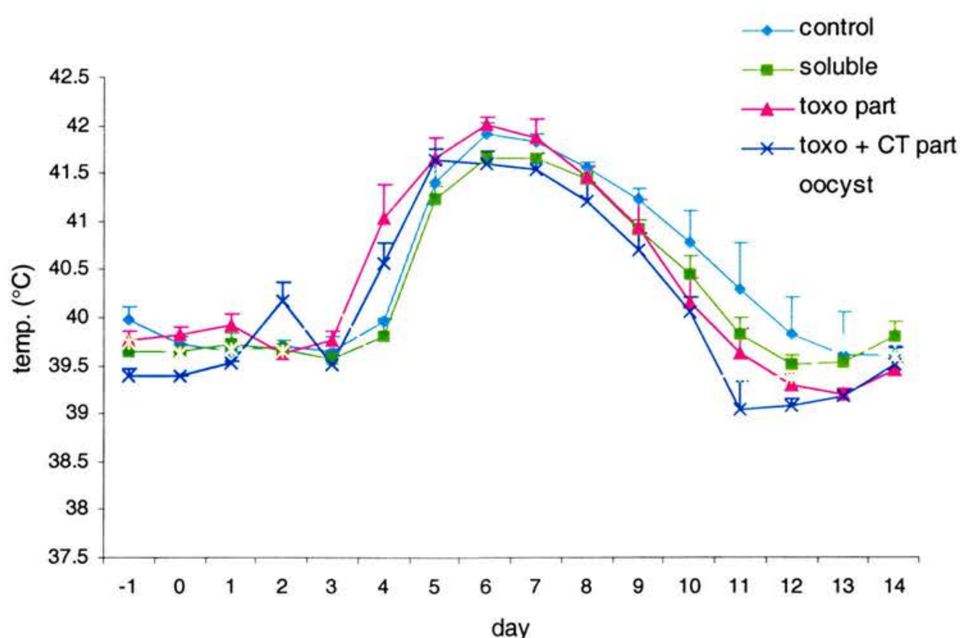


Figure 5.6 *Mean rectal temperatures (±sem) of animals in the different treatment groups in response to an oral infection of toxoplasma oocysts from the day prior to infection to day 14 post-infection*

5.3.3 IgA Antibody Responses

5.3.3.1 Antigen-Specific IgA Antibody in Sera

When sera were titrated no maximum OD values for antigen-specific IgA could be demonstrated, suggesting that only low levels of IgA were present. Therefore the titre at which 50% maximum/minimum OD was produced was not a valid measurement to use, and the following results are presented as the OD@492nm produced for sera at a dilution of 1:10. The mean weekly ODs for antigen-specific serum IgA antibody in each treatment group are demonstrated in Figure 5.7. The results for individual animals are detailed in Appendix 5.3. A sample that produced an OD above 0.1 was considered to be positive. There was a high degree of within group variation in serum IgA levels, and a small number of animals showed high background levels of serum IgA despite efforts to control for this on ELISA plates.

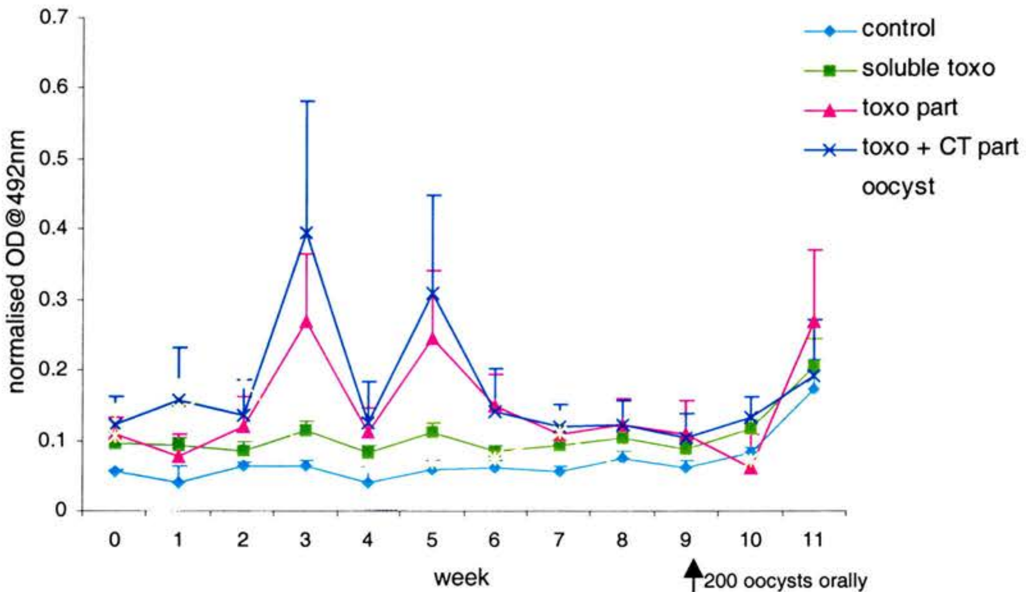


Figure 5.7 *Mean levels of antigen-specific serum IgA antibody (±sem) for each treatment group*

An infective dose of oocysts in naïve animals elicited only a slight increase in levels of antigen-specific IgA antibody in the serum, with peak levels observed at week 2 post-infection, when all four animals had a positive OD above 0.1. Serum IgA ODs quickly dropped down to baseline again after this initial increase for the remainder of the experiment. Re-infection with oocysts 8 weeks later resulted in no

further increase in serum IgA levels. The control animals, immunised with blank particles, showed no increase in serum IgA levels for the duration of the trial. The group immunised with soluble toxoplasma proteins showed only a very small mean increase in serum IgA that was not significantly different from the baseline values shown in the control group of animals.

A marked antigen-specific serum IgA response could be demonstrated in both groups of animals immunised with particulate toxoplasma tachyzoite antigen, characterised by peak IgA levels at weeks 3 and 5, significantly higher than those observed in the other three groups (Wald=113.58, d.f.=35, P<0.001). This represented a biphasic serum IgA response, with each peak occurring 1 week after the second and third immunisation dose. Higher IgA ODs could be measured in the group that had also received cholera toxin, although this difference was not statistically significant. The numbers of animals from each group that produced a serum IgA response are shown in Table 5.6 below. A responder animal was chosen on the basis that IgA levels producing an OD above 0.1 could be detected by ELISA for at least 4 weeks of the trial. A Fisher's Exact Test showed that there was no significant difference in the number of animals responding and not responding to the treatment in each group (P=0.172).

Group	Control	Soluble Toxo	Particulate Toxo	Particulate Toxo + CT	Oocyst
Responder	0	3	3	3	3
Non-Responder	4	5	1	1	1

Table 5.6 *Number of animals from each treatment group that produced a detectable serum IgA response*

When the animals were infected with toxoplasma oocysts there was a general increase in serum IgA levels in all immunised and control animals two weeks post-infection. This increase in IgA was significantly higher in all of these groups than in animals that were re-infected with oocysts (Wald=12.66, d.f.=5, P=0.027). In general this increase in serum IgA was similar to that seen in naïve animals infected with oocysts.

5.3.3.2 Antigen-Specific IgA Antibody in Nasal Secretions

The mean weekly ODs for antigen-specific nasal IgA levels in each treatment group are demonstrated in Figure 5.8. The weekly ODs for individual animals are shown in Appendix 5.4. A sample that produced an OD above 0.1 was considered to be positive. Naïve animals infected with toxoplasma oocysts showed no increase in antigen-specific IgA antibody levels in nasal secretions. The animals in the control group did not produce any local antigen-specific IgA antibody, and ODs remained at baseline for the duration of the experiment. The mean ODs for nasal IgA in animals immunised with soluble toxoplasma tachyzoite antigen did not differ from those in the control group. However, two out of the eight animals in this group showed increases in levels of local IgA antibody in nasal secretions for the last 5-6 weeks of the vaccination trial. Nasal IgA ODs remained consistently very low in all other animals in this group.

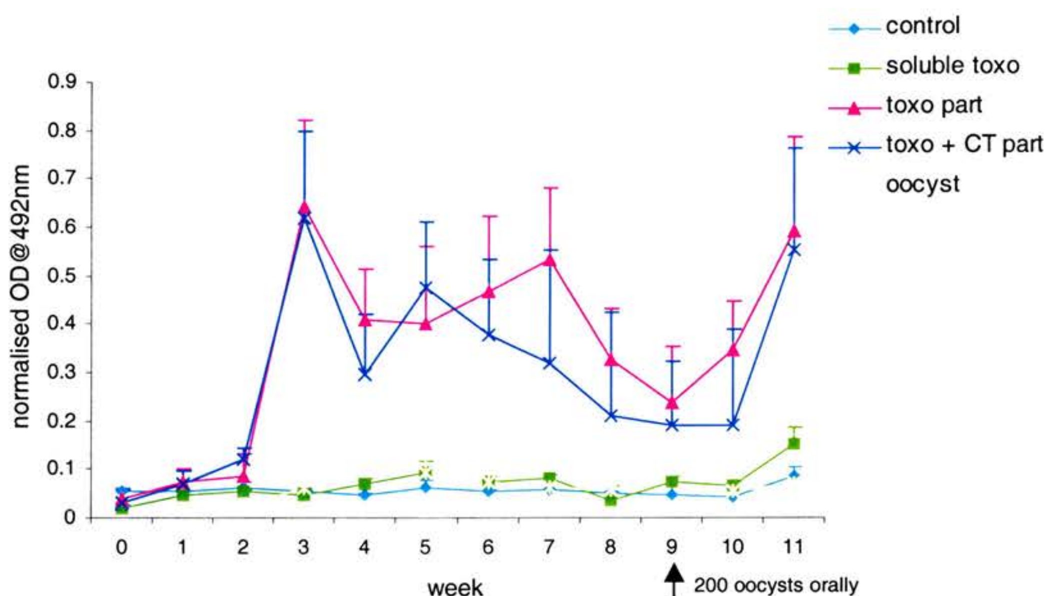


Figure 5.8 *Mean levels of antigen-specific IgA antibody (±sem) in the nasal secretions of the different treatment groups*

High levels of local antigen-specific IgA antibody were observed in the nasal secretions of all animals in both groups immunised with particulate toxoplasma tachyzoite antigen. ODs for IgA were significantly higher in both of these groups than in the other three groups (Wald=112.17, d.f.=33 P<0.001). In both groups nasal

IgA levels became significantly higher in week 3 of the trial, after the second immunisation, and remained higher than the other treatment groups for the rest of the experiment. The use of cholera toxin had no significant effect on nasal IgA levels in the groups immunised with particulate toxoplasma tachyzoite antigen.

The number of animals from each group that produced a nasal IgA response are shown in Table 5.7 below. A responder animal was chosen on the basis that IgA levels producing an OD above 0.1 could be detected by ELISA for at least 4 weeks. A Fisher's Exact Test showed that there were significant differences in the number of animals responding and not responding to the treatment in each group (P=0.002), with proportionally more animals in the 2 groups immunised with particulate toxoplasma tachyzoite antigen responding than in other groups.

Group	Control	Soluble Toxo	Particulate Toxo	Particulate Toxo + CT	Oocyst
Responder	0	2	4	4	1
Non-Responder	4	6	0	0	3

Table 5.7 *Number of animals from each treatment group that produced a detectable nasal IgA response*

When the 2 groups that had been immunised with particulate toxoplasma antigen were infected with toxoplasma oocysts, nasal IgA antibody levels increased sharply by week 2 post-infection in all eight animals, to levels almost as high as the maximum levels produced following immunisation. Nasal IgA ODs in both of these groups were significantly higher than in the other three groups and the naïve animals given an equivalent dose of oocysts (Wald=11.41, d.f.=4, P=0.022). In the group immunised with soluble toxoplasma antigen ODs above 0.1 were produced for IgA in four out of the eight animals. However, when the mean OD for the group was taken, the increase in IgA was not significant. Increase in nasal IgA could only be detected in one animal in the control group following oocyst infection, a similar situation to the naïve animals that were initially infected with oocysts. When these animals were re-infected with oocysts, no further increase in nasal IgA antibody could be detected.

5.3.3.3 Antigen-Specific IgA Antibody in Tissues Collected Post-Mortem

The mean ODs for antigen-specific IgA in each tissue in each treatment group are detailed in Appendix 5.5, and demonstrated in Figures 5.9 and 5.10. In general there appeared to be higher levels of antigen-specific IgA antibody in gut tissues than respiratory tract tissues. There were no significant differences between levels of antigen-specific IgA antibody between any of the groups in any of the tissues taken, except the abomasal fold (F=3.58, P=0.025) where lower levels of IgA were measured in the oocyst-challenged group than all 3 groups immunised with toxoplasma tachyzoite antigen. As a general trend tissues from the group that were initially infected with oocysts contained lower levels of IgA than all other treatment groups. Tissues from the three groups immunised with toxoplasma tachyzoite antigen (particulate or soluble) contained slightly higher levels of antigen-specific IgA than the control group in all tissues sampled, however within-group variation was high.

In the respiratory tract tissues (Figure 5.9), the NALT and pharyngeal tonsil contained lowest levels of antigen-specific IgA antibody, whilst the trachea, bronchus and lung all displayed similar higher levels. Tissues from the group immunised with particulate toxoplasma tachyzoite antigen generally contained highest IgA levels, while the oocyst-infected group contained the lowest IgA levels. The spleen and mesenteric lymph node also contained low levels of antigen- specific IgA antibody in all treatment groups.

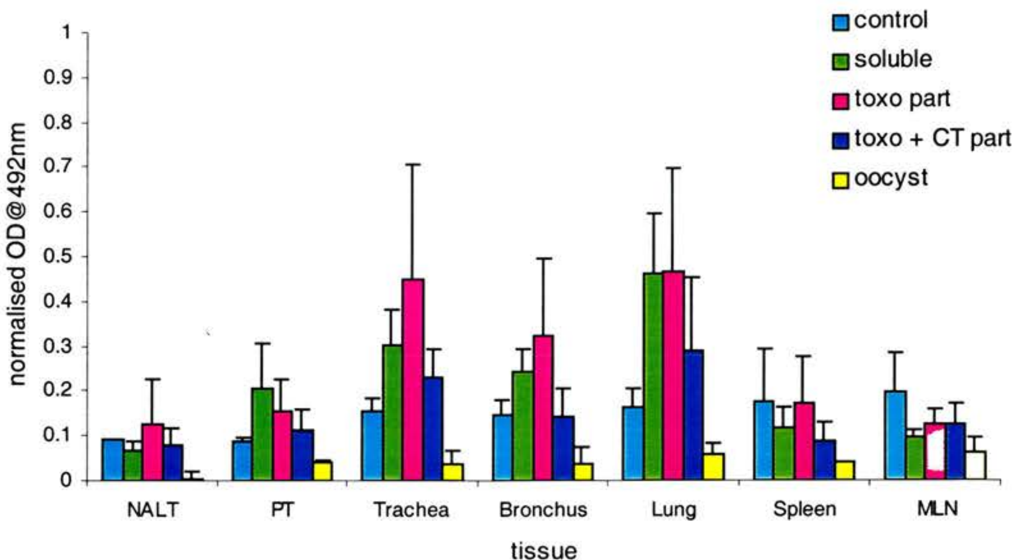


Figure 5.9 *Comparison of mean IgA levels (±sem) in respiratory tract and lymphoid tissue homogenates from each treatment group*

In the gastrointestinal tract (Figure 5.10), highest IgA levels were measured in the duodenum and jejunum, closely followed by the abomasum and ileum, whilst only very low levels were observed in the large intestine and rectum. The IgA levels were very similar in all 5 groups of animals for each tissue apart from the abomasum.

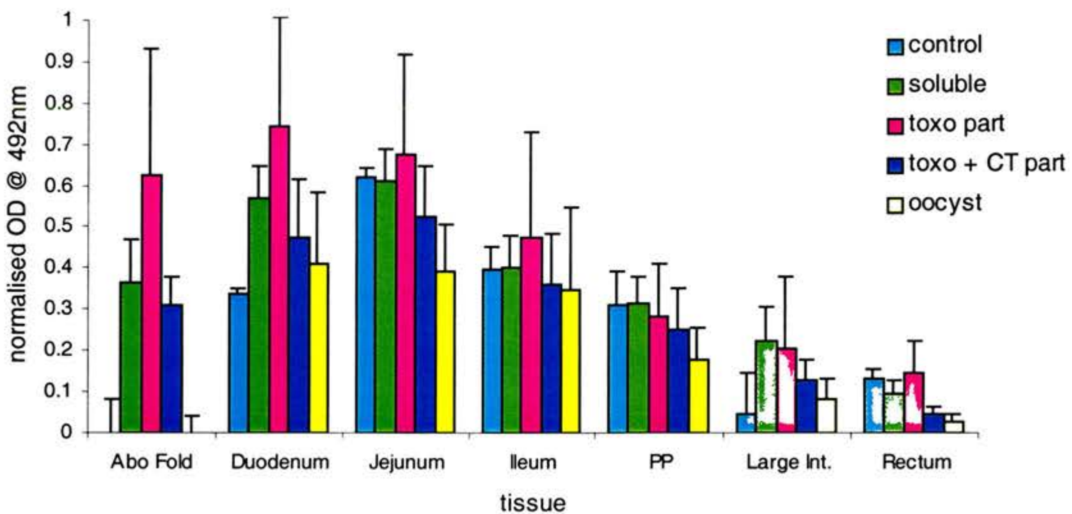


Figure 5.10 *Comparison of mean IgA levels (±sem) in gastrointestinal tract tissue homogenates from each treatment group*

5.3.3.4 Specificity of IgA Antibody

Low levels of background could be observed with day 0 serum samples, likely to be due to a low level of cross-reactivity between SAG1 and the secondary antibody as discussed in Chapter 4. Serum contained IgA specific for a number of toxoplasma proteins following immunisation, but the strongest response was against a protein of approximately 30kDa, the molecular weight of SAG1, as demonstrated in Figure 5.11. Similar proteins were recognised by IgA antibody following immunisation and oocyst infection, demonstrating that the PLG particles contained similar proteins to the soluble tachyzoite extract, and that the route of stimulation did not affect which proteins antibody responses were mounted against. Stronger responses were observed following infection than immunisation in the animals immunised with particulate tachyzoite antigen. However in the naïve animal infected with oocysts (1084N) only a weak antibody response could be detected against SAG1, reflecting the low OD detected by ELISA.

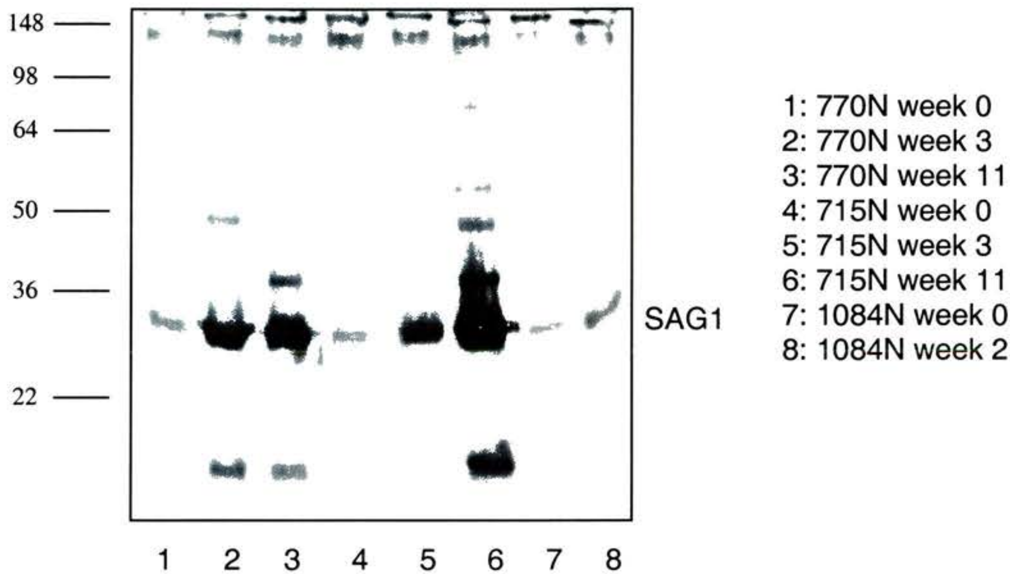


Figure 5.11 *Proteins recognised by IgA antibody from animals following immunisation and oocyst infection*

5.3.4 IgG Antibody Responses

5.3.4.1 Antigen-Specific IgG Antibody in Sera

Antigen-specific IgG antibody levels in the serum were analysed in two different ways. Firstly the OD was measured in sera at a fixed dilution of 1:100 to determine the effects of immunisation on the serum IgG response. Secondly, strongly positive sera were further analysed to quantify the amount of antigen-specific antibody in each sample. Sera were titrated in doubling dilutions and results were expressed as the titres at which 50% maximum/minimum OD was achieved. This measure was only relevant when the antibody response became strong enough to reach a relatively high OD (>0.9), which only occurred after oocyst infection.

The mean weekly ODs for antigen-specific serum IgG antibody in each treatment group for samples diluted to 1:100 are demonstrated in Figure 5.12 and the weekly ODs for individual animals are detailed in Appendix 5.6.

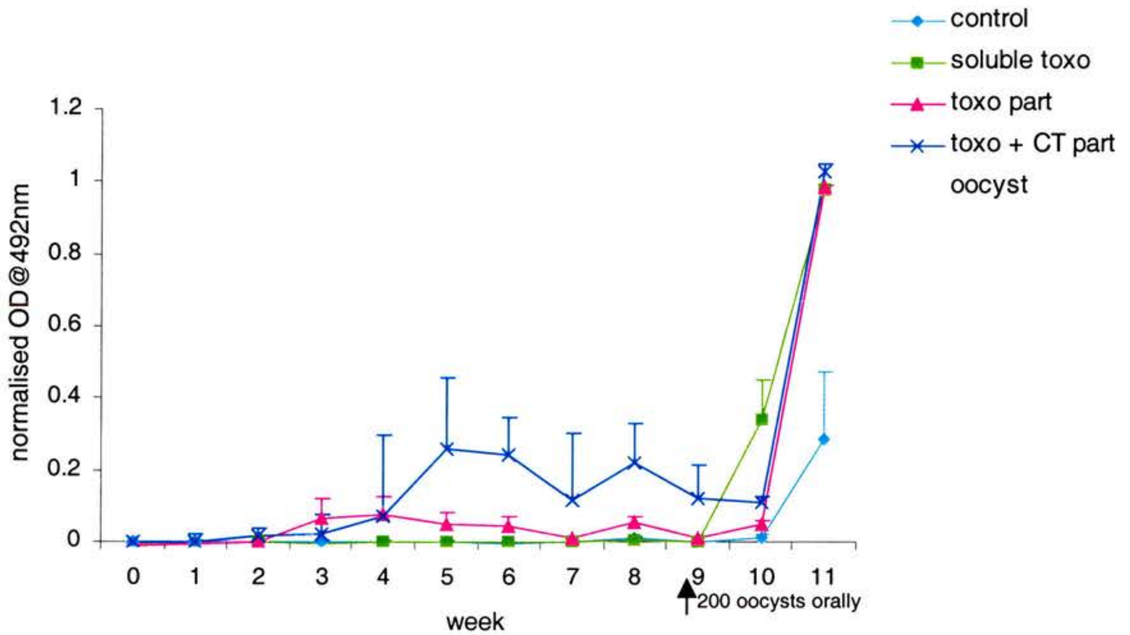


Figure 5.12 *Mean levels of antigen-specific serum IgG antibody (±sem) for each treatment group (results for serum samples diluted to 1:100)*

All four animals that were initially infected with oocysts began to produce detectable levels of antigen-specific IgG at week 2 post-infection. IgG levels then rose quickly until they reached a maximum level at week 5, at which stage an OD of approximately 1.0 at a serum dilution of 1:100 was attained. IgG ODs remained this high for the remainder of the experiment and did not increase further when animals were re-infected with oocysts at week 8 post-infection. None of the animals in the control group or in the group immunised with soluble toxoplasma tachyzoite antigen showed any increases in serum IgG antibody levels following immunisation. Two animals in the group immunised with particulate toxoplasma tachyzoite antigen alone showed very slight increases in serum IgG levels that were not significant. In the group immunised with particulate toxoplasma tachyzoite antigen and cholera toxin two of the animals produced detectable levels of IgG antibody. One of these animals produced only very low levels of antibody, while the other animal produced relatively high levels of IgG, reaching a maximum OD of 0.9 at week 6 of the vaccine trial, making the mean OD values for this group higher than the other immunised groups. Statistical analysis of the data demonstrated higher levels of serum IgG in the group infected with oocysts than all other groups of animals from

week 2 onwards, and that the group immunised with particulate toxoplasma antigen and cholera toxin had higher levels of serum IgG than the other three immunised groups at weeks 5, 6 and 8 (Wald=151.33, d.f.=35, P<0.001).

The number of animals in each group that produced a serum IgG response is shown in Table 5.8 below. A responder animal was chosen on the basis that IgG levels producing an OD above 0.1 could be detected by ELISA for at least 4 weeks. A Fisher's Exact Test showed that there were significant differences in the number of animals responding and not responding to the treatment in each group (P=0.012), with proportionally more animals in the group infected with oocysts responding than in any other groups.

Group	Control	Soluble Toxo	Particulate Toxo	Particulate Toxo + CT	Oocyst
Responder	0	1	2	2	4
Non-Responder	4	7	2	2	0

Table 5.8 *Number of animals from each treatment group that produced a detectable serum IgG response*

Following oocyst infection the negative control group began to mount an IgG response by week 2 post-infection, at similar levels to the naïve animals initially infected with oocysts. By week 2 post-infection much higher levels of serum IgG could be detected in the three groups immunised with toxoplasma tachyzoite antigen. The average ODs were similar between all three groups and at levels as high as the maximum values reached post-infection in the group initially infected with oocysts. IgG levels were significantly higher in all three immunised groups and the re-infected group than the control group and naïve animals initially infected with oocysts (Wald=29.39, d.f.=5, P<0.001). Therefore IgG levels in the three immunised groups increased earlier than in naïve animals infected with oocysts, independent of whether IgG could be detected in the serum following immunisation. Serum samples were thus titrated out so that amounts of antigen-specific IgG antibody could be quantified. These results are shown in Figure 5.13 and detailed in Appendix 5.7.

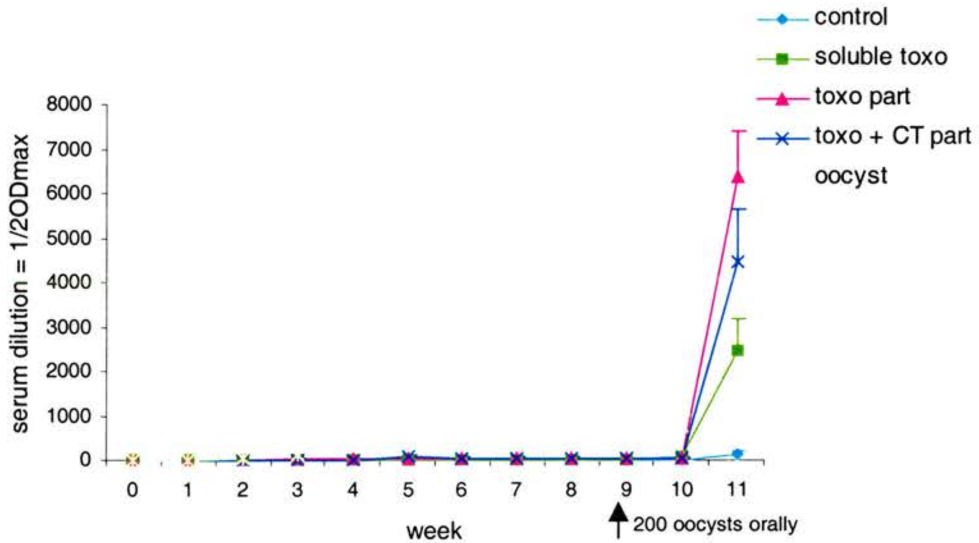


Figure 5.13 *Average serum titre (±sem) at which 50% maximum/minimum OD was reached for IgG in each treatment group*

The titre of serum at which 50% maximum/minimum OD was reached was approximately 1:1000 by week 6 post-infection in naïve animals infected with oocysts. This titre remained constant for the rest of the experiment, and did not increase following re-infection. The serum titre for animals in the control group was only 1:137 by week 2 post-infection, a similar titre to that measured at week 2 in naïve animals infected with oocysts, demonstrating that only very small amounts of antigen-specific IgG antibodies had been produced. In contrast large amounts of IgG antibody were present by week 2 post-infection in the three groups immunised with toxoplasma tachyzoite antigen. The average titre at which 50% maximum/minimum OD was reached for the soluble antigen group was 1:2487, for the particulate group was 1:6376 and for the particulate group that also received cholera toxin was 1:4447. There were significant differences in the average amounts of antigen-specific IgG in the serum in each group post-infection (Wald=59.81, d.f.=5, $P<0.001$). Larger quantities of antigen-specific IgG were produced in the three groups that had been immunised with toxoplasma tachyzoite antigen than in the group initially infected with oocysts.

5.3.4.2 Comparison of Serum IgG1 and IgG2 Levels

In general the ODs produced in the IgG1 and IgG2 specific ELISAs were much lower than for IgG antibody, probably due to few sites being sub-class specific (J. Hopkins, personal communication). Optimisation of the assay may further enhance the sensitivity and the ODs produced. The average ODs for serum IgG1 and IgG2 in each treatment group week 2 post-infection are shown in Figure 5.14, and data for individual animals are shown in Appendix 5.8.

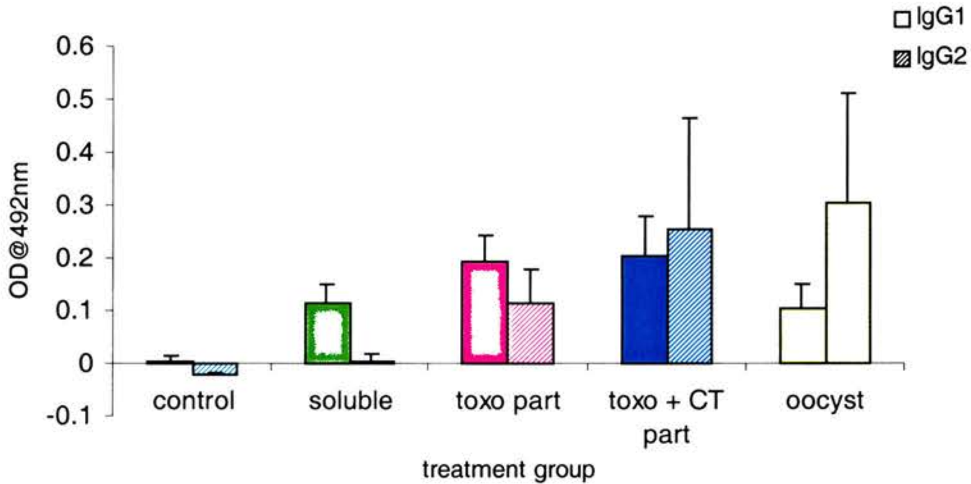


Figure 5.14 *Comparison of antigen-specific serum IgG1 and IgG2 antibody levels (±sem) between treatment groups 2 weeks post-infection with oocysts*

IgG1 levels were higher in the 3 groups immunised with toxoplasma tachyzoite antigen than the control group (F=4.58, d.f.=4, P=0.011). IgG1 ODs tended to be higher in animals immunised with particulate than soluble antigen, and IgG1 ODs in the oocyst-infected group tended to be in between those in the immunised groups and the control group. No serum IgG2 was detected in the control group or the group immunised with soluble toxoplasma tachyzoite antigen. Serum IgG2 ODs were generally higher in animals immunised with particulate toxoplasma tachyzoite antigen, and highest in animals infected with oocysts. These differences were not statistically significant due to high within group variation.

5.3.4.3 Antigen-Specific IgG Antibody in Nasal Secretions

The mean weekly ODs for antigen-specific IgG in nasal secretions in each treatment group are demonstrated in Figure 5.15, and the weekly ODs for individual animals are shown in Appendix 5.9. In general, naïve animals that were infected with oocysts began to produce detectable levels of IgG antibody in their nasal secretions by week 3 post-infection. IgG levels then rose steadily until week 6 when they began to level out. When animals were re-infected with oocysts, nasal IgG levels increased further. There was considerable variation in the amount of antigen-specific nasal IgG produced between animals in this group.

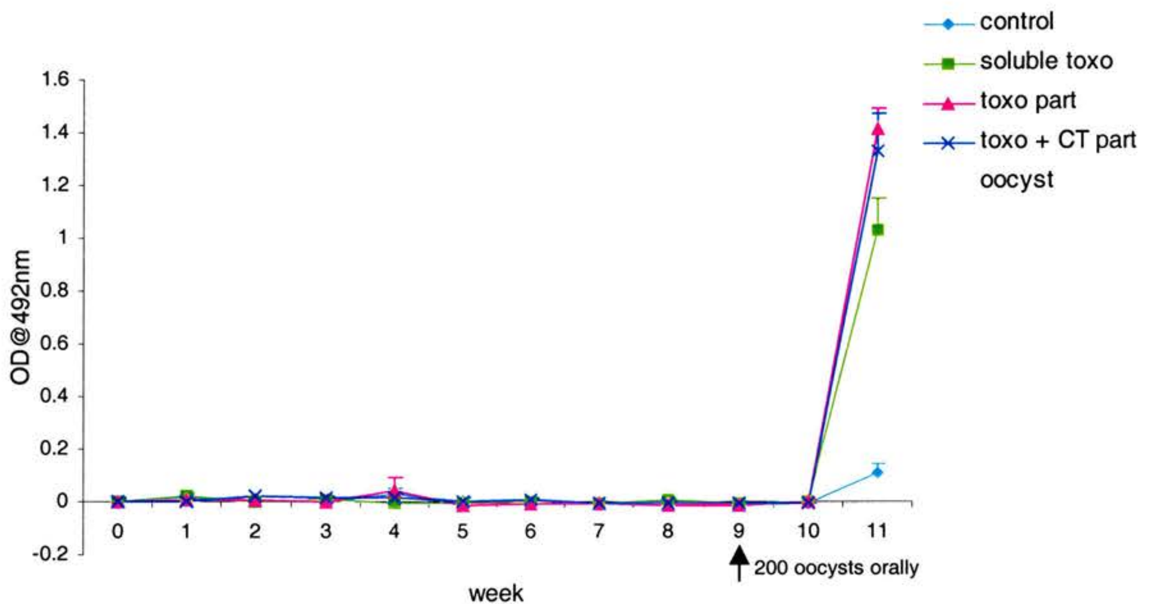


Figure 5.15 *Mean levels of antigen-specific IgG antibody (±sem) in nasal secretions in each treatment group*

The oocyst-infected group had significantly higher levels of IgG in nasal secretions than any of the other groups from week 3 onwards (Wald=55.49, d.f.=33, P=0.008). No IgG antibody could be detected in any immunised or control groups until animals were infected with oocysts. By week 2 post-infection IgG could be detected in the nasal secretions of the majority of animals. Very low ODs were produced in the nasal secretions of control animals, similar to those measured at the same time in naïve animals infected with oocysts. Nasal secretions from the three groups of animals that had been immunised with toxoplasma tachyzoite antigen contained much higher levels of IgG than naïve animals infected with oocysts

(Wald=123.35, d.f.=4, P<0.01). As a general trend animals immunised with particulate antigen produced higher levels of nasal IgG than animals immunised with soluble antigen, but this difference was not significant. In all three immunised groups nasal IgG was produced more quickly and in greater amounts than in naïve animals given an equivalent dose of oocysts.

5.3.4.4 Comparison of Nasal IgG1 and IgG2 Levels

The average ODs for antigen-specific IgG1 and IgG2 antibody in nasal secretions in each treatment group at week 2 post-infection are shown in Figure 5.16, and data for individual animals are detailed in Appendix 5.8.

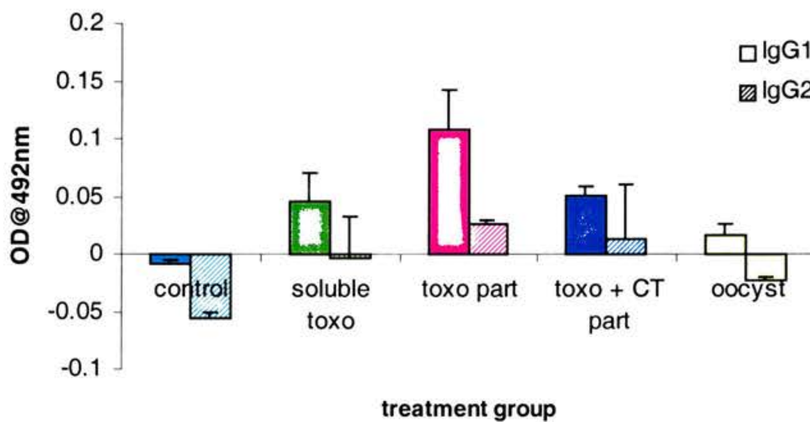


Figure 5.16 *Comparison of antigen-specific nasal IgG1 and IgG2 antibody levels (±sem) between treatment groups 2 weeks post-infection*

No statistically significant differences could be detected in nasal IgG1 and IgG2 ODs between the treatment groups. The two groups immunised with particulate toxoplasma tachyzoite antigen tended to have higher levels of IgG1 in nasal secretions than the other groups. IgG1 ODs were low in the groups immunised with soluble toxoplasma antigen and the group infected with oocysts, and no IgG1 could be detected in the control group. IgG2 ODs were very low in the nasal secretions of all animals.

5.3.4.5 Antigen-Specific IgG Antibody in Tissues Collected Post-Mortem

The mean ODs for antigen-specific IgG in each tissue in each treatment group are detailed in Appendix 5.10, and demonstrated in Figures 5.17 and 5.18 overleaf. In general similar levels of antigen-specific IgG antibody were detected in gut and respiratory tract tissue homogenates. Lymph nodes contained slightly higher levels of IgG. There were significant differences between groups in levels of antigen-specific IgG antibody in all tissues sampled. The F and P values for the comparison of IgG in each tissue between groups are shown in Table 5.9.

Tissue	F value	P value
NALT	24.41	<0.001
Pharyngeal Tonsil	16.33	<0.001
Trachea	10.69	<0.001
Bronchus	21.18	<0.001
Lung	38.00	<0.001
Abomasal Fold	43.15	<0.001
Duodenum	25.77	<0.001
Jejunum	33.07	<0.001
Ileum	14.35	<0.001
Peyer's Patch	26.00	<0.001
Large Intestine	26.37	<0.001
Rectum	13.59	<0.001
Spleen	18.75	<0.001
Mesenteric Lymph Node	19.06	<0.001

Table 5.9 *Statistical analyses of between group differences in antigen-specific IgG antibody in gut and respiratory tract tissues*

In general, tissues from the control group contained significantly lower levels of antigen-specific IgG than all other groups for all tissues sampled. The group that were initially infected with oocysts tended to have lower levels of IgG in all tissues sampled than the 3 groups immunised with toxoplasma tachyzoite antigen, however this difference was only significant in the large intestine and the rectum. IgG levels were highest in animals from the three groups immunised with toxoplasma tachyzoite antigen in all tissues sampled.

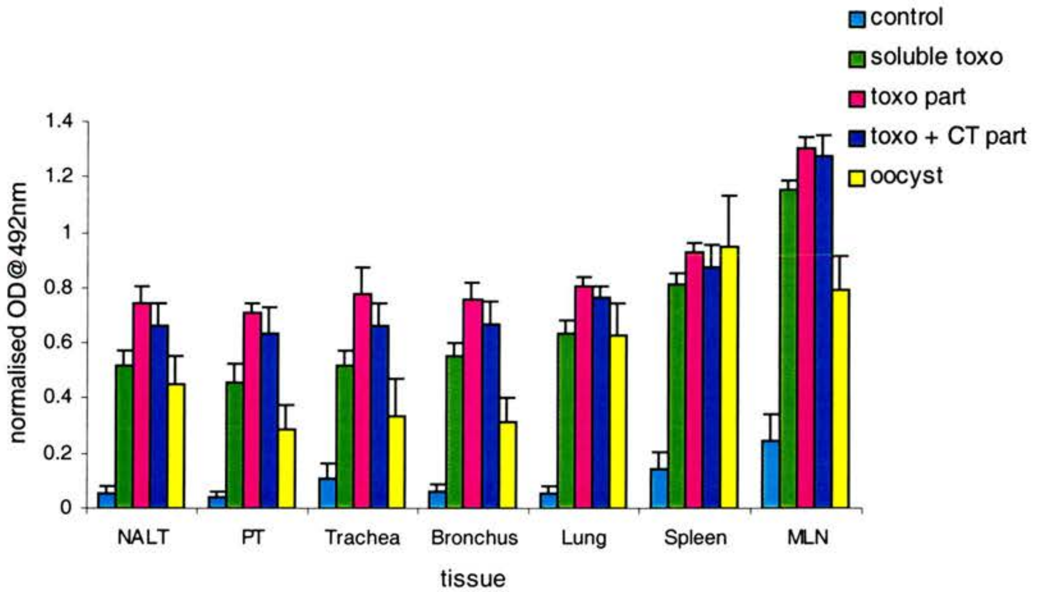


Figure 5.17 *Comparison of mean IgG levels (±sem) in respiratory tract and lymphoid tissue homogenates from each treatment group*

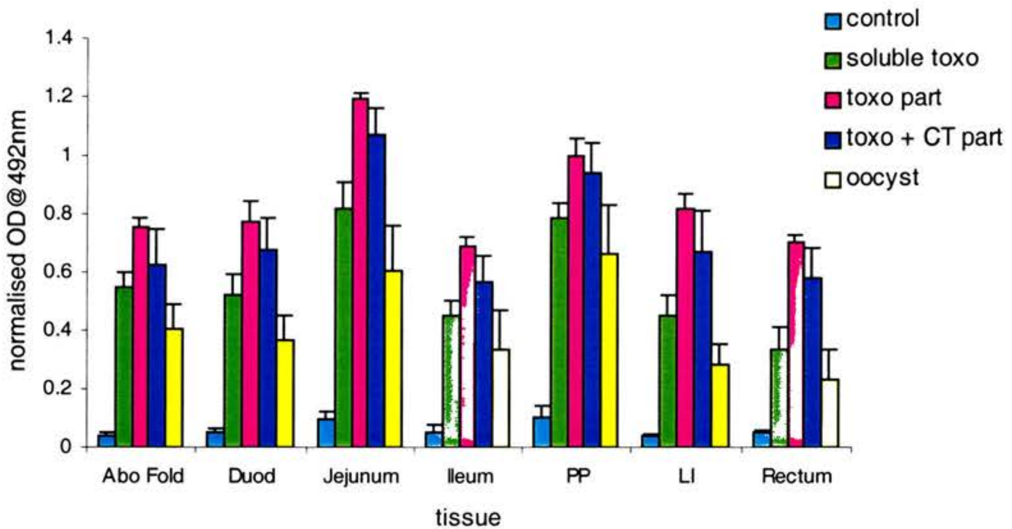


Figure 5.18 *Comparison of mean IgG levels (±sem) in gastrointestinal tract tissue homogenates from each treatment group*

5.3.4.6 Specificity of IgG Antibody

Low levels of background could be observed on day 0 serum samples, likely to be due a low level of cross-reactivity between SAG1 and the secondary antibody as discussed in Chapter 4. However, serum from animals that had a strong IgG response following immunisation reacted strongly against a number of bands of protein in the toxoplasma extract on the western blot, demonstrated in Figure 5.19. Serum from all 3 animals contained antigen-specific IgG against a number of toxoplasma proteins but the strongest response was against a protein of approximately 30kDa, the molecular weight of SAG1. Serum IgG in the immunised animals reacted against more bands of protein from the toxoplasma extract following infection with oocysts. Antigen-specific responses were stronger following immunisation than infection. The serum IgG from animals only infected with oocysts appeared to be against SAG1 alone.

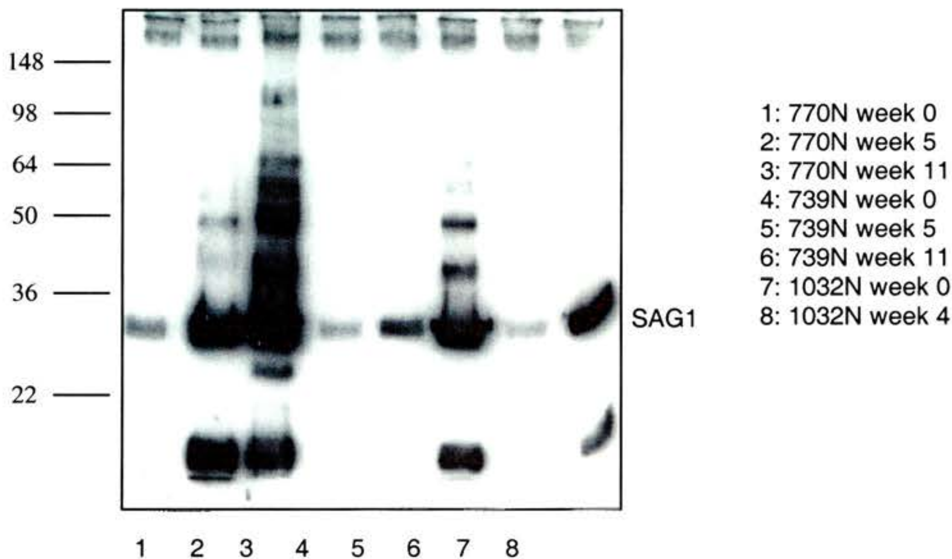


Figure 5.19 *Proteins recognised by IgG antibody from animals following immunisation and oocyst infection*

5.3.4.7 Antigen-Specific IgE Antibody

No detectable levels of antigen-specific IgE antibody were measured in the serum or nasal secretions from any animals in any treatment group.

5.3.5 Cell-Mediated Responses

5.3.5.1 PBMCs

PBMCs were tested for their ability to respond to toxoplasma tachyzoite antigen *in vitro* to determine whether peripheral blood contained antigen-specific lymphocytes following immunisation. Stimulation indices (SI) were determined by dividing the arithmetic median of counts per minute (cpm) pulsed with ^{3}[H]-thymidine, by the arithmetic median of cpm of the background medium control cultures. All results are discussed in terms of the logarithmic transformations of the data (Log_{10}SI), since cell proliferation occurs in a logarithmic manner. Actual cpm values for proliferative responses to medium controls, vero cell antigen controls, Con A positive controls, and toxoplasma antigen are presented in Appendices 5.11-5.15. The proliferative responses of PBMCs to the toxoplasma antigen were highly variable between animals within each treatment group, and even for the same animal in different weeks, and the weekly responses for individual animals are illustrated in Figures 5.20 – 5.24. The test is considered positive when the SI has a value above 2.5-3 according to Kristensen *et al.* (1982). This is represented in the graphs by a continuous line at $Log_{10}3=0.47$.

No animals presented SI values above 3 for toxoplasma antigen prior to infection or immunisation. All animals produced a strong proliferative response to the mitogen Con A, and were negative for the medium control and the vero cell antigen, demonstrating that PBMCs were not proliferating in a non-specific manner. The proliferative responses were very similar to both concentrations of toxoplasma antigen, and for the purpose of statistical analysis the response to the higher concentration of antigen (2μg/ml) was used. Positive proliferation in response to toxoplasma tachyzoite antigen was demonstrated in PBMCs from all four animals that were orally infected with oocysts at week 2 post-infection, demonstrated in Figure 5.20. Two of the animals continued to show positive proliferation to the antigen for the remainder of the experiment, and proliferation levels did not increase further when the animals were re-infected with oocysts. The other two animals demonstrated a positive response until week 5, after which SIs fluctuated between positive and negative.

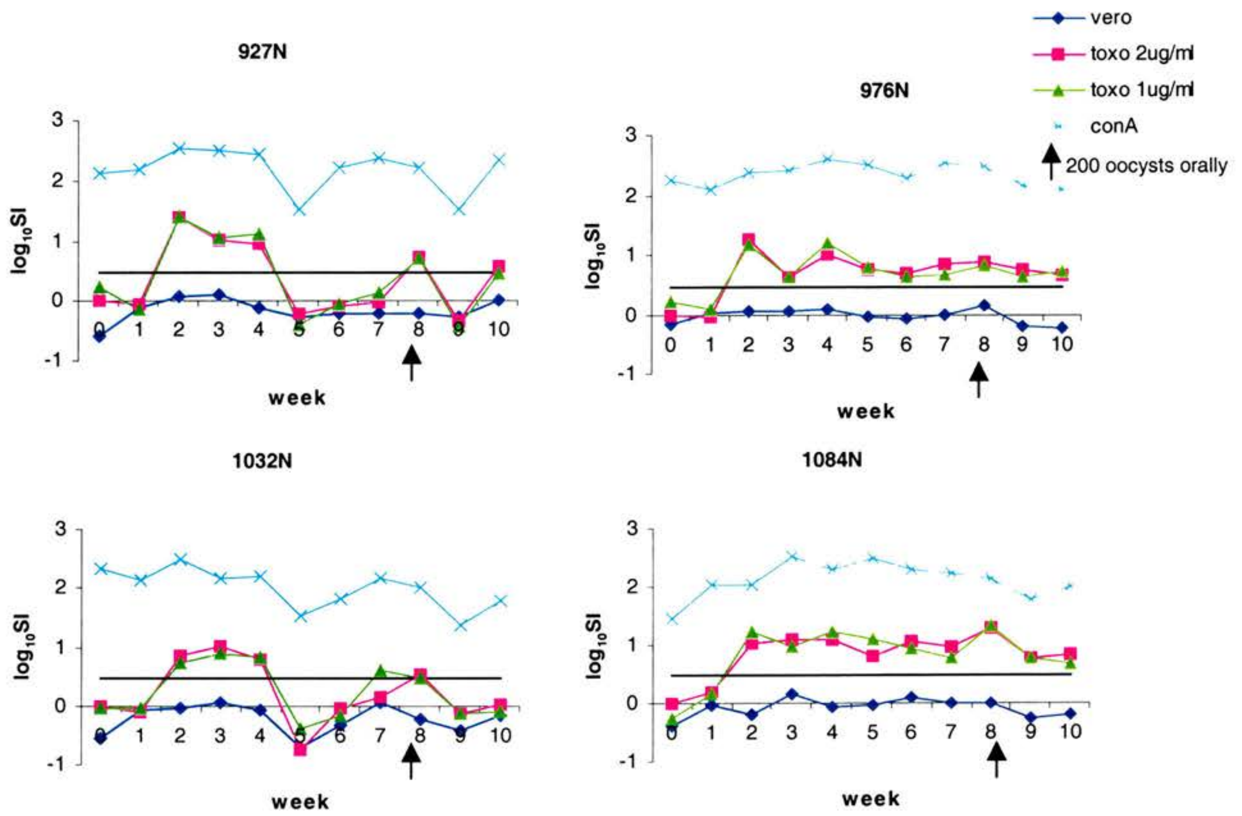


Figure 5.20 *Proliferation of PBMC from sheep infected with toxoplasma oocysts following stimulation with with vero cell antigen,* T. gondii *tachyzoite antigen at 1µg/ml and 2µg/ml, or Con A*

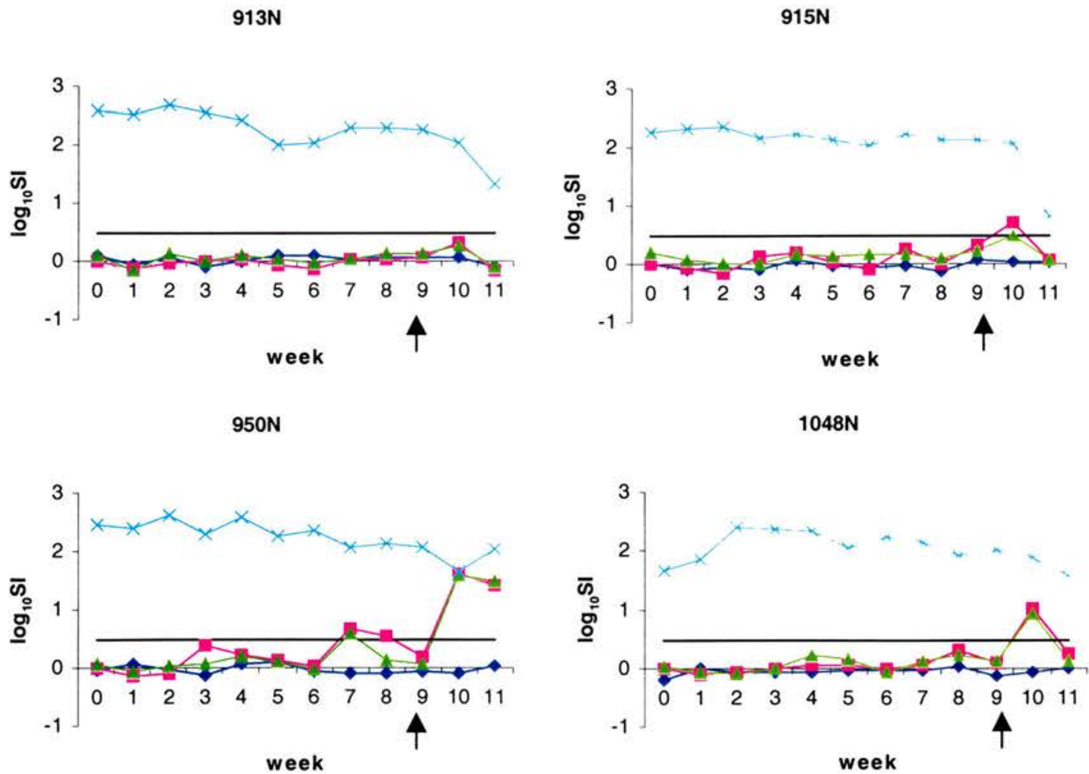


Figure 5.21 *Proliferation of PBMC from sheep in control group immunised with blank particles following stimulation with with vero cell antigen,* T. gondii *tachyzoite antigen at 1µg/ml and 2µg/ml, or Con A*

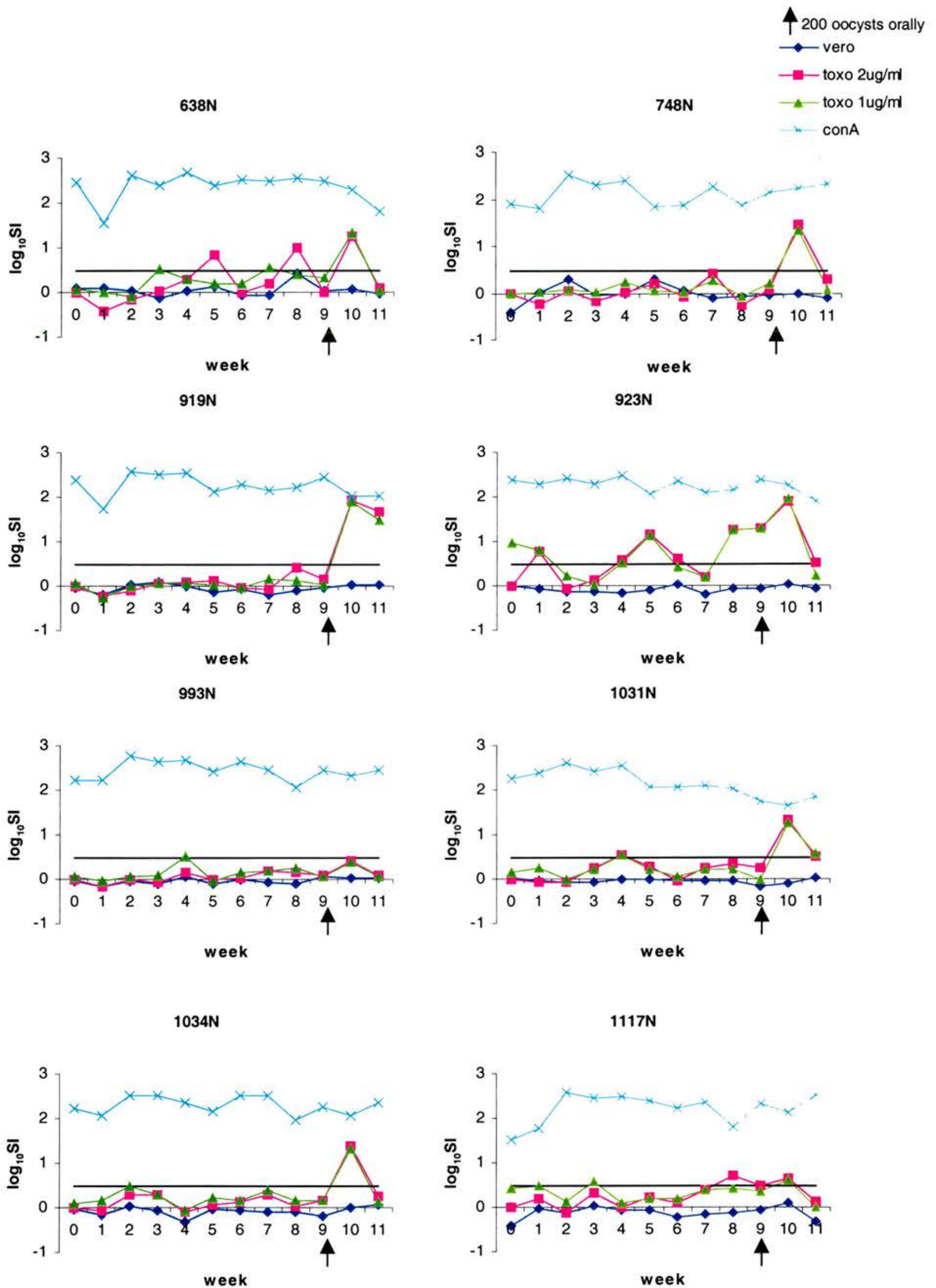


Figure 5.22 *Proliferation of PBMC from sheep immunised intra-nasally with soluble toxoplasma tachyzoite antigen following stimulation with vero cell antigen,* T. gondii *tachyzoite antigen at 1μg/ml and 2μg/ml, or Con A*

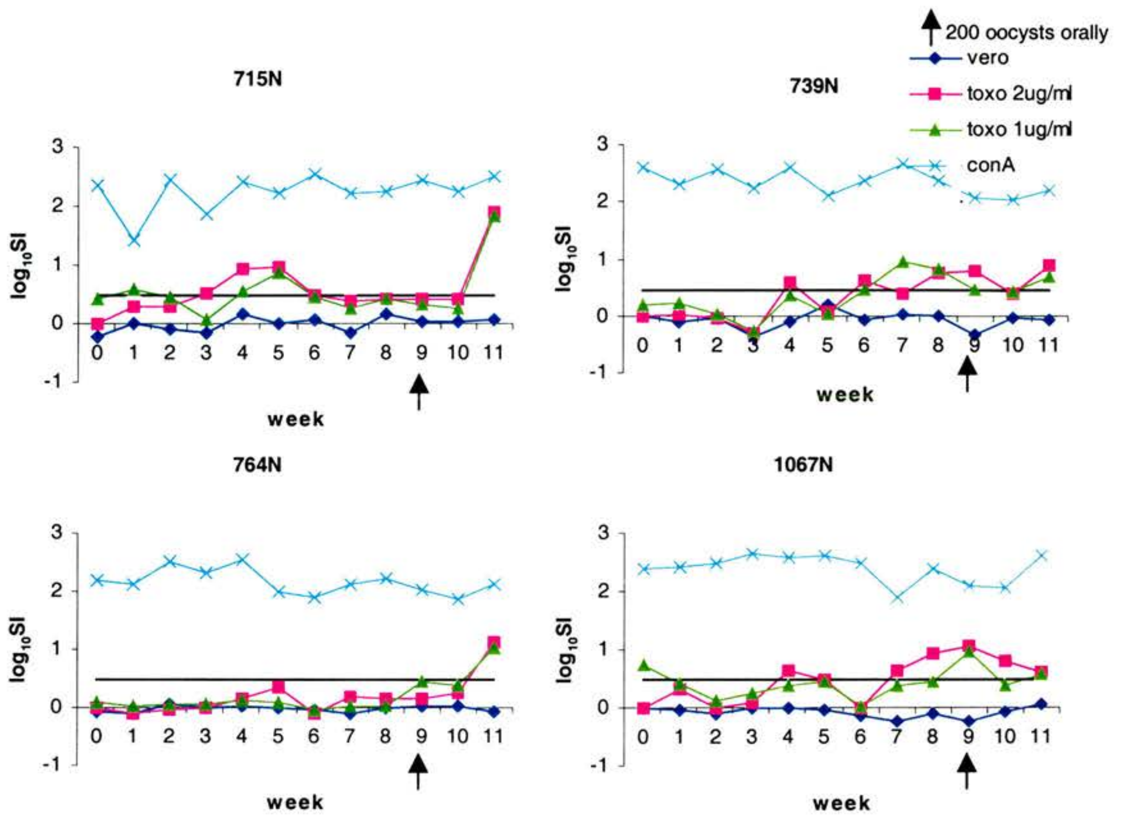


Figure 5.23 *Proliferation of PBMC from sheep immunised intra-nasally with particulate toxoplasma tachyzoite antigen following stimulation with vero cell antigen,* T. gondii *tachyzoite antigen at 1μg/ml and 2μg/ml, or Con A*

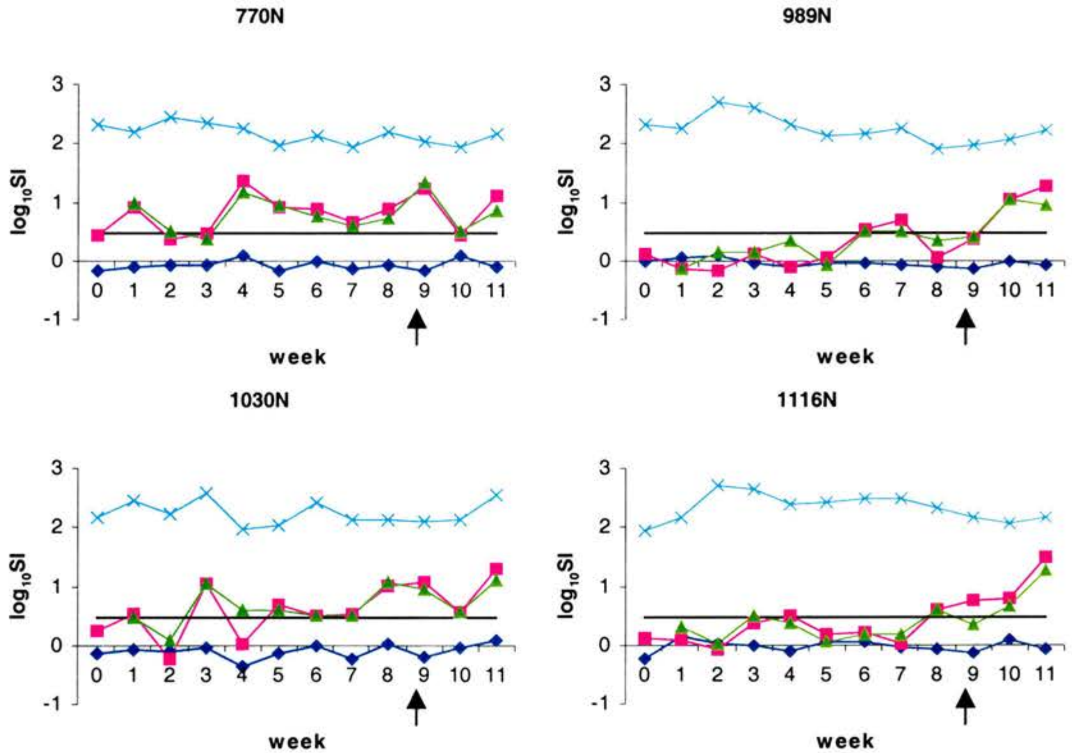


Figure 5.24 *Proliferation of PBMC from sheep immunised intra-nasally with particulate toxoplasma tachyzoite antigen and cholera toxin following stimulation with vero cell antigen,* T. gondii *tachyzoite antigen at 1μg/ml and 2μg/ml, or Con A*

PBMCs collected from animals in the control group that had been immunised with blank particles showed no positive proliferation to toxoplasma antigen for the duration of the experiment, apart from animal 950N, in which a borderline response could be measured at weeks 7 and 8 (Figure 5.21). When the control group was infected with oocysts three out of the four animals produced positive proliferative responses to the antigen by week 2 post-infection. In general, PBMCs from animals immunised with soluble toxoplasma tachyzoite antigen showed very low levels of proliferation to toxoplasma antigen *in vitro,* demonstrated in Figure 5.22. No proliferation could be detected in PBMCs from five out of the eight animals in the group following immunisation. Of the remaining three animals, one produced a borderline positive proliferative response and the other two animals fluctuated between positive and negative proliferative activity to the antigen. Following oocyst infection positive proliferation to the antigen was measured in PBMCs from six out of the eight animals, some responses reaching almost as high as those observed to Con A.

In comparison a greater proportion of animals, immunised with particulate toxoplasma tachyzoite antigen, showed positive proliferation to the antigen *in vitro.* In the group immunised with particulate antigen alone (Figure 5.23) three out of the four animals showed positive proliferative activity to the antigen starting at weeks 3 or 4 after the first immunisation, and fluctuating between positive and negative thereafter. A similar situation occurred in the animals that were immunised with particulate antigen and cholera toxin (Figure 5.24), with PBMCs from 3 out of the 4 animals proliferating in response to the antigen as early as the first week after vaccination, and the other animal showing borderline responses. Two of the animals that responded in this group showed proliferative activity as high as the levels seen in animals infected with oocysts. Following oocyst infection positive proliferative activity to the antigen could be demonstrated in PBMCs from all 8 animals in these two groups.

When the average responses for each treatment group were compared before challenge infection, highly significant differences in the proliferative responses to the antigen were observed (Wald=100.09, d.f.=35, $P<0.001$). The group infected with oocysts showed increased levels of proliferation at weeks 2, 3, 4 and 8 post-infection,

which were, on average, significantly higher than the levels seen in the control and soluble antigen group but not the 2 particulate groups. Higher responses than the control group could be demonstrated in the particulate group in weeks 4-5 and 8-9, and in the cholera toxin group in weeks 3-4 and 8-9, however these increases in proliferation were just below the level of significance due to high within group variation.

The number of animals from each group that showed proliferative PBMC responses to toxoplasma antigen are shown in Table 5.10 below. A responder animal was chosen on the basis that an SI over 3 could be measured for at least 4 weeks of the trial. A Fisher's Exact Test showed that there were significant differences in the number of animals responding and not responding to the treatments in each group (P=0.044). More animals showed positive proliferation in the two particulate groups and the group infected with oocysts.

Group	Control	Soluble Toxo	Particulate Toxo	Particulate Toxo + CT	Oocyst
Responder	0	3	3	3	4
Non-Responder	4	5	1	1	0

Table 5.10 *Number of animals in each treatment group that produced a positive proliferative response to toxoplasma antigen* in vitro

For the 2 weeks post-infection with oocysts, significant differences could be observed between the treatment groups (Wald=83.89, d.f.=5, P<0.001). At week 2 post-infection the two groups immunised with particulate antigen and the group initially infected with oocysts demonstrated higher levels of proliferation than the control, soluble or group re-infected with oocysts. During the first week post-infection the control and soluble groups showed the highest proliferative responses to the antigen.

5.3.5.2 Lymph Nodes, Tonsils and Spleen Collected Post-Mortem

Table 5.11 overleaf demonstrates whether positive proliferation to the tachyzoite antigen could be measured in single cell suspensions from the lymph nodes, tonsils and spleen collected post-mortem. Data are presented in Appendices 5.16-5.18. There were no significant differences between the treatment groups. In general, positive responses were most frequently measured in cells from the pre-

femoral lymph node and the spleen, more commonly in lymph nodes from animals that were immunised with particulate toxoplasma antigen with or without cholera toxin. Only one positive response was detected in PBMCs from animals in the control group.

Group	Animal	RP LN	P-F LN	MLN	Med LN	PT	Spleen
control	913N	-	-	+/-	-	-	-
	915N	-	-	-	-	-	++
	950N	-	-	-	-	-	-
	1048N	-	-	-	-	-	-
i.n. soluble toxoplasma	638N	-	+	++	-	-	+/-
	748N	-	-	-	-	-	+++
	919N	-	++	-	-	-	-
	923N	++	+	+	-	-	+++
	993N	-	-	-	-	-	+
	1031N	-	-	-	-	-	-
	1034N	-	-	+	-	-	-
	1117N	-	+/-	-	-	-	-
i.n. particulate toxoplasma	715N	-	-	+/-	-	-	+
	739N	+/-	+++	-	+++	-	+++
	764N	+	+/-	+	+/-	-	+++
	1067N	-	++	-	++	-	-
i.n. particulate toxoplasma + CT	770N	+/-	++	+/-	-	-	+++
	989N	-	++	-	-	+/-	+
	1030N	-	-	+/-	-	-	+
	1116N	+/-	++	+	-	-	-
oocyst	927N	-	+++	-	++	-	-
	976N	-	-	-	-	ns	++
	1032N	-	ns	-	-	-	++
	1084N	-	-	+/-	-	ns	+

Table 5.11 *Positive (+), negative (-), and borderline (+/-) proliferative responses to toxoplasma tachyzoite antigen in cells from lymph nodes, tonsil and spleen collected post-mortem SI = stimulation index.*
- SI<3, **+/-** SI 3-5, **+** SI 5-10, **++** SI 10-20, **+++** SI >20, **ns** no sample

5.3.6 Interferon Gamma Production

5.3.6.1 PBMCs

Cell free supernatants from PBMCs stimulated *in vitro* with toxoplasma tachyzoite antigen, ConA, vero cell antigen or medium alone were collected and analysed for the presence of IFNγ by ELISA. The amount of IFNγ (pg/ml) detected

in these samples was calculated using standards of recombinant bovine IFNγ. The correlation coefficient for the relationship between the concentration of IFNγ produced in pg/ml and the stimulation index from the lymphocyte proliferation assay was 0.64. Mean IFNγ concentrations for each group are demonstrated in Figures 5.25 - 5.29, and actual data are detailed in Appendices 5.19 – 5.21.

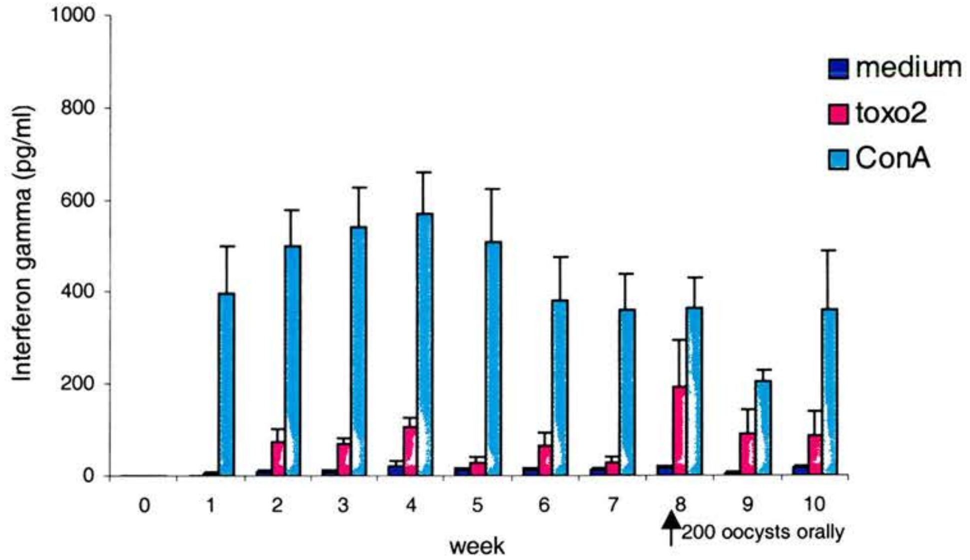


Figure 5.25 *Mean IFNγ production (±sem) in animals infected with toxoplasma oocysts*

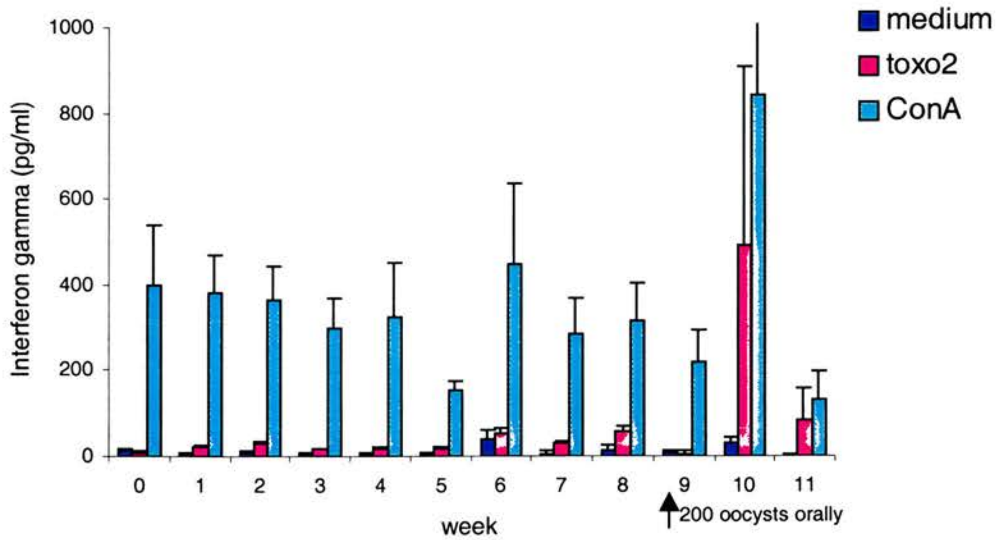


Figure 5.26 *Mean IFNγ production (±sem) in the control group immunised with blank microparticles*

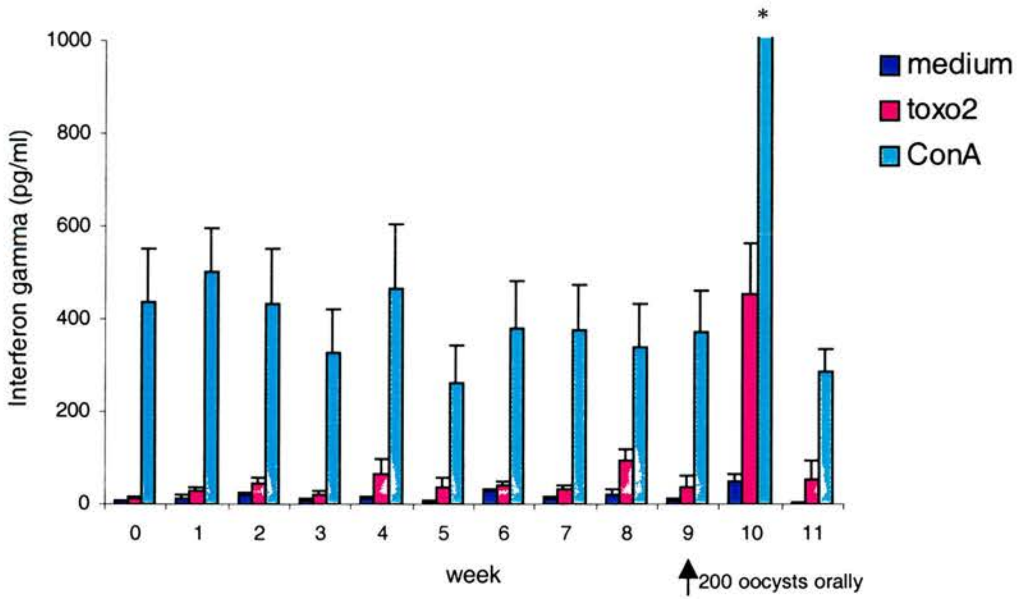


Figure 5.27 *Mean IFNγ production (±sem) In animals immunised with soluble toxoplasma tachyzoite antigen*

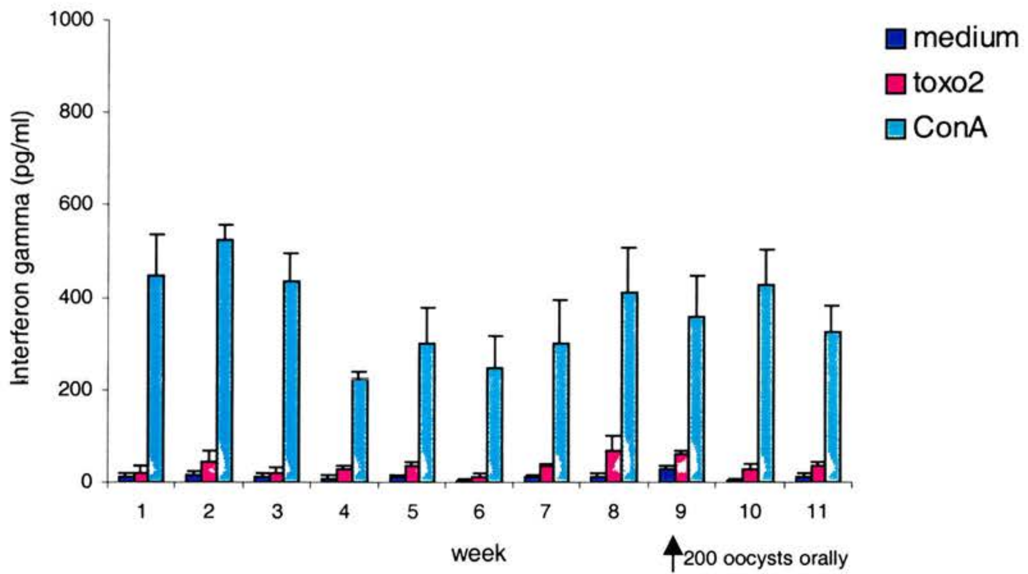


Figure 5.28 *Mean IFNγ production (±sem) in animals immunised with particulate toxoplasma tachyzoite antigen*

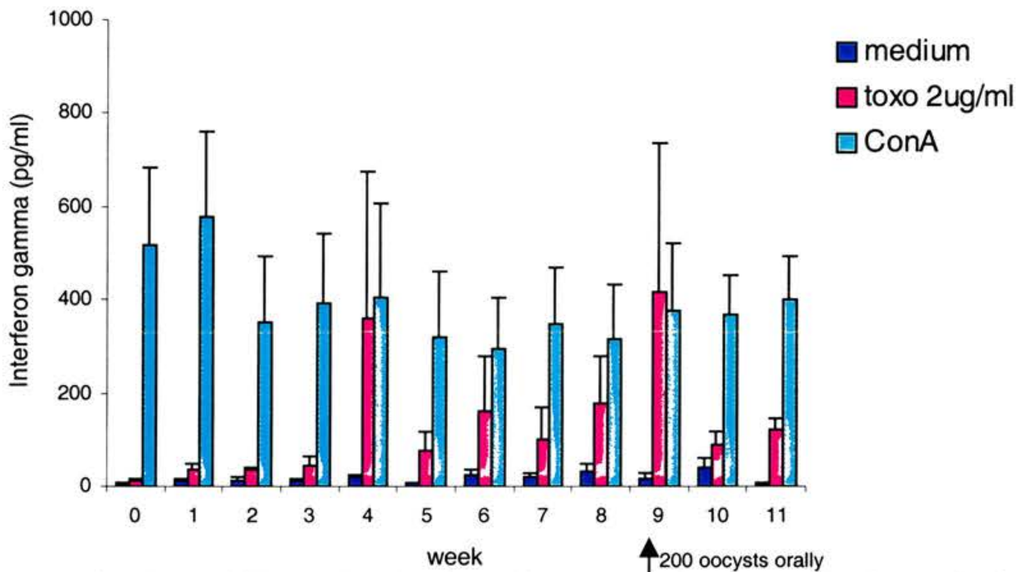


Figure 5.29 *Mean IFNγ production (±sem) in animals immunised with particulate toxoplasma tachyzoite antigen and cholera toxin*

Supernatant samples from cells cultured in medium alone or with vero cell antigen were consistently negative for IFNγ (background values of 0-50pg/ml could be detected). PBMCs cultured with Con A consistently produced high concentrations of IFNγ, up to 2000pg/ml in cell-free supernatants in high-responding animals. In general, supernatants from PBMCs collected from naïve animals that were infected with oocysts contained increased levels of IFNγ from week 2 post-infection onwards, demonstrated in Figure 5.25. However, IFNγ was only produced in small quantities in response to antigen following infection, with an average concentration of only 80pg/ml in cell-free supernatants. No further increases in IFNγ levels were detected after animals were re-infected with oocysts. No IFNγ was produced by cells stimulated by toxoplasma tachyzoite antigen *in vitro* from animals in the control group until they were infected with oocysts at the end of the experiment, demonstrated in Figure 5.26.

Increased levels of IFNγ were produced by PBMCs stimulated by tachyzoite antigen *in vitro* from two of the animals in the group immunised with soluble toxoplasma antigen, demonstrated in Figure 5.27. No IFNγ could be detected in cell-free supernatants from the other animals in this group, therefore the mean IFNγ concentrations for the group were relatively low. Increased IFNγ could only be

detected in supernatants from PBMCs from one of the animals in the group immunised with particulate toxoplasma antigen (Figure 5.28), and two animals in the group that were immunised with particulate tachyzoite antigen and cholera toxin (Figure 5.29). PBMCs from one animal in this group consistently produced very high concentrations of IFNγ, much higher than those seen in animals infected with oocysts. The mean IFNγ concentrations for this group were significantly increased from week 2 onwards.

Statistical analysis revealed only differences within groups across the time period of the experiment, but not between the different treatment groups at any point in the first nine weeks of the experiment (Wald=66.93, d.f.=1.97, P<0.001). However, following oocyst infection, cell-free supernatants contained much higher levels of IFNγ in the control group and the group immunised with soluble protein in the first week post-infection, and in the two groups immunised with particulate antigen in the second week post-infection (Wald=62.51, d.f.=5, P<0.001).

The numbers of animals from each group that produced increased IFNγ in cell-free supernatants following *in vitro* stimulation with toxoplasma tachyzoite antigen are shown in Table 5.12 below. A responder animal was chosen on the basis that IFNγ levels in supernatants from cells cultured with the antigen were significantly higher than those from cells cultured with medium alone. A Fisher's Exact Test showed that there were more animals producing an IFNγ response in the group infected with oocysts (P=0.038).

Group	Control	Soluble Toxo	Particulate Toxo	Particulate Toxo + CT	Oocyst
Responder	0	2	1	2	4
Non-Responder	4	6	3	2	0

Table 5.12 *Number of animals from each treatment group that produced a detectable IFNγ response in cell-free supernatants*

5.3.6.2 Lymph Nodes, Tonsils and Spleen Collected Post-Mortem

Table 5.13 below demonstrates whether increased production of IFNγ occurred in single cell suspensions from lymph nodes, tonsils and spleen collected post-mortem, in response to stimulation with tachyzoite antigen. Data are presented in Appendices 5.19-5.21. The stimulation index was calculated as in the lymphocyte

proliferation assays and an IFNγ response was considered positive when this value was higher than 3. When no medium response had been measured a response greater than 20 was considered to be positive. The results were highly variable but in general increased IFNγ levels could be detected more often in cells from lymph nodes than the pharyngeal tonsil. Increased production of IFNγ occurred more frequently in cells from lymph nodes collected from animals immunised with toxoplasma tachyzoite antigen, more commonly with particulate antigen. In particular, six or seven out of the eight animals immunised with particulate antigen were positive in the mesenteric lymph node, pre-femoral lymph node and spleen. Lymph nodes from animals in the control group were generally negative for IFNγ production, as were those from animals initially infected with oocysts. However, this may have been due to the high degree of fungal contamination observed in these cell cultures.

Group	Animal	RP LN	P-F LN	MLN	Med LN	PT	Spleen
control	913N	-	-	-	-	-	-
	915N	-	-	-	-	-	+
	950N	-	+	-	-	-	+
	1048N	-	+/-	-	-	-	-
i.n. soluble toxoplasma	638N	+/-	-	+	+	-	+
	748N	-	+/-	-	-	-	+
	919N	-	+	+	-	-	-
	923N	+	+	+	+/-	+/-	+
	993N	-	-	-	-	-	+
	1031N	ns	-	-	-	-	-
	1034N	+/-	-	+	-	-	-
	1117N	+	+	-	-	-	-
i.n. particulate toxoplasma	715N	-	-	-	-	+/-	+
	739N	+	+	+	+	-	+
	764N	+	+	+	+	-	+
	1067N	-	+	+	+	-	-
i.n. particulate toxoplasma + CT	770N	-	+	+	+	+/-	+
	989N	-	+	+	-	-	+
	1030N	+	+	+	+/-	-	+
	1116N	+	+	+	-	+/-	-
oocyst	927N	-	-	-	-	+	-
	976N	-	ns	-	+/-	ns	+
	1032N	ns	ns	-	+/-	ns	-
	1084N	ns	ns	+	ns	ns	-

Table 5.13 *Positive (+), negative (-), and borderline (+/-)IFNγ responses to* in vitro *stimulation with toxoplasma tachyzoite antigen in cells from lymph nodes, tonsil and spleen collected post-mortem* **ns** = no sample

5.3.7 Summary of Results

The following tables summarise the immune responses produced following immunisation and challenge infection in each treatment group.

Group	1	2	3	4	5
Treatment	control	i.n. soluble toxo	i.n. toxo part	i.n. toxo part + CT	oocyst
Serum IgA	-	-	+	+	+/-
Nasal IgA	-	-	+	+	-
Serum IgG	-	-	-	+/-	+
Nasal IgG	-	-	-	-	+
T cell	-	+/-	+	+	+
IFNγ	-	+/-	+/-	+	+

Table 5.14 *Summary of immune responses produced in each treatment group following immunisation or oocyst infection*

Group	1	2	3	4	5
Treatment	control	i.n. soluble toxo	i.n. toxo part	i.n. toxo part + CT	oocyst
Serum IgA	+	+	+	+	-
Nasal IgA	-	-	+	+	-
Serum IgG	-	++	++	++	+
Serum IgG1	-	+	+	+	+
Serum IgG2	-	-	+	+	+
Nasal IgG	-	+	+	+	+
Nasal IgG1	-	+/-	+	+	-
Nasal IgG2	-	-	-	-	-
Tissue IgG	+/-	+	+	+	+
T cell	+	+	+	+	+
IFNγ	+	+	+/-	+	+

Table 5.15 *Summary of immune responses produced in each treatment group following challenge infection*

5.4 DISCUSSION

Mucosal immunisation in sheep using the intranasal route of administration has been demonstrated for the first time to be efficient in the induction of *T. gondii* specific immune responses. Both cell-mediated and humoral responses were stimulated following immunisation, including both systemic and mucosal humoral responses. Encapsulation of toxoplasma tachyzoite antigen into a biodegradable particulate delivery system considerably enhanced these responses, but in this initial

study they were not sufficient to protect against infection. Further, as yet undefined, factors may also be required for protection.

The major obstacles encountered in the vaccination trial were the high degree of between animal variability and the small numbers of animals in each treatment group, due to cost and sample handling restrictions. The inter-individual variability in this study was likely to be due to either the natural range of responsiveness in large outbred animal populations, or uneven delivery of the particles or antigen to NALT in different animals. Within each treatment group, variation between animals occurred, but in general, animals that responded strongly for one parameter produced responses for other parameters, whilst in other animals no immune responses were detected.

In order for an ELISA to be quantitative each sample must be titrated out so that the titre at which the 50% maximum/minimum OD is produced on the sigmoid curve can be compared. However, in these studies maximum OD levels could only be demonstrated following infection with oocysts. Sigmoid titration curves could not be obtained following immunisation, suggesting that only low levels of antibody were present. Therefore antibody responses prior to infection are discussed in terms of the OD produced at a fixed dilution of sample. This provides a qualititative measure of antibody with information on the presence or absence of antibody but no indication of the amounts of antibody present.

Infection of sheep with M3 oocysts led to strong antigen-specific serum IgG antibody responses and cell-mediated immune responses. *T. gondii* is known to stimulate both humoral and cellular mechanisms in sheep, but protection is thought to be largely cell-mediated (Buxton & Innes, 1995; Buxton, 1998). Antigen-specific IgG antibody could be detected in the serum of infected animals 2 weeks post-infection, and reached maximum values shortly afterwards. A similar IgG response profile has been previously demonstrated following infection with M3 oocysts (Buxton *et al.*, 1991, 1993). Antibody titres to complete isolates of *T. gondii* are thought to rise to high values within 4 weeks of infection and to remain high for many months (McColgan *et al.*, 1988) or years (Buxton *et al.*, 1991). This may be due to the repeated antigenic challenge that may occur if tissue cysts were to break down at intervals (Conley & Jenkins, 1981). The role of IgG antibody in immunity is

unclear but a number of studies have led to the general assumption that serum antibody responses play a partial role in protection against *T. gondii* infection (Johnson *et al.*, 1983; Buxton & Innes, 1995), particularly secondary infections. Specific antibody can destroy tachyzoites in the presence of complement (Sabin & Feldman, 1948), may facilitate killing by macrophages (Joiner *et al.*, 1990) or may inhibit the invasion of cells by blocking the activity of secretory-excretory substances that enhance host cell penetration (Schwartzman, 1986). Immunoblotting revealed that the serum IgG response following infection was predominantly against a protein of 30kDa, likely to be SAG1, confirming its role as an immunodominant antigen. The IgG1 and IgG2 antibody isotypes have not been previously studied in sheep in response to *T. gondii* infection, but it would appear from these studies that both serum IgG1 and IgG2 antibodies were involved in the response, although it was not possible to compare the quantity or relevance of each isotype. The present study is also the first demonstration that antigen-specific IgG antibody is present in ovine nasal secretions following oral oocyst infection. Serum derived and locally produced IgG may reach the mucosal surfaces by passive diffusion between epithelial cells or leakage through minor breaks in the mucosal epithelium, a mechanism that is enhanced by inflammatory processes of the nasal mucosa (Brandtzaeg, 1984). It is unclear whether the nasal IgG was locally or systemically produced, but it seems likely that high levels of nasal IgG may be a reflection of the high levels present in the serum.

IgA levels following infection with *T. gondii* have not been previously investigated in sheep, and this study demonstrates that antigen-specific IgA antibody is not produced locally or systemically in the serum or in the nasal cavity following oocyst infection. This may not be surprising as IgA functions at mucosal surfaces (Mestecky & McGhee, 1987) and is likely to be produced at the gut surface following oral infection in response to penetration of the parasite into the epithelium. However, there are considerable difficulties with measuring local IgA production in the ovine gut following infection and a response here may not be reflected in increased systemic IgA antibody. No serum IgE antibody was detected following oocyst infection.

Protective immunity against *T. gondii* infection is generally considered to be cell mediated, with a special involvement of CD8+ T cells and IFNγ (Parker *et al.*, 1991; Khan *et al.*, 1994; Gazzinelli *et al.*, 1991, 1993). Proliferation of PBMCs to the tachyzoite antigen occurred *in vitro* following infection, indicative of the cellular immune response that may be central in resolving primary *T. gondii* infection. However, proliferation in response to the antigen was at relatively low levels and variable between animals. Previous studies have demonstrated low variable responses similar to those presented here in sheep infected with much higher doses of 10^5 oocysts (Esteban-Redondo, 1997). More reproducible and stronger cellular responses have been demonstrated in efferent lymphatic cannulation studies following infection (Innes *et al.*, 1995a). In such studies lymphocyte stimulation assays can be performed on cells responding to the antigen locally. This suggests that proliferation of ovine PBMCs *in vitro* in response to *T. gondii* antigen may offer a poor reflection of the status of the animal *in vivo*. It is thought that proliferation is greater when animals are actively responding to the antigen *in vivo* (weeks 2-4), after which time proliferative responses decline as fewer memory T cells specific for the antigen circulate systemically (Innes *et al.*, 1995).

In general increased IFNγ production was observed following *in vitro* stimulation with tachyzoite antigen, but the amounts of IFNγ produced were relatively low. Ovine recombinant IFNγ is known to inhibit *T. gondii* replication within ovine fibroblasts and macrophages *in vitro* (Oura *et al.*, 1993). Previous studies have similarly shown only low levels of IFNγ production by PBMCs stimulated *in vitro* following infection with 10^5 oocysts (Esteban-Redondo, 1997). Determination of IFNγ levels in efferent lymph offers a more accurate reflection of the kinetics of IFNγ production in response to oocyst infection. IFNγ appears in the lymph of sheep 2-4 days following infection and persists for 6-9 days, and cells can be demonstrated to produce IFNγ in supernatants following stimulation with *T. gondii* antigen from day 6-15 (Innes *et al.*, 1995b).

In group 5 no further increases in cellular or humoral responses were observed following a second challenge infection eight weeks after the primary infection with oocysts. This may have been due to the short time interval between infective doses if responses to the first infective dose were ongoing. Also no

differences were detected between the immune responses produced to 200 and 500 oocysts. Following primary infection animals are immune to *T. gondii*, and do not develop pyrexia when re-infected (McColgan *et al.*, 1988). Immune sheep re-infected in this study showed a slight early increase in temperature lasting one day at day 3 post-infection, but no sustained febrile responses were produced. Similarly, slight increases in temperature can be observed following infection in sheep immunised with the live S48 *T. gondii* vaccine (Buxton *et al.*, 1991, 1993).

Animals in the control group that were immunised with blank microparticles and infected with oocysts at the end of the study produced very similar immune responses to those in the naïve animals infected with oocysts, suggesting they had had no previous exposure to *T. gondii*. Prior to this, no immune responses were detected in these animals following immunisation with blank microparticles demonstrating that no non-specific responses were produced.

In general, both cell-mediated and humoral responses could be demonstrated following intranasal immunisation with toxoplasma tachyzoite antigen encapsulated into a PLG microparticle delivery system. The initial aims of the project were thus achieved, as systemic cell-mediated immunity and strong antigen-specific IgA responses could be demonstrated following intranasal immunisation with particulate antigen. The most striking difference between immune responses in immunised and infected animals was that in infected animals IgG was the predominant antibody isotype whilst IgA was predominant in immunised animals. Thus in the latter a largely mucosal antibody response was produced, with a systemic antibody response in the former. IgA was present in both the serum and nasal secretions suggesting it may have been both locally and systemically produced. Serum IgA antibody was detected transiently following the second and third immunisation, perhaps due to the short half-life that IgA antibody has in the circulation (Mestecky & McGhee, 1987)). Nasal IgA levels dramatically increased in all animals immunised with particulate antigen, and persisted at high levels for several weeks, suggesting that antibody may have been continually locally produced. The local IgA antibody response is normally short-lived after either naturally acquired infection or mucosal vaccination (Kazanji *et al.*, 1994; Keren *et al.*, 1982), but has been prolonged in this study by repeated immunisation. Immunoblotting with sera from immunised animals revealed that the

prominent antigenic band recognised by the IgA antibody at 30kDa was likely to be SAG1, demonstrating that SAG1 is immunogenic following immunisation and a relevant vaccine candidate. Similar specificity was demonstrated for the IgG antibody produced following infection. SAG1 has been implicated in the stimulation of both humoral and cellular immune responses in mice (Khan *et al.*, 1988; McLeod *et al.*, 1991). Similarly, oral immunisation of mice with a toxoplasma sonicate and CT has been shown to result in increased intestinal IgA, largely against the SAG1 protein (Bourguin *et al.*, 1991), and intranasal immunisation of mice with SAG1 plus CT leads to increased intestinal IgA antibody and IgA in nasal secretions (Debard *et al.*, 1996; Bonenfant *et al.*, 2001). In this study, IgA levels increased further following infection both locally and systemically, reflecting activation of the immune system in response to the challenge infection.

The other major finding was that increased proliferation of PBMCs or lymph node cells to the antigen could be observed *in vitro* in the majority of animals following immunisation with particulate antigen, and that the responses in some were as high as those measured in infected animals. Proliferative responses tended to fluctuate, perhaps demonstrating the transient presence of antigen-specific lymphocytes in the circulation as they migrated from the mucosal inductive site to effector sites following each immunisation. Proliferative responses have been demonstrated in spleen and MLN cells but not PBMCs in mice following intranasal immunisation with SAG1 plus CT (Debard *et al.*, 1996; Bonenfant *et al.*, 2001). Increased IFNγ production also occurred in cells from a number of immunised animals in response to antigenic stimulation, and in one animal (770N) levels of IFNγ production were much higher than those noted in animals infected with oocysts. In general, IFNγ levels were relatively low, as observed in oocyst-infected animals. Similarly, only very low or barely detectable levels of IFNγ production have been demonstrated in lymph node cells from mice intranasally immunised with SAG1 plus CT (Debard *et al.*, 1996; Velge-Roussel *et al.*, 2000). In one study increased IL-2 and IL-5 production was noted. IFNγ production and proliferative responses also tended to be higher in lymph nodes from animals immunised with particulate toxoplasma antigen, perhaps reflecting some priming of the cell-mediated immune response, resulting in earlier and stronger responses following infection.

There was a high degree of variability in proliferative responses and IFNγ production between animals in each group. In the future, it may be useful to examine the local cell-mediated responses that occur following immunisation to further characterise the kinetics of the cellular immune response.

In the present study, very little antigen-specific IgG antibody was produced in response to intranasal immunisation with particulate antigens, and no IgG could be detected in nasal secretions, reflecting the low levels in the serum. In contrast, high levels of serum IgG antibodies were detected in protected mice following intranasal immunisation with SAG1 plus CT (Debard *et al.*, 1996). Only one animal immunised with particulate antigen produced a significant IgG response, which was demonstrated to be largely against a protein of 30kDa, likely to be SAG1. Following infection, rapid increases in both serum and nasal IgG antibody could be measured. The amount of antigen-specific IgG that was produced both locally and systemically was much larger and occurred much more rapidly than in naïve animals that had been infected with oocysts. The kinetics of the IgG antibody response suggests that the immune system of immunised animals had been primed to the antigen despite the fact that only very low levels of IgG production had been demonstrated following immunisation. Supporting this observation, levels of IgG antibody were higher in all the tissues collected post-mortem in animals immunised with particulate antigen than all other treatment groups. It is thought that IgG may have a more predominant role in secondary than primary response to *T. gondii* infection because it is present early in large quantities that can target the parasite before it enters host cells (Innes & Wastling, 1995). The high levels of IgG antibody present in nasal secretions following infection reflect the high levels present in the serum. It appeared that more IgG1 was present in nasal secretions in animals immunised with particulate toxoplasma antigen than the other treatment groups, perhaps reflecting the mucosal IgG1 response that is known to participate in ruminant mucosal immunity (Butler, 1998). It is unclear whether this antibody was produced locally or systemically.

It is unclear from the present study as to whether cholera toxin has been effective as a mucosal adjuvant for the toxoplasma tachyzoite antigen in conjunction with the PLG delivery system. Similar immune responses were detected in animals immunised with particulate antigen with or without CT, suggesting that a mucosal

adjuvant was not essential for the generation of humoral and cell-mediated immunity. The inherent adjuvanticity of the PLG particles may have been sufficient for the induction of immune responses, or the quantities of CT present may have been insufficient. Similarly, in mice, oral co-immunisation with soluble cholera toxin and peptides from measles virus nucleoprotein encapsulated in 50:50 PLG microparticles resulted in no further enhancement of the observed CTL responses to the encapsulated peptide alone (Partidos *et al.*, 1999). The responses produced in animals immunised with CT tended to be slightly higher than in those immunised with particulate antigen alone for most parameters measured, but differences were not significant and the small animal numbers in each treatment group did not allow significant comparisons to be made. However it is intriguing that an impact on the febrile response produced to infection was noted only in animals that had been immunised with antigen and cholera toxin, although the significance of this response is not currently clear. In contrast, in mouse studies using soluble antigen, CT has been shown to considerably enhance immune responses to oral immunisation with whole toxoplasma sonicate or intranasal immunisation SAG1 (Bourguin *et al.*, 1991, 1993; Debard *et al.* 1996). The adjuvant effect of CT may be strongly dependent on the antigen dose (Lycke & Holmgren, 1986; Bourguin *et al.*, 1991), thus the dose of tachyzoite antigen used in this study may not have been optimal. Future studies may usefully further investigate the potential of cholera toxin as an adjuvant using larger numbers of animals. It may also be interesting to compare the adjuvant effect of both encapsulated and soluble CT, or the adjuvant effect of CT on soluble tachyzoite antigen. Quantification of the amount of CT associated with the particles is also necessary.

The use of CT as an adjuvant may have an influence on whether a Th1 or Th2 type of response is produced following immunisation, since a Th1 type of response involving IFNγ is desirable for protection against *T. gondii* (Subauste & Remington, 1991). Although *in vitro* studies have clearly demonstrated that CT stimulates Th2 clones and inhibits Th1 clones (Munoz *et al.* 1990), some debate exists as to whether CT as a mucosal adjuvant favours the development of Th2-type responses or not. Some investigators report consistently higher frequencies of antigen-specific Th2-type cells, whereas others observed induction of both Th1 and Th2 lymphokines

(Hornquist & Lycke, 1993; Marinaro *et al.*, 1995; Vajdy & Lycke, 1993; Xu-Amano *et al.*, 1993). The intranasal administration of CT with SAG1 in mice suggested that both Th1 and Th2 type response had been produced (Debard *et al.*, 1996).

The responses to intranasal immunisation with soluble toxoplasma tachyzoite antigen were variable. No immune responses to immunisation were observed for the majority of animals in this group. This study confirms findings that in general soluble antigens are poor mucosal immunogens (Waldo *et al.*, 1994). Soluble antigen may not be taken up by M cells, or may be processed or presented to the immune system in different ways. However, the fact that these animals showed similar immune responses to those induced by particulate antigen demonstrates that there is some potential to stimulate both local and systemic immune responses following intranasal administration of soluble antigen in sheep under appropriate conditions. Particulate antigen, however, is preferable and superior for the generation of more frequent and stronger immune responses. The variability in this treatment group may be explained by differences in the ability of animals to respond to the soluble antigen, or the amount of antigen that reached the appropriate area of NALT or the underlying mucosal immune system. In general, poor immune responses have been produced following oral immunisation of mice with soluble *T. gondii* sonicate alone (Bourguin *et al.*, 1991, 1993) or intranasal administration of soluble SAG1 alone (Debard *et al.*, 1996). Responses to these antigens were enhanced and protective when CT was used as an adjuvant, however intranasal immunisation in ewes with soluble SAG1 plus CT did not lead to protection (D. Bout, personal communication). Despite the fact that no immune responses could be detected in the majority of animals following immunisation, considerable increases in serum and nasal IgG antibody could be detected in all animals following oocyst infection, much more rapidly and in much larger quantities than in naïve animals. In addition, levels of IgG antibody were higher in all the tissues collected post-mortem in animals immunised with soluble antigen than negative control animals or animals initially infected with oocysts. This strongly suggests that some systemic priming of the immune system had occurred in all animals following intranasal administration of soluble antigen, as demonstrated in the animals immunised with particulate antigen. Rapid increases in

cell-mediated immunity were also observed in some animals in this group one week post-infection.

Despite the increased mucosal IgA antibody and cell-mediated responses following infection, and the priming of the IgG antibody response, immunised animals were not protected against a challenge infection of oocysts, as assessed by the febrile response. All animals demonstrated pyrexia, much higher than the normal body temperature of 39.3°C, for several days following infection. This suggested that the immune responses produced were not in sufficient quantity or quality for protection. A febrile response around 4-6 days following infection of susceptible sheep with *T. gondii* is a very consistent clinical finding (Miller *et al.*, 1982; Dubey, 1984; McColgan *et al.*, 1988; Buxton *et al.*, 1991, 1994) and is correlated with a detectable parasitaemia (Buxton, 1990). Pyrexia is thought to be due to the induction of IL-1 (an endogenous pyrogen) and prostaglandin E_2 (involved in suppression of a variety of relevant immune factors) following macrophage activation by *T. gondii* (Roitt & Delves, 2001). One of the negative control animals succumbed to acute toxoplasmosis following infection, even though infective doses were low. It is interesting to note that no immunised animals were affected in the same way.

An interesting increase in temperature, very similar to that observed in immune animals, was measured in animals that had been immunised with particulate tachyzoite antigen and cholera toxin. It is tempting to suggest that this early rise in temperature might indicate the release of lymphokines by activated lymph node cells demonstrating recognition of the parasite by the immune system. However, the responses mounted were not able to clear the infection at this point and pyrexia followed two days later. It is also interesting to note that the maximum temperature reached tended to be lower in this group than in the negative control animals, and that the temperature returned to baseline one day earlier. Thus intranasal immunisation with particulate antigen plus CT had an impact on the febrile response, despite the fact that protection could not be demonstrated. In mice, protection may be assessed by the degree of reduction in the number of tissue cysts following infection. This method is unlikely to produce meaningful results using such small infective doses in large animals such as sheep, because accurate detection of tissue cyst

numbers would be difficult. A reduction of 85% in tissue cyst numbers in mouse brain following infection with 100 cysts of the 76K strain, for at least 5 months, has been demonstrated following intranasal immunisation with SAG1 plus CT (Debard *et al.*, 1996), however in these murine studies febrile responses following infection were not monitored. It may have been interesting to compare the febrile response in protected mice with that produced in sheep immunised with particulate antigen and CT. In the future pregnant ewe studies would allow assessment of protection by a reduction in the number of abortions, and therefore direct comparison with human infection.

In summary this pilot study has shown that there is considerable potential for intranasal immunisation against *T. gondii* in sheep, demonstrated by the high levels of mucosal and systemic IgA antibody, and increased proliferation in response to the antigen in conjunction with increased production of IFNγ.

CHAPTER 6

General Discussion

This study has clearly demonstrated that the intranasal immunisation route in sheep offers potential for the stimulation of both mucosal and systemic immunity, involving both cell-mediated and humoral immune responses, and the original hypothesis has been proved. These results are extremely encouraging and demonstrate that mucosal immunisation in sheep has potential as a strategy for the control of infectious disease. By stimulating an effective mucosal IgA response at mucosal surfaces it is hoped that a barrier to infection may be created. A systemic response is also stimulated to target pathogenic organisms that enter the circulation. The majority of current vaccinations in sheep are delivered via the systemic route, and although this may be effective at stimulating a systemic immune response that targets the organism once it has entered the body, the prevention of pathogen entry may be more effective for the control of infection. Previous studies that target the ovine mucosal immune system are scarce, and this is the first demonstration that immune responses can be produced following intranasal delivery of an antigen encapsulated into PLG particles. Another study has demonstrated the potential of an ISCOMATRIX® adjuvanted intranasal influenza vaccine in sheep to stimulate antibody responses (Coulter *et al.*, 2003). Similar to the present study strong mucosal IgA responses could be detected in nasal secretions following immunisation, as well as strong serum IgG responses. These responses were enhanced compared to subcutaneous administration of unadjuvanted vaccine, but whether these responses were protective was not assessed.

In addition to the fully competent mucosal immune system that exists in the ovine jejunal Peyer's patches (Larsen & Landsverk, 1986; Hein *et al.*, 1989; Mutwiri *et al.*, 2000), the present study has demonstrated that there is a fully developed arm of the mucosal immune system in the ovine nasopharyngeal tract, similar to the NALT described in other species. This lymphoid tissue has the characteristics of a mucosal immune inductive site fully capable of the induction of a mucosal immune response. In addition it appears that M cells are present in the epithelium overlying this tissue that are fully capable of the uptake of particulate antigen and delivery to the underlying lymphoid tissue. The nasal route of immunisation is preferable to the oral route in sheep and other ruminants, due to the harsh conditions of pH and enzymatic activity that antigen must encounter in the rumen before reaching MALT

in the jejunal PP. However, intranasal immunisation may only be preferable if sheep have a common mucosal immune system, so that stimulation of NALT results in the production of generalised mucosal immune responses at other mucosal surfaces. A number of studies in rodent models have suggested that the recirculation of activated lymphocytes from NALT to GALT does occur following intranasal immunisation (Wu & Russell, 1993, 1998; Velge-Roussel *et al.*, 2000). However, in contrast there is some evidence in both mice and pigs that although recirculation may occur from GALT to NALT, migration of activated lymphocytes from NALT to GALT is negligible (Sminia *et al.*, 1989; Nadal *et al.*, 1991; VanCott *et al.*, 1994; Saif, 1996). In the sheep there appears to be differential expression of homing molecules on recirculating lymphocytes from the gut and the lung, suggesting that compartmentalisation between the respiratory tract and gut-associated lymphoid tissues may exist (Abitorabi *et al.*, 1996).

Despite the mucosal and cell-mediated responses that were produced to immunisation in this study, protection against oral infection with *T. gondii* oocysts was not achieved. Similarly, some authors have suggested that intranasal administration of antigens may induce mucosal and systemic immune responses but may fail to trigger a strong protective immunity (Bonenfant *et al.*, 2001), the reasons for which are unclear. One possible reason for this may have been that the challenge infection of *T. gondii* oocysts was delivered via the oral rather than the intranasal route. It would have been interesting to determine whether intranasal administration of the same dose of oocysts would have afforded better protection, since strong local immune responses were detected in the nasal cavity. However, infection with *T. gondii* generally occurs via the oral route and triggers a cellular response in the gut (Chardes *et al.*, 1990, 1993) and intranasal delivery of a challenge infection of oocysts would not offer a realistic reflection of the natural route of infection. Nevertheless, 85% protection against oral infection with *T. gondii* has been demonstrated in similar intranasal immunisation studies in mice (Debard *et al.*, 1996). In the future it may be interesting to perform a similar immunisation study against a respiratory pathogen or a model infectious agent that uses the nasal route as the natural route of infection.

Tolerogenic responses were not investigated following immunisation, but the finding that animals were primed in response to a challenge infection, rather than suppressed, suggests that tolerance had not been produced. Thus there is no evidence that tolerance was induced but this matter will require further investigation in future studies.

A drawback of this study was that it was not possible to determine whether local immune responses were produced in the gut as well as the nasal cavity following intranasal immunisation, and thus the degree of compartmentalisation of the ovine mucosal immune system remains unknown. The aim of this initial experiment was to determine whether protective immunity had been stimulated by the immunisation regime, which required all animals to be dosed with a live challenge of the organism. When gut samples were collected post-mortem the immune responses that had been stimulated following immunisation were masked by the responses produced to infection. Following oral infection, toxoplasma sporozoites penetrate epithelial cells and stimulate the mesenteric lymph node and the systemic immune system (Dubey, 1984), as was reflected in the lymph nodes and spleens collected post-mortem. The fact that protective immunity against an oral infection could not be achieved suggests that some compartmentalisation of the ovine mucosal immune system does exist, or alternatively that intranasal immunisation did not result in the production of a sufficiently robust mucosal immune response at the gut surfaces. Despite this, it was interesting to demonstrate priming of the systemic IgG response to infection in gut and respiratory tract tissues from immunised animals. Systemic lymph nodes collected post-mortem from animals immunised with particulate antigen were also able to respond better to *in vitro* stimulation with the antigen, both in terms of proliferation and IFNγ production. Studies in mice have suggested that immune responses are generated both in the gut and the nasal cavity following intranasal immunisation with SAG1 (Velge-Roussel *et al.*, 2000). However in these murine studies, animals were anaesthetised before intranasal administration of antigen, which may have led to some antigen being swallowed, resulting in direct stimulation of the GALT as well as the NALT.

Future studies should thus not only confirm the stimulation and priming of the ovine mucosal and systemic immune responses, but investigate the degree of

compartmentalisation of the mucosal immune system. For this purpose immune responses in the nasal mucosa and at other mucosal sites to an inert antigen could be monitored. Lymphatic cannulation experiments may be performed on afferent and efferent lymph from lymph nodes draining both NALT and GALT to study lymphocyte activation at local and more distant mucosal sites. ELISPOT and lymphocyte stimulation assays could determine the degree of both B and T lymphocyte activation. Although it may not be possible to cannulate the retropharyngeal lymph node draining NALT, due to its location in the head, it may be possible to cannulate the cervical lymph nodes or the pre-scapular lymph node. The mesenteric lymph node may be cannulated to provide information on the activation of cells in the GALT. In addition, activated lymphocytes could be collected from the lymph, labelled, re-transfused and their presence monitored in efferent lymph to determine recirculation and migration pathways. Such studies would be difficult in small rodents, but similar experiments have been previously performed in pigs (Rothkotter *et al.*, 1999) and this is one of the advantages of employing larger animals in these studies. Moreover, it is not possible to preclude the overspill of intranasally administered antigen to the bronchus or the gut, but this is likely to be minimal in large animals compared to rodent models.

One of the major problems encountered in large animal studies is the high degree of between animal variability that is often encountered in immune responses. In this study some animals could be shown to mount a number of different responses following immunisation, whereas others failed to mount any response. It now remains to be determined whether this was a genuine reflection of the range of between animal variability, or whether a more sophisticated delivery system would result in more even distribution of the antigen to NALT. There may be differences between animals in the ability to take up or respond to particulate antigen, perhaps due to the number of M cells present in the FAE. Some authors have shown that the number of M cells increase following antigen exposure (Savidge *et al.*, 1991; Meynell *et al.*, 1999), and thus the number of M cells present and the ability of these cells to take up particulate antigen may be due to the degree of previous antigenic exposure in each animal. Differences in the quantity of antigen that was presented to the immune system would be reflected in the immune responses produced. When

inbred strains of laboratory animals are used, results are often more uniform but are less likely to reflect the situation in large animal models and humans.

The PLG polymer microparticle delivery system has been shown to offer good potential for the mucosal delivery of antigen in sheep and other ruminants, demonstrated by the enhanced immune responses that were produced to encapsulated antigen in this study. This system was chosen because it is the most widely used strategy to optimise antigen delivery to MALT, the most well-defined in terms of the characterisation of the immune responses produced following immunisation, and has been shown to generate protective mucosal immune responses for a number of pathogens in mouse models. It may also protect antigen on passage through the rumen in future investigation of the oral immunisation route. This study has demonstrated that nanoparticles that may optimise M cell uptake (Brayden & Baird, 2001) can be easily produced in large quantities. Results presented here also offer useful information on the effect of a number of parameters on protein encapsulation into sub-micron particles, although methods must be tailored to individual proteins. Most importantly, the ability to encapsulate a mixture of biologically active and functional proteins has been demonstrated, since most previous studies have focused on the encapsulation of one model protein, and this is the first demonstration of encapsulation of a soluble toxoplasma tachyzoite extract into particles made from the PLG polymer. In addition, the demonstration of the co-encapsulation of SAG1 and CT offers the possibility of simultaneous presentation of the mucosal adjuvant with the antigen to the immune system, which is likely to be preferable to using a soluble adjuvant alongside the particulate delivery system. The retained functional activity of encapsulated LLO is also extremely encouraging and demonstrates that functionality and antigenicity of proteins may be retained despite the harsh conditions of encapsulation.

The theory that particulate antigen was taken up by M cells overlying the NALT was central to these studies. However, the cells responsible for particle uptake were not defined and future work should focus on further characterisation of M cells in this region in the sheep. The investigation of M cell specificity and development may allow optimisation of the uptake and delivery of antigenic material to NALT. A method of isolating or culturing ovine M cells *in vitro* would be extremely useful for

such studies, but to date efforts to culture M cells from other species or sites have been largely unsuccessful.

T. gondii was the model disease chosen for the investigation of the potential of intranasal immunisation in sheep because of the well-defined systems to measure immune responses to the antigen, and protectivity of these responses by the febrile response to infection. However, a subunit vaccine for the stimulation of protective immunity in sheep is highly desirable for *T. gondii* in sheep, since the live attenuated vaccine that currently exists (Buxton *et al.*, 1991) has many associated drawbacks. An effective vaccine in sheep may also offer potential as a control strategy for human toxoplasmosis, since the pregnant ewe offers a good model of the human disease. Vaccination with live organisms in humans is unlikely due to possible reversion to virulence or the risk of vaccinating immunosuppressed individuals, and mucosal delivery of subunit vaccines, particularly by the intranasal route, offers good potential for the stimulation of protective immunity (Debard *et al.*, 1996). It has been proposed that an acceptable and effective human vaccine would have to carry the optimised synthetic vaccine (subunit or DNA) plus an appropriate adjuvant, and to target the mucosal dendritic cells by means of an inert delivery system such as polymer microparticles, which can be endocytosed by M cells of the gut or nasal-associated lymphoid tissues (Bout *et al.*, 2002). This study has provided initial encouraging results towards this ultimate aim in sheep, which may now be further developed. Strong antigen-specific local and systemic IgA responses were produced following intranasal immunisation, as were cell-mediated responses with some IFNγ production. These are thought to be the most effective components of the immune system in the control of toxoplasmosis (Subauste & Remington, 1991; Gazzinelli *et al.*, 1991, 1993), and offer real potential for protection against the disease.

It is clear that a great deal of further research is now required to investigate the stimulation of protective immunity using the intranasal immunisation route in sheep, since protection was not achieved in this pilot study. Protection against infection is the obvious goal of any vaccination strategy, and if future improvements to the techniques presented here could lead to the generation of fully effective

immune responses this immunisation strategy could offer huge benefits to the farming industry, not only for the control of toxoplasmosis, but for a wide range of infectious agents. To be widely applicable an intranasal vaccine for sheep must be developed to facilitate easy and quick immunisation of a large number of animals with little technical expertise. In addition, the development of a single dose vaccine to preclude the need for booster vaccinations is highly desirable. The possibility for such a vaccine lies in the mucosal delivery of a combination of particles made from different PLG polymers, with different release characteristics, to prolong antigen release and stimulation of the immune system. Optimisation of the type and dose of mucosal adjuvant is one of the main ways in which protective immune responses may be stimulated, as is manipulation of the antigen and delivery system. Further to this, there is potential to use this route of immunisation for DNA vaccination, and PLG particles can be developed to encapsulate or surface-bind DNA. In addition, it would be useful to use a viral or bacterial respiratory pathogen as a model system in future studies to determine whether protective responses at the natural site of infection could be detected.

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Appendices

Appendix 5.1 *Comparison of temperature response to oral infection with 200 and 500 toxoplasma oocysts*

Oocyst Dose	Animal Number	Day															
		-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
		Temperature (°C)															
200	927N	39.51	39.34	39.73	39.11	39.41	40.31	42.09	42.13	41.45	40.6	40.44	39.55	39.35	39.21	39.39	39.25
200	976N	39.6	39.72	39.94	39.93	39.72	39.4	40.86	41.67	41.95	41.21	41.25	40.11	39.07	39.27	39.69	39.64
500	1032N	39.49	39.63	39.62	39.56	39.26	39.29	41.54	42.08	41.78	41.57	41.4	40.25	39.4	39.18	39.62	39.23
500	1084N	39.56	39.48	39.5	39.65	39.55	39.48	40.83	41.72	41.82	41.07	40.02	39.27	39.43	38.99	39.48	39.33

Appendix 5.2 *Temperature responses following oral infection with 200 toxoplasma oocysts*

Treatment Group	Animal Number	Day -1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
		Temperature (°C)															
1. control	913N	39.75	39.72	39.51	39.66	39.4	39.94	41.69	42.27	42.02	41.71	41.14	40.91	39.42	39.29	38.88	39.49
	915N	39.82	39.63	39.75	39.87	39.71	39.95	40.88	41.77	41.81	41.59	41.33	41.06	40.14	39.82	39.31	39.74
	950N	40.33	39.62	39.57	39.58	39.46	39.94	41.33	41.85	41.88	41.47	40.96	39.8	39.96	39.29	39.31	*
	1048N	40.06	39.93	39.76	39.68	40.05	40.03	41.71	41.77	41.67	41.47	41.48	41.32	41.69	40.92	40.94	*
2. i.n. soluble toxo	638N	39.51	39.39	39.23	39.64	39.38	39.46	40.68	41.59	41.65	41.14	40.57	39.95	39.79	39.33	39.08	39.92
	748N	39.84	39.93	39.67	39.7	39.68	39.75	41.34	41.46	41.8	41.54	41.06	40.39	40.05	39.74	39.66	40.2
	919N	39.48	39.49	39.51	39.24	39.27	39.35	40.75	41.41	41.58	41.73	41.02	40.77	39.66	39.23	39.25	*
	923N	39.43	39.46	39.52	39.72	39.29	39.71	41.33	41.89	41.46	41.47	40.99	40.72	39.64	39.52	39.34	39.35
	993N	39.94	39.77	40.08	39.8	39.79	39.73	41.34	41.7	41.79	41.26	40.65	40.57	39.93	39.88	39.73	40.34
	1031N	39.36	40.01	39.89	39.79	39.51	41.02	41.07	41.5	41.41	41	40.53	39.46	39.21	39.11	40.32	*
	1034N	39.54	39.49	39.9	39.58	39.72	39.53	41.76	41.98	41.68	41.92	41.05	40.54	39.54	39.28	39.01	39.54
	1117N	40.16	39.59	40.09	39.86	39.99	39.95	41.59	41.79	41.86	41.52	41.44	41.24	40.84	39.95	39.9	40.37
3. i.n. toxo particle	715N	39.88	40.09	40.04	39.86	39.92	41.16	41.93	42.19	41.96	41.48	41.54	40.78	39.99	39.2	39.27	39.57
	739N	39.92	39.78	40.19	39.48	39.52	41.97	42.12	42.06	41.81	41.8	41.36	39.74	38.97	39.11	39.08	39.23
	764N	39.75	39.67	39.78	39.68	39.93	40.52	41.1	42	42.32	41.21	40.41	40.38	40.09	39.63	39.11	39.56
	1067N	39.52	39.72	39.67	39.48	39.68	40.51	41.46	41.8	41.43	41.38	40.46	39.76	39.43	39.24	39.32	39.45
4. i.n. toxo particle + CT	770N	39.2	39.39	39.58	40.08	39.69	40.39	41.36	41.57	41.59	41.09	40.4	40.16	39.37	39.25	39.13	39.15
	989N	39.48	39.37	39.52	40.38	39.51	41.13	41.64	41.37	41.37	41.34	40.61	39.91	38.64	38.9	39.07	39.7
	1030N	39.51	39.41	39.51	40.07	39.35	40.19	41.91	41.87	41.67	41.19	41.09	40.1	39.1	39.09	39.34	39.7
	1116N	39.55	39.43	39.48	39.45	39.32	40.23	41.37	41.89	42.21	41.95	41.12	40.68	40.04	39.24	39.22	39.04
5.oocyst challenge	927N	39.81	39.78	39.73	39.46	40.22	39.01	39.48	39.97	39.34	39.93	39.19	39.43	39.72	39.45	39.94	39.99
	976N	39.69	39.63	39.76	39.75	40.44	39.85	39.9	39.88	39.56	39.75	39.56	39.52	39.46	39.38	39.61	39.78
	1032N	39.72	39.61	39.67	39.87	39.97	39.81	39.54	39.52	39.6	39.69	39.31	39.42	39.34	39.5	39.78	39.25
	1084N	39.73	39.47	39.53	39.5	40.07	39.14	39.16	39.31	39.46	39.48	39.17	39.12	39.04	39.14	39.47	39.31

Appendix 5.3 *Serum IgA responses*

Treatment Group	Animal Number	Normalised OD @ 492nm											
		Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11
1. control	913N	0.054	-0.031	0.050	0.050	-0.021	0.041	0.040	0.046	0.050	0.050	0.108	0.099
	915N	0.061	0.065	0.081	0.071	0.039	0.055	0.067	0.071	0.094	0.039	0.064	0.122
	950N	0.042	0.054	0.052	0.051	0.042	0.042	0.053	0.041	0.061	0.064	0.077	0.187
	1048N	0.063	0.071	0.069	0.084	0.098	0.095	0.089	0.065	0.092	0.092	0.078	0.288
2. i.n. soluble toxo	638N	0.090	0.083	0.092	0.103	0.097	0.110	0.096	0.116	0.143	0.105	0.177	0.122
	748N	0.183	0.134	0.126	0.138	0.075	0.091	0.068	0.074	0.073	0.059	0.145	0.162
	919N	0.064	0.064	0.071	0.117	0.100	0.074	0.093	0.085	0.084	0.122	0.080	0.232
	923N	0.053	0.045	0.045	0.192	0.099	0.195	0.107	0.080	0.091	0.075	0.139	0.357
	993N	0.134	0.108	0.134	0.116	0.117	0.142	0.108	0.148	0.169	0.133	0.145	0.163
	1031N	0.052	0.099	0.059	0.060	0.042	0.118	0.062	0.037	0.091	0.064	0.071	0.110
	1034N	0.115	0.136	0.105	0.120	0.085	0.084	0.068	0.113	0.102	0.075	0.077	0.116
	1117N	0.072	0.075	0.058	0.070	0.056	0.082	0.085	0.089	0.079	0.071	0.096	0.387
3. i.n. toxo particle	715N	0.135	0.098	0.122	0.540	0.184	0.501	0.261	0.133	0.157	0.179	0.068	0.461
	739N	0.067	-0.003	0.059	0.090	0.046	0.068	0.060	0.064	0.063	0.026	0.052	0.083
	764N	0.066	0.056	0.059	0.203	0.057	0.135	0.093	0.053	0.062	0.032	-0.053	0.108
	1067N	0.167	0.155	0.239	0.251	0.159	0.281	0.187	0.189	0.212	0.203	0.178	0.426
4. i.n. toxo particle + CT	770N	0.240	0.378	0.287	0.943	0.304	0.695	0.329	0.209	0.222	0.203	0.212	0.389
	989N	0.067	0.055	0.060	0.121	0.048	0.076	0.063	0.051	0.060	0.040	0.092	-0.005
	1030N	0.078	0.078	0.089	0.289	0.055	0.317	0.081	0.122	0.113	0.087	0.099	0.180
	1116N	0.106	0.116	0.106	0.229	0.091	0.154	0.089	0.100	0.098	0.081	0.136	0.203
5. oocyst challenge	927N	0.238	0.268	0.173	0.134	0.110	0.099	0.106	0.127	0.099	0.094	0.106	*
	976N	0.054	0.078	0.188	0.073	0.059	0.066	0.063	0.059	0.067	0.078	0.034	*
	1032N	0.062	0.114	0.087	0.054	0.052	0.064	0.075	0.069	0.082	0.070	0.046	*
	1084N	0.076	0.115	0.292	0.077	0.032	0.052	0.050	0.191	-0.018	0.049	0.052	*

Appendix 5.4 *Nasal IgA responses*

Treatment Group	Animal Number	Normalised OD @ 492nm											
		Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11
1. control	**913N**	0.047	0.055	0.040	0.050	0.046	0.053	0.054	0.050	0.048	0.050	0.046	0.070
	915N	0.052	0.054	0.062	0.057	0.049	0.050	0.051	0.055	0.048	0.046	0.048	0.074
	950N	0.043	0.049	0.046	0.037	0.035	0.036	0.054	0.055	0.050	0.041	0.024	0.140
	1048N	0.075	0.057	0.099	0.067	0.054	0.108	0.065	0.074	0.059	0.047	0.051	0.065
2. i.n. soluble toxo	**638N**	0.043	0.047	0.047	0.052	0.069	0.108	0.069	0.077	0.051	0.056	0.039	0.062
	748N	0.021	0.047	0.051	-0.054	0.068	0.057	0.077	0.077	0.063	0.061	0.056	0.086
	919N	0.045	0.040	0.051	0.061	0.056	0.064	0.063	0.059	-0.162	0.056	0.054	0.170
	923N	-0.015	0.040	0.040	0.124	0.032	0.139	0.107	0.121	0.078	0.124	0.073	0.311
	993N	0.020	0.044	0.058	0.051	0.129	0.155	0.114	0.118	0.083	0.120	0.124	0.299
	1031N	0.044	0.052	0.066	0.050	0.084	0.180	0.082	0.061	0.059	0.063	0.074	0.082
	1034N	-0.015	0.042	0.077	0.035	0.067	0.000	0.017	0.076	0.060	0.046	0.041	0.045
	1117N	0.011	0.051	0.058	0.055	0.058	0.060	0.055	0.058	0.055	0.068	0.065	0.148
3. i.n. toxo particle	**715N**	0.054	0.067	0.126	1.028	0.780	0.713	0.899	1.130	0.978	0.640	0.946	1.167
	739N	0.063	0.075	0.053	0.179	0.269	0.081	0.145	0.177	0.096	0.073	0.119	0.221
	764N	0.079	0.149	0.121	0.789	0.377	0.306	0.419	0.143	0.096	0.110	0.146	0.635
	1067N	-0.034	-0.001	0.041	0.575	0.214	0.499	0.399	0.684	0.137	0.130	0.184	0.345
4. i.n. toxo particle + CT	**770N**	0.071	0.147	0.259	1.146	0.573	0.901	0.808	0.746	0.516	0.533	0.471	1.035
	989N	0.012	0.053	0.060	0.347	0.094	0.203	0.115	0.104	0.061	0.060	0.056	0.107
	1030N	0.052	0.058	0.112	0.526	0.178	0.549	0.431	0.303	0.188	0.114	0.180	0.408
	1116N	-0.007	0.016	0.058	0.461	0.333	0.246	0.156	0.121	0.071	0.049	0.050	0.656
5. oocyst challenge	**927N**	*	*	0.045	0.009	0.056	0.037	0.059	0.055	0.051	0.056	0.048	*
	976N	*	*	0.026	0.103	0.251	0.207	0.130	0.062	0.066	0.049	0.127	*
	1032N	*	*	*	0.072	0.085	0.071	0.059	0.065	0.060	0.059	0.056	*
	1084N	*	*	0.004	0.062	0.067	0.060	0.053	0.063	0.053	0.051	0.049	*

Appendix 5.5 *Tissue IgA responses*

Treatment Group	Animal Number	Normalised OD @ 492nm													
		NALT	PT	Trachea	Bronchus	Lung	Abo Fold	Duodenum	Jejenum	Ileum	PP	LI	Rectum	Spleen	MLN
1. control	913N	0.086	0.109	*	0.076	0.043	-0.210	0.311	0.564	0.303	0.184	0.236	0.156	0.062	0.079
	915N	0.086	0.090	0.214	0.150	0.190	0.198	0.331	0.676	0.327	0.225	-0.230	0.078	0.028	0.121
	950N	0.096	0.067	0.100	0.141	0.227	-0.003	0.377	0.604	0.423	0.269	0.048	0.111	0.081	0.127
	1048N	0.097	0.088	0.152	0.227	0.201	0.013	0.330	0.635	0.543	0.556	0.134	0.178	0.535	0.464
2. i.n. soluble toxo	638N	0.009	0.044	0.104	0.073	-0.056	0.153	0.421	0.560	0.210	0.234	0.081	0.012	0.053	0.066
	748N	-0.009	0.892	0.196	0.134	0.352	0.917	0.951	1.020	0.844	0.739	0.136	0.035	0.090	0.067
	919N	0.197	0.168	0.178	0.183	0.274	0.211	0.544	0.679	0.443	0.330	0.087	0.081	0.124	0.083
	923N	0.112	0.134	0.509	0.392	0.376	0.449	0.445	0.504	0.473	0.270	0.792	0.125	0.104	0.182
	993N	0.088	0.047	0.275	0.442	0.828	0.591	0.848	0.754	0.403	0.231	0.248	0.283	0.051	0.122
	1031N	0.027	0.063	0.766	0.357	1.007	0.097	0.257	0.333	0.108	0.112	0.046	0.066	0.037	0.017
	1034N	0.027	0.088	0.104	0.097	0.102	0.069	0.554	0.444	0.444	0.334	0.224	0.062	0.040	0.083
	1117N	0.078	0.229	0.291	0.266	0.815	0.453	0.534	0.614	0.304	0.271	0.171	0.118	0.444	0.145
3. i.n. toxo particle	715N	0.409	0.372	1.208	0.831	1.126	1.006	0.917	0.595	0.392	0.355	0.735	0.372	0.478	0.232
	739N	0.009	0.071	0.337	0.269	0.351	0.255	0.401	0.572	0.226	0.137	0.044	0.088	0.063	0.088
	764N	-0.051	0.051	0.095	0.066	0.058	-0.035	0.242	0.182	0.068	0.035	0.031	0.020	0.041	0.061
	1067N	0.137	0.121	0.156	0.124	0.328	1.270	1.412	1.349	1.211	0.611	0.007	0.112	0.113	0.116
4. i.n. toxo particle + CT	770N	0.194	0.239	0.402	0.337	0.772	0.343	0.747	0.777	0.536	0.522	0.089	0.073	0.208	0.152
	989N	0.061	0.050	0.173	0.062	0.090	0.198	0.115	0.187	0.074	0.033	0.062	0.016	0.037	0.063
	1030N	0.039	0.120	0.098	0.068	0.078	0.484	0.407	0.596	0.596	0.278	0.280	0.076	0.069	0.249
	1116N	0.033	0.041	0.248	0.102	0.223	0.221	0.639	0.541	0.234	0.165	0.074	0.017	0.048	0.034
5. oocyst challenge	927N	-0.022	0.037	0.084	0.050	0.060	0.021	0.430	0.601	0.936	0.369	0.223	-0.017	0.041	0.159
	976N	0.048	0.039	0.089	-0.042	0.056	0.088	0.895	0.574	0.242	0.229	0.053	0.039	0.040	0.037
	1032N	-0.018	0.055	-0.019	0.010	-0.007	0.003	0.071	0.133	0.088	0.043	0.046	0.031	0.038	0.029
	1084N	0.012	0.042	-0.004	0.137	0.121	-0.150	0.240	0.259	0.126	0.076	0.009	0.065	0.044	0.026

Appendix 5.6 *Serum IgG responses at a fixed dilution of 1:100*

Treatment Group	Animal Number	Normalised OD @ 492nm											
		Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11
1. control	**913N**	-0.001	-0.002	-0.006	0.000	0.002	0.005	0.001	0.000	0.005	0.003	0.018	0.838
	915N	-0.004	0.011	-0.005	0.005	-0.004	-0.008	-0.010	-0.001	0.003	-0.007	0.027	0.134
	950N	-0.003	-0.001	0.001	-0.006	0.001	-0.005	-0.004	-0.002	0.025	0.001	-0.009	0.149
	1048N	0.002	-0.002	0.008	0.004	0.006	0.001	-0.001	-0.003	0.011	-0.003	0.012	0.015
2. i.n. soluble toxo	**638N**	-0.017	-0.004	0.000	-0.006	0.000	-0.008	-0.002	0.000	0.010	-0.001	0.703	0.979
	748N	-0.007	-0.008	-0.007	-0.013	-0.006	-0.005	-0.003	-0.001	-0.003	-0.004	0.096	0.956
	919N	-0.002	-0.003	-0.002	-0.006	-0.008	-0.009	-0.007	-0.001	0.011	-0.001	0.043	0.953
	923N	-0.001	-0.004	0.000	0.007	0.012	0.016	0.010	0.004	0.024	-0.003	0.548	1.039
	993N	-0.008	0.000	-0.004	-0.010	0.010	0.003	-0.004	0.007	0.010	-0.002	0.133	0.964
	1031N	-0.004	-0.003	-0.002	-0.012	-0.003	-0.009	-0.006	-0.010	-0.008	-0.002	0.750	0.984
	1034N	-0.009	-0.002	0.001	-0.006	0.001	0.012	-0.003	-0.002	0.001	0.002	0.466	1.015
	1117N	-0.009	-0.004	0.005	-0.005	0.004	-0.004	-0.003	-0.003	0.014	0.002	-0.005	0.968
3. i.n. toxo particle	**715N**	-0.014	-0.003	-0.007	-0.006	-0.004	0.000	0.003	0.001	0.048	0.005	0.056	0.968
	739N	-0.018	-0.002	0.000	0.225	0.221	0.129	0.091	0.020	0.080	0.018	0.047	0.984
	764N	0.007	0.000	0.005	0.037	0.085	0.067	0.083	0.018	0.081	0.020	0.078	0.994
	1067N	-0.014	-0.008	-0.005	0.005	0.003	0.005	0.003	0.000	0.007	0.001	0.014	0.994
4. i.n. toxo particle + CT	**770N**	0.010	0.006	0.077	0.087	0.235	0.930	0.839	0.434	0.781	0.459	0.388	1.048
	989N	0.000	0.001	-0.006	0.019	0.038	0.016	0.032	0.003	0.014	0.003	0.007	1.065
	1030N	0.000	0.000	-0.002	-0.004	0.012	0.097	0.103	0.015	0.078	0.028	0.047	1.037
	1116N	-0.011	-0.006	-0.006	-0.005	0.003	-0.006	-0.005	0.001	0.003	0.000	-0.008	0.973
5. oocyst challenge	**927N**	0.270	0.314	0.674	1.028	0.974	1.031	1.079	1.040	1.001	1.000	1.070	*
	976N	0.005	0.002	0.270	0.930	0.958	1.027	0.988	0.994	0.992	0.988	0.995	*
	1032N	0.000	-0.004	0.299	0.951	0.955	0.975	1.045	0.980	0.973	0.970	0.995	*
	1084N	0.028	0.001	0.047	0.616	0.800	0.926	0.934	0.941	0.883	0.907	0.870	*

Appendix 5.7 *Titre of serum at which 50% maximum/minimum OD was reached for IgG*

Treatment Group	Animal Number	Normalised OD @ 492nm											
		Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11
1. control	913N	0	0	0	0	0	0	0	0	0	0	50.0	399.6
	915N	0	0	0	0	0	0	0	0	0	0	0	65.0
	950N	0	0	0	0	0	0	0	0	0	0	0	35.8
	1048N	0	0	0	0	0	0	0	0	0	0	0	50.0
2. i.n. soluble toxo	638N	0	0	0	0	0	0	0	0	0	0	121.9	3965.9
	748N	0	0	0	0	0	0	0	0	0	0	50.0	1504.0
	919N	0	0	0	0	0	0	0	0	0	0	40.0	1243.5
	923N	0	0	0	30.0	40.0	40.0	40.0	50.0	50.0	40.0	142.8	6888.7
	993N	0	0	0	0	0	0	0	0	0	0	60.0	1317.6
	1031N	0	0	0	0	0	0	0	0	0	0	125.2	1990.1
	1034N	0	0	0	0	0	0	0	0	0	0	43.4	1651.7
	1117N	0	0	0	0	0	0	0	0	0	0	0.0	1340.5
3. i.n. toxo particle	715N	0	0	0	0	0	0	0	0	0	0	0	3756.9
	739N	0	0	0	37.4	70.0	70.0	60.0	70.0	40.0	40.0	30.0	7150.0
	764N	0	0	0	50.0	50.0	50.0	60.0	50.0	50.0	50.0	50.0	8663.2
	1067N	0	0	0	0	0	0	0	0	0	0	40.0	5935.8
4. i.n. toxo particle + CT	770N	0	0	40.0	50.0	50.0	227.5	131.9	102.5	156.2	42.5	38.6	7308.8
	989N	0	0	0	0	0	0	0	0	0	0	0	4447.0
	1030N	0	0	0	0	0	77.1	65.0	65.0	65.0	50.0	50.0	4661.1
	1116N	0	0	0	0	0	0	0	0	0	0	0	1372.2
5. oocyst challenge	927N	28.8	35.7	75.4	188.5	575.1	835.3	1139.2	1144.0	1229.2	1386.3	1248.2	*
	976N	0	0	70.0	125.2	303.6	607.7	1192.8	1182.8	1160.1	734.5	699.8	*
	1032N	0	0	50.0	200.0	322.5	782.2	1121.4	1293.0	971.9	1179.7	1077.1	*
	1084N	0	0	20.0	77.1	123.6	163.5	130.4	158.8	373.2	189.6	113.4	*

Appendix 5.8 *IgG1 and IgG2 responses at week 2 post-infection with oocysts*

Treatment Group	Animal Number	Antibody			
		SERUM IgG1	SERUM IgG2	NASAL IgG1	NASAL IgG2
		Normalised OD @ 492nm			
1. control	913N	-0.005	-0.0275	-0.001	-0.067
	915N	-0.011	-0.0170	-0.013	-0.057
	950N	0.003	-0.0145	-0.010	-0.046
	1048N	0.031	-0.0290	*	*
2. i.n. soluble toxo	638N	0.096	-0.006	0.098	-0.020
	748N	0.066	-0.003	0.003	-0.053
	919N	0.311	-0.024	0.011	-0.056
	923N	0.205	0.087	0.196	-0.040
	993N	0.045	0.001	0.008	-0.059
	1031N	0.068	-0.008	0.010	0.020
	1034N	0.089	0.002	0.029	0.240
	1117N	0.047	-0.008	0.014	-0.055
3. i.n. toxo particle	715N	0.056	0.222	0.015	0.016
	739N	0.211	0.228	0.106	0.031
	764N	0.297	0.023	0.182	0.030
	1067N	0.214	-0.009	0.130	0.028
4. i.n. toxo particle + CT	770N	0.432	0.878	0.064	0.153
	989N	0.162	0.073	0.067	-0.019
	1030N	0.129	0.069	0.044	-0.031
	1116N	0.097	-0.003	0.032	-0.053
5. oocyst challenge	927N	0.234	0.059	0.016	-0.026
	976N	0.051	0.930	*	*
	1032N	0.107	0.093	0.033	-0.027
	1084N	0.020	0.126	0.002	-0.017

Appendix 5.9 *Nasal IgG responses*

Treatment Group	Animal Number	Normalised OD @ 492nm											
		Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11
1. control	913N	-0.011	0.002	0.003	-0.008	-0.010	-0.022	-0.015	-0.007	-0.013	-0.019	-0.016	0.190
	915N	0.006	-0.002	0.002	0.018	-0.015	0.045	0.000	-0.002	0.014	-0.010	-0.002	0.043
	950N	0.014	0.015	0.009	0.004	0.049	-0.001	0.008	0.002	0.020	0.002	0.010	0.084
	1048N	-0.011	0.045	0.001	-0.013	0.085	-0.022	-0.015	-0.023	-0.010	-0.019	-0.016	*
2. i.n. soluble toxo	638N	-0.002	-0.009	-0.001	-0.004	-0.007	-0.011	-0.010	-0.007	0.006	-0.002	-0.011	1.399
	748N	0.010	-0.002	0.005	0.014	-0.008	-0.011	-0.005	-0.011	-0.004	-0.005	-0.005	0.519
	919N	-0.001	-0.002	-0.004	-0.005	-0.013	-0.014	-0.004	-0.008	-0.011	-0.019	-0.007	0.768
	923N	0.004	0.075	-0.006	0.045	-0.017	-0.010	-0.009	-0.006	-0.009	-0.009	-0.007	1.499
	993N	-0.008	-0.012	0.014	-0.016	-0.015	-0.018	-0.001	-0.021	-0.013	-0.010	-0.010	0.875
	1031N	0.008	0.003	0.013	0.005	0.006	0.002	0.004	-0.009	0.088	-0.006	0.007	0.924
	1034N	0.017	0.023	-0.001	0.013	-0.005	-0.012	0.023	0.002	0.002	0.002	0.022	1.348
	1117N	-0.007	0.085	-0.006	-0.010	-0.020	-0.007	-0.012	-0.020	-0.014	-0.001	-0.002	0.906
3. i.n. toxo particle	715N	0.002	0.004	0.011	-0.001	0.187	-0.017	0.001	-0.004	-0.005	-0.006	0.020	1.182
	739N	-0.010	-0.011	0.004	0.003	0.008	-0.015	-0.008	-0.005	-0.014	-0.011	0.002	1.494
	764N	0.005	0.034	-0.005	-0.008	-0.007	-0.020	-0.008	-0.004	-0.011	-0.015	-0.007	1.497
	1067N	-0.002	-0.004	0.011	*	-0.011	-0.020	-0.015	-0.016	-0.019	-0.014	-0.013	1.481
4. i.n. toxo particle + CT	770N	0.025	0.015	0.027	0.033	-0.005	0.034	0.024	0.018	0.005	0.004	-0.003	1.527
	989N	-0.006	-0.006	0.017	*	0.006	0.012	0.010	-0.006	-0.013	-0.008	-0.011	1.508
	1030N	-0.012	-0.009	0.013	-0.004	-0.014	-0.023	-0.012	-0.020	-0.011	-0.011	-0.012	1.365
	1116N	0.006	0.008	0.017	0.008	0.067	-0.011	0.001	-0.013	-0.003	-0.002	-0.001	0.917
5. oocyst challenge	927N	*	*	0.1945	0.139	0.177	0.271	0.853	0.6155	0.397	0.706	0.6305	*
	976N	*	*	0.0235	0.425	0.466	1.1865	1.3295	1.1965	1.209	1.2765	1.5255	*
	1032N	*	*	*	0.55	0.3485	0.4525	0.6335	1.231	0.99	1.1195	1.191	*
	1084N	*	*	0.018	0.047	0.119	0.106	0.179	0.079	0.1565	0.1045	0.2365	*

Appendix 5.10 *Tissue IgG responses*

Treatment Group	Animal Number	Normalised OD @ 492nm													
		NALT	PT	Trachea	Bronchus	Lung	Abo Fold	Duodenum	Jejenum	Ileum	PP	LI	Rectum	Spleen	MLN
1. control	**913N**	0.133	0.096	0.272	0.153	0.132	0.065	0.087	0.156	0.121	0.215	0.038	0.052	0.323	0.487
	915N	0.042	0.032	0.062	0.035	0.035	0.036	0.058	0.068	0.016	0.061	0.035	0.062	0.087	0.111
	950N	0.037	0.033	0.062	0.035	0.041	0.038	0.044	0.113	0.047	0.094	0.051	0.053	0.122	0.302
	1048N	0.016	0.013	0.029	0.019	0.017	0.018	0.019	0.044	0.017	0.038	0.023	0.046	0.032	0.072
2. i.n. soluble toxo	**638N**	0.746	0.700	0.688	0.661	0.761	0.547	0.546	1.075	0.628	0.969	0.709	0.331	0.972	1.267
	748N	0.435	0.422	0.486	0.500	0.612	0.579	0.453	0.654	0.360	0.735	0.278	0.265	0.664	1.054
	919N	0.350	0.248	0.370	0.441	0.569	0.385	0.364	0.504	0.445	0.655	0.228	0.070	0.780	1.066
	923N	0.781	0.780	0.743	0.816	0.847	0.870	0.882	1.232	0.728	0.964	0.807	0.824	1.005	1.334
	993N	0.462	0.287	0.416	0.496	0.575	0.386	0.468	0.766	0.455	0.694	0.347	0.205	0.720	1.080
	1031N	0.476	0.533	0.632	0.600	0.708	0.584	0.682	0.902	0.389	0.887	0.456	0.500	0.799	1.144
	1034N	0.521	0.302	0.538	0.560	0.609	0.523	0.535	0.840	0.319	0.803	0.465	0.204	0.752	1.209
	1117N	0.376	0.389	0.291	0.356	0.415	0.503	0.256	0.597	0.284	0.606	0.313	0.267	0.810	1.081
3. i.n. toxo particle	**715N**	0.564	0.680	0.522	0.573	0.711	0.764	0.750	1.160	0.603	0.959	0.695	0.720	0.908	1.190
	739N	0.784	0.783	0.829	0.789	0.837	0.782	0.870	1.241	0.691	1.163	0.861	0.762	1.024	1.342
	764N	0.860	0.761	0.987	0.808	0.834	0.804	0.892	1.169	0.756	0.987	0.919	0.643	0.935	1.390
	1067N	0.764	0.604	0.777	0.861	0.843	0.656	0.589	1.211	0.703	0.900	0.810	0.692	0.840	1.301
4. i.n. toxo particle + CT	**770N**	0.863	0.854	0.849	0.845	0.850	0.871	0.877	1.195	0.556	1.073	0.970	0.570	1.008	1.365
	989N	0.680	0.685	0.699	0.720	0.809	0.689	0.786	1.167	0.755	1.134	0.695	0.786	0.917	1.332
	1030N	0.654	0.608	0.655	0.658	0.753	0.657	0.659	1.124	0.630	0.898	0.721	0.670	0.935	1.351
	1116N	0.466	0.398	0.438	0.441	0.643	0.286	0.384	0.809	0.324	0.663	0.300	0.300	0.625	1.066
5. oocyst challenge	**927N**	0.516	0.317	0.654	0.463	0.759	0.392	0.481	0.978	0.361	0.975	0.340	0.543	1.143	0.955
	976N	0.469	0.324	0.141	0.308	0.700	0.465	0.503	0.609	0.246	0.603	0.298	0.109	1.013	0.755
	1032N	0.651	0.461	0.468	0.414	0.768	0.590	0.355	0.604	0.696	0.852	0.418	0.146	1.226	1.001
	1084N	0.156	0.039	0.084	0.078	0.292	0.176	0.125	0.235	0.047	0.239	0.068	0.130	0.412	0.447

Appendix 5.11 *Proliferative responses of PBMC from Group 1 (control)*

Animal	Treat-ment	Median counts per minute (cpm)											
		Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11
913N	M	60.0	75.2	44.0	65.9	77.9	190.0	195.0	127.5	86.0	144.0	143.5	70.2
	V	77.5	65.2	50.3	53.7	76.0	234.5	235.5	141.5	99.4	163.0	167.0	57.5
	T2	77.0	57.0	40.9	66.0	81.5	167.0	147.5	138.0	91.5	162.5	295.0	50.2
	T1	*	52.2	60.7	65.9	95.5	198.5	182.5	140.5	114.2	189.0	254.0	58.3
	C	22968.0	23826.5	21025.5	23654.0	21207.0	18871.5	21097.5	24493.5	16873.5	25977.5	15889.5	1464.0
915N	M	141.0	139.5	85.7	124.0	101.0	192.0	156.5	127.5	195.0	174.5	154.5	165.5
	V	136.5	109.5	75.7	102.9	113.5	177.5	138.0	121.0	140.0	209.5	172.5	176.5
	T2	225.5	121.0	57.9	169.0	155.5	210.5	124.0	224.5	193.0	356.5	784.0	190.5
	T1	*	166.0	85.5	124.0	144.0	255.0	225.5	185.01	247.0	283.5	470.5	190.5
	C	25431.5	29476.0	19343.0	18307.5	16688.5	25247.0	17206.0	21450.5	25309.5	23094.0	18578.5	1023.5
950N	M	85.0	92.0	66.7	138.5	44.5	94.2	96.7	218.0	145.0	149.0	391.5	147.5
	V	80.0	103.0	62.7	101.5	50.7	120.0	83.4	178.0	116.0	129.5	302.0	156.5
	T2	100.2	68.4	53.9	348.0	77.7	127.5	101.5	1017.5	529.5	225.5	16054.5	3917.5
	T1	*	80.5	71.9	157.0	69.9	116.5	87.4	812.0	194.0	168.0	15433.0	4654.0
	C	23863.0	22884.0	26479.0	26874.5	16340.0	17064.0	21437.5	26149.5	20163.5	16775.0	17696.5	16145.0
1048N	M	617.0	344.5	118.0	128.0	96.9	206.5	142.0	198.0	293.5	222.0	329.0	291.5
	V	401.5	337.0	102.4	108.5	79.5	189.5	131.5	178.0	314.5	166.5	283.5	278.5
	T2	676.0	265.0	101.9	128.0	113.0	243.5	142.5	229.5	605.0	270.0	3307.5	514.0
	T1	*	291.5	95.5	125.5	156.5	285.5	120.5	254.5	479.0	299.0	2809.0	390.5
	C	27062.5	24274.5	30159.0	29686.0	21008.0	21991.5	24743.5	27118.0	23649.5	22712.0	25374.0	10314.0

M = medium control; **V** = vero cell control; **T2** = toxoplasma tachyzoite antigen at 2μg/ml; **T1** = toxoplasma tachyzoite antigen at 1μg/ml; **C** = Con A positive control

Appendix 5.12 *Proliferative responses of PBMC from Group 2 (i.n. immunisation with soluble antigen)*

Animal	Treat -ment	Median counts per minute (cpm)											
		Week 0	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**	**Week 7**	**Week 8**	**Week 9**	**Week 10**	**Week 11**
638N	**M**	114.0	462.0	73.3	111.5	53.3	94.0	70.2	97.9	57.3	79.5	116.5	62.4
	V	142.5	563.5	81.5	83.7	58.8	127.5	61.7	86.5	154.9	84.5	139.0	56.2
	T2	129.5	177.0	50.5	118.0	100.7	670.5	67.9	158.0	554.5	81.7	2158.5	76.0
	T1	*	455.0	60.4	354.0	107.0	148.5	113.7	356.5	135.4	164.0	2365.0	68.0
	C	32373.5	15981.0	30240.0	27404.0	25949.5	22775.0	23571.0	30675.0	20575.0	24296.5	22483.0	3944.5
748N	**M**	405.0	508.0	127.5	168.0	132.0	426.5	326.0	184.0	443.0	217.0	171.5	166.0
	V	159.0	533.5	241.5	148.0	130.5	842.5	370.5	147.5	359.0	195.5	165.5	132.5
	T2	389.5	308.0	143.5	117.0	140.0	684.5	280.0	494.0	249.5	259.0	5117.0	335.5
	T1	*	532.5	154.0	177.5	220.0	486.0	336.0	329.5	362.5	345.0	3689.0	203.5
	C	31290.0	31959.5	40087.5	33573.5	33085.0	28924.0	23705.0	32915.0	31182.0	29012.5	28868.0	33760.0
919N	**M**	139.0	555.5	58.4	90.2	68.7	168.5	116.0	218.5	138.0	96.2	160.0	147.0
	V	127.5	343.5	62.0	116.5	66.5	121.5	102.5	138.5	111.5	88.7	168.5	160.5
	T2	159.0	326.5	47.9	107.0	87.7	228.5	111.0	187.5	351.0	141.0	13778.5	6826.5
	T1	*	300.0	59.4	106.4	82.4	184.0	111.0	325.5	186.5	106.0	12815.5	4479.0
	C	34842.0	29690.5	22567.0	30537.0	23669.5	23214.5	23224.5	31218.5	23306.0	27158.0	17270.0	15979.5
923N	**M**	117.5	150.5	82.2	72.5	82.7	181.0	100.0	137.5	146.0	128.5	149.0	162.5
	V	120.5	126.5	60.9	54.4	56.4	146.0	106.0	89.5	122.5	113.5	163.5	144.5
	T2	1122.5	904.5	71.2	97.0	325.0	2647.5	424.0	215.5	2691.0	2456.5	12183.5	551.0
	T1	*	954.5	137.0	79.2	262.5	2472.0	270.5	216.5	2619.5	2589.0	13390.0	276.5
	C	29248.0	28710.5	21843.5	14166.5	25133.0	20811.0	23245.5	17010.0	21726.0	30339.5	26393.5	12578.5
993N	**M**	150.0	164.0	55.2	65.2	49.0	90.4	52.8	101.2	137.0	83.7	112.5	92.0
	V	134.5	116.0	52.2	52.5	58.7	73.7	51.9	85.2	111.5	94.0	118.5	101.9
	T2	170.0	110.5	55.4	57.0	69.0	91.2	55.7	155.5	205.5	108.0	285.0	116.0
	T1	*	150.0	62.9	81.2	155.5	80.9	74.9	154.5	255.5	97.0	275.5	105.5
	C	24317.0	28267.0	31810.0	28108.0	23415.5	23329.5	23147.0	29382.0	16477.5	24441.5	23536.5	25216.0

1031N	M	175.5	135.5	72.4	103.2	64.7	186.5	204.5	253.0	220.5	384.0	531.0	63.9
	V	190.0	127.0	64.2	89.2	65.0	185.0	189.5	227.5	200.5	270.0	430.0	67.9
	T2	258.5	120.5	61.7	190.5	231.5	370.5	188.5	449.0	518.0	707.0	11257.5	216.5
	T1	*	248.0	74.0	173.0	222.5	312.5	239.0	414.0	379.5	391.0	9878.0	247.5
	C	31239.0	31847.5	29780.0	26198.0	23016.5	22232.0	23260.0	30651.0	24628.0	21734.5	24024.5	4469.5
1034N	M	153.0	300.5	104.5	89.5	113.0	149.5	85.9	119.0	251.5	139.5	246.5	147.5
	V	146.5	212.0	114.0	78.7	55.5	135.5	73.0	98.2	194.5	87.9	238.5	172.5
	T2	194.0	264.5	199.0	173.5	87.5	173.0	113.0	231.5	273.0	206.5	6005.0	260.5
	T1	*	448.0	320.0	171.0	97.7	254.0	127.5	300.5	374.0	202.0	5346.0	193.0
	C	25170.0	33953.0	34048.5	30385.0	24919.5	22043.5	27296.5	40230.0	22885.0	24981.0	28226.0	32743.5
1117N	M	989.0	518.5	61.9	113.0	90.5	189.5	153.5	177.5	414.0	132.0	189.0	74.2
	V	380.0	498.5	47.2	123.0	78.5	167.5	91.9	119.5	314.0	115.0	233.5	36.0
	T2	2508.5	793.5	47.7	238.0	93.5	327.5	190.0	429.5	2082.5	389.0	865.0	103.4
	T1	*	1609.5	81.7	442.0	116.5	295.0	232.5	426.5	1109.5	289.5	758.0	76.7
	C	32259.5	31416.5	24351.0	31534.0	27411.0	47212.0	25455.5	40453.0	25865.5	28707.0	24808.0	24824.0

M = medium control; **V** = vero cell control; **T2** = toxoplasma tachyzoite antigen at 2μg/ml; **T1** = toxoplasma tachyzoite antigen at 1μg/ml; **C** = Con A positive control

Appendix 5.13 *Proliferative responses of PBMC from Group 3 (i.n. immunisation with particulate toxoplasma antigen)*

Animal	Treat -ment	Median counts per minute (cpm)											
		Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11
715N	M	155.5	1181.0	157.0	448.5	93.4	166.0	78.2	196.0	174.0	125.5	244.0	82.8
	V	90.5	1140.0	127.5	311.0	137.4	166.0	93.9	134.0	254.5	130.5	270.5	93.4
	T2	410.0	2376.5	304.5	1501.0	813.5	1495.0	234.5	489.0	442.5	341.0	652.5	6607.5
	T1	*	4631.5	430.5	518.0	335.5	1198.0	217.0	342.5	447.5	269.0	429.5	5758.5
	C	34078.0	30215.0	44547.5	33224.5	24921.0	28600.5	27360.0	32709.5	31149.5	35444.5	43782.5	26484.0
739N	M	81.9	136.5	77.4	136.5	60.5	119.5	91.4	63.7	107.5	230.0	217.5	148.0
	V	78.4	106.5	69.3	60.0	47.7	181.0	78.9	66.7	109.4	101.9	190.5	129.0
	T2	128.7	144.5	73.0	69.4	241.5	135.0	371.5	160.0	599.5	1399.0	533.5	1150.5
	T1	*	234.5	84.4	73.0	135.9	129.5	268.5	565.0	713.0	663.0	592.0	739.5
	C	31639.5	27654.5	28516.5	24167.0	24729.5	15419.0	21295.0	29710.5	25076.5	28239.0	23576.0	23108.5
764N	M	185.5	227.5	87.9	127.5	67.0	240.5	197.0	206.0	134.0	222.0	260.5	219.5
	V	160.5	180.0	98.4	129.0	71.0	235.5	183.5	165.5	130.5	234.5	274.5	191.0
	T2	233.0	184.5	79.2	127.0	100.7	560.5	162.5	332.5	196.0	327.5	467.5	3031.0
	T1	*	247.0	100.4	144.0	91.8	297.0	189.0	221.5	147.0	646.5	638.5	2316.0
	C	29727.5	29562.5	28865.5	26518.5	23936.0	23712.0	15564.0	28383.0	22219.5	24378.5	19686.0	28560.0
1067N	M	122.0	126.5	105.5	65.9	62.0	109.5	86.0	433.0	130.0	185.0	230.0	73.4
	V	119.0	119.0	83.5	65.9	59.9	103.2	62.4	264.0	105.5	113.5	198.0	82.8
	T2	657.5	262.5	105.7	84.0	265.5	336.5	83.9	1845.5	1120.5	2179.0	1467.0	292.0
	T1	*	334.5	137.5	121.0	148.0	303.5	92.0	1052.5	377.0	1685.5	575.0	279.0
	C	28825.5	34126.0	31296.5	29878.5	22763.5	44881.0	26923.0	35434.5	32064.5	22929.5	26337.0	29450.5

M = medium control; **V** = vero cell control; **T2** = toxoplasma tachyzoite antigen at 2μg/ml; **T1** = toxoplasma tachyzoite antigen at 1μg/ml; **C** = Con A positive control

Appendix 5.14 *Proliferative responses of PBMC from Group 4 (i.n. immunisation with particulate toxoplasma antigen + CT)*

Animal	Treat -ment	Median counts per minute (cpm)											
		Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11
770N	M	163.5	197.0	101.7	127.5	137.0	234.0	161.5	337.0	164.5	256.5	234.5	101.9
	V	114.0	156.0	85.8	109.5	165.5	157.5	157.0	247.0	140.5	174.0	290.0	78.5
	T2	466.5	1647.0	239.5	375.0	3180.5	1932.0	1233.0	1570.0	1229.5	4400.5	665.0	1341.0
	T1	*	1858.0	313.5	303.5	2017.5	2052.5	917.5	1334.5	901.5	5491.5	756.0	751.5
	C	35627.0	31414.5	29298.0	29868.0	25104.0	22109.5	22570.0	30725.5	26416.0	28365.5	20654.5	15255.0
989N	M	140.5	164.0	39.8	83.4	53.5	157.0	156.0	152.5	315.0	298.5	251.5	187.0
	V	134.0	193.5	50.0	78.5	43.7	145.0	144.0	134.0	239.5	219.0	242.0	156.5
	T2	179.5	120.0	27.2	112.5	41.7	181.5	550.5	757.5	359.5	718.5	2707.5	3512.0
	T1	*	121.5	57.2	119.0	117.2	134.5	496.0	483.5	681.5	769.5	2721.5	1637.0
	C	30596.0	30180.0	20177.5	33878.5	11394.5	22045.0	22507.0	27178.5	26184.5	28719.0	30229.0	31252.5
1031N	M	182.5	94.3	57.5	74.2	128.9	183.0	88.0	195.0	158.0	185.0	151.5	77.0
	V	131.5	80.2	43.7	68.0	57.4	136.5	84.0	113.5	172.0	113.5	140.5	91.2
	T2	325.0	324.0	33.0	851.5	136.0	893.0	276.0	655.5	1642.5	2179.0	577.5	1512.0
	T1	*	270.0	71.7	853.5	521.0	741.5	275.5	620.5	1945.5	1685.5	543.0	966.5
	C	27040.0	27911.0	9547.0	29376.0	11777.0	20389.5	23799.0	27522.0	21817.5	22929.5	21044.5	26687.0
1116N	M	279.5	199.0	61.5	71.5	87.0	88.9	76.0	82.2	139.0	179.0	220.0	214.5
	V	159.0	282.5	66.2	71.5	67.9	98.2	88.0	76.2	115.5	133.0	262.0	185.0
	T2	357.0	241.0	52.5	172.0	277.0	139.5	123.5	87.9	568.5	1041.0	1391.0	6798.0
	T1	*	406.5	62.9	229.0	206.0	101.5	115.5	124.0	544.5	407.5	1031.5	3912.5
	C	23870.0	29103.5	31697.5	30650.0	21560.0	24263.0	23448.5	25295.5	30065.5	25492.5	26118.0	32442.0

M = medium control; **V** = vero cell control; **T2** = toxoplasma tachyzoite antigen at 2μg/ml; **T1** = toxoplasma tachyzoite antigen at 1μg/ml; **C** = Con A positive control

Appendix 5.15 *Proliferative responses of PBMC from Group 5 (oocyst challenge)*

Animal	Treat-ment	Median counts per minute (cpm)										
		Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
927N	M	194.5	140.0	88.5	68.0	111.4	664.0	137.0	89.7	130.5	650.0	134.0
	V	50.2	106.5	104.0	86.0	86.3	352.5	83.7	56.9	79.5	333.0	133.0
	T2	322.5	121.0	2136.5	713.0	989.0	420.0	109.0	85.2	727.5	299.0	509.0
	T1	*	102.4	2271.5	731.5	1472.5	269.5	122.5	125.5	664.5	232.5	383.5
	C	25998.0	21629.0	29128.5	21307.5	29506.0	22663.5	22495.5	20710.0	21598.0	21023.0	28570.5
976N	M	72.0	155.0	123.0	83.4	92.0	76.0	156.0	83.5	71.8	160.5	205.5
	V	46.7	157.5	140.5	96.0	112.5	68.0	125.5	78.7	103.0	96.3	123.0
	T2	112.0	142.5	2252.5	351.0	907.5	437.5	752.5	580.5	537.5	873.5	935.0
	T1	*	186.0	1807.5	345.0	1425.0	449.5	646.0	382.5	476.5	655.0	1097.0
	C	12526.5	19189.5	29039.0	21102.5	35310.5	23253.0	28332.0	28221.5	21251.0	22517.5	24165.0
1032N	M	95.5	191.5	99.9	123.0	159.5	808.5	317.5	124.0	171.5	920.0	213.5
	V	26.5	160.0	88.9	143.5	131.0	156.5	151.5	140.5	98.9	346.0	146.5
	T2	85.7	146.0	724.0	1234.5	966.0	144.5	295.0	173.0	600.5	689.5	227.0
	T1	*	179.0	517.0	986.0	1095.5	318.5	208.0	485.0	522.5	670.5	174.0
	C	20030.5	25755.0	29789.5	17535.5	25763.0	27878.0	20661.5	18514.5	17260.0	21483.5	12516.0
1084N	M	738.0	279.5	252.0	102.5	160.5	109.5	142.5	140.0	146.5	343.5	317.0
	V	299.5	264.0	159.0	144.5	139.5	98.9	183.0	135.0	152.0	195.5	204.0
	T2	409.0	428.5	2641.5	1278.0	1933.0	721.5	1636.5	1291.0	2779.5	2013.5	2140.5
	T1	*	400.5	4246.5	987.5	2616.0	1377.0	1271.0	810.0	3153.5	2092.0	1556.0
	C	20716.0	29519.5	27246.0	34428.0	30307.5	33218.0	27619.0	22896.0	20053.5	20290.0	31655.5

M = medium control; **V** = vero cell control; **T2** = toxoplasma tachyzoite antigen at 2μg/ml; **T1** = toxoplasma tachyzoite antigen at 1μg/ml; **C** = Con A positive control

Appendix 5.16 *Proliferative responses of lymph node cells from Groups 1 and 5*

GROUP 1 Animal	T	MLN	RP LN	P-F LN	MedLN	PT	spleen
		Lymph Node Median cpm					
913N	M	43.5	149.5	23.5	43.0	15.7	30.0
	V	71.4	190.0	22.0	49.9	22.0	47.5
	T2	232.0	313.0	40.9	101.4	23.9	63.5
	T1	216.0	372.0	31.7	58.4	24.7	57.0
	C	14732.0	22332.5	8500.5	10463.5	821.5	2169.0
915N	M	72.2	57.4	50.9	48.3	25.7	46.7
	V	64.8	46.4	56.0	51.0	105.0	53.0
	T2	84.0	42.4	36.9	60.7	42.7	1284.0
	T1	87.9	57.4	62.7	50.9	93.3	465.0
	C	5779.5	4708.0	10952.5	5734.5	4552.0	924.5
950N	M	16.5	27.4	17.3	24.0	30.3	114.4
	V	13.4	15.2	22.9	18.0	49.4	61.4
	T2	20.9	14.2	23.7	18.7	39.9	421.5
	T1	23.0	16.7	19.3	23.2	51.2	240.5
	C	1266.5	85.5	2572.5	25.7	3634.5	841.0
1048N	M	165.5	47.9	18.5	14.2	31.0	43.9
	V	150.5	43.0	17.8	19.0	22.3	39.4
	T2	331.0	45.7	24.5	19.5	20.4	35.9
	T1	166.5	48.7	23.2	24.2	16.2	38.8
	C	9756.0	4699.0	93.2	3212.0	1215.5	2229.5

GROUP 5 Animal	T	MLN	RP LN	P-F LN	MedLN	PT	spleen
		Lymph Node Median cpm					
927N	M	277.0	144.0	21.7	27.0	85.5	39.2
	V	738.0	1620.5	34.5	22.2	135.5	209.5
	T2	183.0	351.0	710.0	551.5	61.0	55.4
	T1	169.0	104.0	489.0	181.5	110.5	34.5
	C	5335.0	4765.0	1480.5	1021.5	8766.0	50.0
976N	M	44.4	116.0	39.0	38.5	*	126.5
	V	42.7	84.4	108.4	100.4	*	61.4
	T2	123.0	152.0	125.0	37.9	*	2384.0
	T1	78.9	106.5	59.2	*	*	1784.5
	C	6904.0	7216.5	7236.5	*	*	18252.0
1032N	M	86.8	97.0	*	86.7	848.5	46.9
	V	188.0	184.5	*	64.0	1288.5	244.0
	T2	229.0	200.0	*	159.0	673.0	601.5
	T1	179.5	83.0	*	71.2	702.5	514.5
	C	*	3496.0	*	2899.0	8772.0	2919.0
1084N	M	116.5	393.0	185.5	55.0	*	26.0
	V	93.2	358.0	179.0	101.0	*	23.8
	T2	612.0	1266.0	1127.0	63.2	*	226.0
	T1	596.0	979.5	17.7	61.9	*	97.2
	C	12423.5	7332.0	*	*	*	1460.5

T = *in vitro* treatment: M = medium control; **V** = vero cell control; **T2** = toxoplasma tachyzoite antigen at 2µg/ml; **T1** = toxoplasma tachyzoite antigen at 1µg/ml; **C** = Con A positive control

Appendix 5.17 *Proliferative responses of lymph node cells from Group 2*

GROUP 2 Animal	T	MLN	RP LN	P-F LN	MedLN	PT	spleen
		Lymph Node (Median cpm)					
638N	M	97.0	112.0	239.5	57.5	492.0	64.5
	V	175.5	68.2	237.0	57.2	491.0	57.9
	T2	1513.5	421.5	2300.5	32.0	516.5	355.0
	T1	841.0	204.5	1691.5	33.0	622.5	189.0
	C	14533.5	17724.0	20325.0	9377.5	12878.0	5808.0
748N	M	58.8	72.4	25.0	39.4	133.5	343.0
	V	53.2	77.3	23.5	73.4	141.0	290.5
	T2	54.2	76.7	34.4	32.8	136.0	8670.0
	T1	53.0	88.7	24.7	52.7	176.0	7156.5
	C	9561.0	19534.0	4284.0	1812.5	10341.0	12955.5
919N	M	53.3	43.9	19.2	42.0	21.0	19.5
	V	96.2	35.2	27.4	30.2	26.2	16.9
	T2	227.0	43.0	147.5	26.2	31.5	36.9
	T1	132.5	33.0	365.5	46.4	29.8	31.7
	C	11922.5	3849.5	11346.5	7024.0	645.0	709.5
923N	M	763.5	379.5	940.0	85.0	749.0	108.0
	V	536.5	304.5	992.5	70.0	700.5	105.9
	T2	6012.0	5055.0	9000.5	221.5	1161.5	8882.0
	T1	4407.5	4102.0	11027.0	106.7	999.0	6895.5
	C	17637.5	19392.0	18920.0	3333.0	13594.5	14786.5

GROUP 2 Animal	T	MLN	RP LN	P-F LN	MedLN	PT	spleen
		Lymph Node (Median cpm)					
993N	M	34.5	63.9	43.5	51.7	61.7	55.3
	V	28.0	39.0	66.3	51.2	129.0	46.5
	T2	20.3	51.3	69.0	30.0	73.7	904.5
	T1	28.4	52.4	72.0	32.2	*	190.0
	C	4261.5	6349.0	18056.5	7792.0	1497.0	4949.0
1031N	M	7.5	30.2	7.5	24.7	14.8	26.7
	V	10.0	30.7	8.9	6.3	13.5	27.0
	T2	6.2	12.9	8.7	4.7	12.8	29.2
	T1	12.0	24.3	17.3	7.5	15.3	26.9
	C	24.3	42.0	33.7	25.7	257.5	214.0
1034N	M	308.0	120.5	59.5	48.0	147.0	49.8
	V	296.0	95.7	67.8	33.5	217.0	38.7
	T2	2575.0	379.0	157.5	34.5	155.0	183.2
	T1	3255.5	135.0	145.5	36.0	290.5	114.4
	C	13591.0	7528.0	16760.0	5652.5	17597.5	1507.5
1117N	M	77.0	348.0	702.0	74.7	1060.5	66.5
	V	91.8	268.0	694.5	62.4	981.5	48.2
	T2	79.5	502.0	2733.5	41.2	802.5	45.5
	T1	90.7	626.0	2693.5	47.5	917.0	53.4
	C	11757.5	22578.5	20639.0	17633.5	15546.5	10674.0

T = *in vitro* treatment: M = medium control; **V** = vero cell control; **T2** = toxoplasma tachyzoite antigen at 2μg/ml; **T1** = toxoplasma tachyzoite antigen at 1μg/ml; **C** = Con A positive control

Appendix 5.18 *Proliferative responses of lymph node cells from Groups 3 and 4*

GROUP 3 Animal	T	Lymph Node MLN	RP LN	P-F LN	MedLN	PT	spleen
		Median cpm					
715N	M	48.7	66.4	42.5	58.8	94.0	39.3
	V	32.7	25.7	43.2	27.0	79.2	26.4
	T2	226.5	155.0	114.9	28.7	110.5	332.5
	T1	201.5	92.0	106.4	35.4	84.4	165.0
	C	17940.5	15239.5	20525.0	19995.0	18480.0	2916.5
739N	M	1646.5	687.5	144.5	143.0	863.0	169.0
	V	1472.5	570.0	179.0	106.2	518.0	152.0
	T2	3776.5	4330.5	3013.0	4356.0	786.0	4452.0
	T1	2987.5	2502.5	3174.0	3225.5	1125.0	4377.0
	C	17384.0	15223.5	15748.5	23911.5	15247.0	168.5
764N	M	517.0	918.0	1606.0	1049.0	4189.0	128.0
	V	368.5	1075.5	1624.5	928.5	3966.5	136.0
	T2	4391.5	5700.0	8258.0	5654.0	3175.0	8755.5
	T1	3724.5	4736.5	7248.0	4449.5	3815.0	7076.5
	C	11671.0	16539.0	16931.0	16514.5	13475.5	8714.0
1067N	M	108.0	129.5	124.0	94.7	135.0	54.0
	V	132.5	153.5	136.0	109.0	93.4	38.7
	T2	311.0	152.5	2371.5	2218.0	85.2	55.8
	T1	255.0	148.0	1259.0	1086.5	127.5	51.5
	C	15692.0	15964.0	19125.0	18266.0	11405.0	3118.0

GROUP 4 Animal	T	Lymph Node MLN	RP LN	P-F LN	MedLN	PT	spleen
		Median cpm					
770N	M	2795.5	3485.5	923.5	393.5	1193.0	126.0
	V	2537.5	3394.5	1075.5	408.5	1582.5	84.5
	T2	10473.0	10159.5	15110.0	622.5	3345.5	4622.5
	T1	8164.0	14111.5	14190.0	318.0	2897.5	5013.5
	C	13993.5	13720.0	17045.0	18065.5	10021.5	9679.5
989N	M	95.4	219.5	159.0	173.0	136.5	84.9
	V	77.7	277.0	154.0	144.0	210.0	76.3
	T2	212.5	197.5	2749.0	70.0	463.0	713.0
	T1	255.5	222.5	2625.0	101.7	529.0	660.5
	C	14812.0	15113.5	17360.0	10705.0	16957.5	12011.0
1030N	M	297.0	139.0	50.9	62.5	1083.0	63.0
	V	218.0	151.0	67.4	44.9	324.0	65.4
	T2	1014.0	119.0	117.2	37.4	297.5	627.0
	T1	1221.5	131.7	84.9	145.4	519.0	606.5
	C	16013.0	5424.5	14297.0	6385.5	8535.0	2113.0
1116N	M	248.5	504.5	293.0	548.0	1131.5	35.2
	V	285.5	587.5	493.0	377.0	1181.0	28.5
	T2	3051.5	2528.5	5845.5	458.0	2221.0	20.2
	T1	2458.0	1288.5	4822.0	376.0	2045.5	33.0
	C	20420.0	19578.0	19371.0	19463.0	17330.5	1175.5

T = *in vitro* treatment: M = medium control; **V** = vero cell control; **T2** = toxoplasma tachyzoite antigen at 2µg/ml; **T1** = toxoplasma tachyzoite antigen at 1µg/ml; **C** = Con A positive control

Appendix 5.19 *Interferon gamma production* in vitro *in groups 1 and 5*

Animal	T	Week												Lymph Node					
		0	1	2	3	4	5	6	7	8	9	10	11	RP LN	P-F LN	Med LN	MLN	PT	spleen
		IFNγ (pg/ml)																	
GROUP 1. CONTROL GROUP																			
913N	M	14.4	0.0	0.0	0.0	0.0	0.0	13.5	2.4	2.7	10.8	25.6	0.0	23.3	0	0	2.9	0	7.3
	T2	12.8	17.5	19.3	12.8	17.0	6.1	55.4	19.3	40.4	20.3	52.5	7.6	43.24	0.0	9.1	0.0	0.0	10.1
	C	584.1	641.9	411.2	347.5	690.3	203.4	1003	443.3	575.7	421.2	861.5	75.4	493.7	123.7	244.8	223.6	0.0	115.8
915N	M	19.3	14.0	28.5	13.9	19.0	11.5	39.1	21.0	55.7	20.5	49.3	10.0	0.0	0.0	33.1	0.8	0.0	17.1
	T2	0.0	20.6	31.4	22.3	23.8	13.0	39.5	25.6	93.0	4.2	64.6	10.5	0.0	0.0	45.7	0.0	9.1	187.6
	C	116.1	255.5	134.3	149.3	119.2	95.0	370.8	109.6	209.9	185.0	489.4	37.4	62.5	238.2	109.9	68.7	57.8	252.5
950N	M	0.0	0.0	0.0	0.0	0.0	12.6	97.0	0.0	0.0	0.0	49.9	0.0	7.6	4.0	0.0	14.9	48.2	2.6
	T2	10.8	30.3	24.2	18.9	12.8	36.1	80.6	44.6	58.8	0.0	1731	307.5	23.0	63.8	9.3	18.6	5.6	171.6
	C	699.3	334.5	468.1	457.8	324.7	150.6	234.2	403.9	284.5	210.7	1308	325.6	22.4	33.0	16.3	49.6	*	250.3
1048N	M	17.4	0.0	0.0	0.0	0.0	0.0	13.9	2.4	0.0	0.0	0.0	0.0	54.7	10.8	37.8	55.9	13.5	39.9
	T2	11.7	20.7	39.4	9.5	18.3	13.0	42.4	24.9	41.2	0.0	153.8	9.5	17.5	9.2	33.9	45.4	0.0	69.4
	C	191.0	301.8	443.5	245.9	159.8	156.8	180.5	181.7	190.0	63.4	703.3	90.3	*	0.0	64.4	91.4	46.8	79.3
GROUP 5. OOCYST CHALLENGE GROUP																			
927N	M	*	0.0	0.0	9.6	48.2	12.7	15.2	16.7	27.9	9.9	21.2	*	15.8	27.0	20.0	25.7	4.4	20.7
	T2	*	0.0	85.3	62.7	66.9	28.6	57.1	47.0	124.0	30.4	25.4	*	18.2	20.0	20.0	29.7	26.8	8.3
	C	*	221.8	402.1	530.6	566.6	429.1	314.1	334.4	378.3	172.0	248.9	*	38.1	23.5	48.0	35.5	36.1	6.6
976N	M	*	0.0	0.0	10.3	22.1	10.6	15.2	13.5	15.7	12.8	20.0	*	*	*	10.3	13.0	*	17.6
	T2	*	0.0	41.7	34.6	92.9	33.8	41.9	53.4	94.7	75.7	66.0	*	10.0	*	31.0	16.7	*	633.3
	C	*	384.0	460.9	381.3	597.4	526.9	386.4	384.1	441.2	253.6	305.7	*	59.9	*	*	32.4	*	293.1
1032N	M	*	0.0	20.5	10.5	0.0	18.9	15.3	0.0	6.4	0.0	8.2	*	*	*	*	0.0	*	17.1
	T2	*	9.0	10.3	92.1	108.7	0.0	11.5	0.0	48.7	17.4	0.0	*	*	*	19.7	22.2	*	28.0
	C	*	283.4	398.9	473.4	329.2	266.9	193.9	171.7	174.9	149.9	158.1	*	*	*	45.7	*	*	35.6
1084N	M	*	0.0	8.1	9.6	16.1	9.5	8.0	17.4	12.3	0.0	15.4	*	*	*	*	*	*	9.1
	T2	*	8.8	150.7	83.3	160.4	54.1	144.6	20.8	499.7	245.4	246.1	*	*	*	*	77.7	*	9.5
	C	*	693.7	732.4	782.8	780.2	811.3	637.4	552.7	460.2	244.0	729.8	*	*	*	*	93.1	*	21.3

T = *in vitro* treatment: M = medium control; **T2** = toxoplasma tachyzoite antigen at 2μg/ml; **C** = Con A

Appendix 5.20 *Interferon gamma production* in vitro *in group 2*

Animal	T	Week												Lymph Node					
		0	1	2	3	4	5	6	7	8	9	10	11	RP LN	P-F LN	Med LN	MLN	PT	spleen
		IFNγ (pg/ml)																	
GROUP 2. INTRANASAL IMMUNISATION WITH SOLUBLE TOXOPLASMA ANTIGEN																			
638N	M	0.0	9.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.0	31.8	2.9	0.0	0.0	0.0
	T2	6.0	53.6	103.5	51.4	302.9	65.8	19.3	19.7	109.4	5.5	333.9	7.1	30.3	62.6	17.4	229.9	0.0	177.4
	C	923.2	749.0	975.4	880.4	1353	743.1	981.2	965.8	807.1	958.8	1429	258.0	362.3	327.5	151.0	446.9	81.6	666.0
748N	M	16.5	48.9	23.2	11.3	23.3	2.8	20.7	25.1	48.4	1.6	0.0	6.3	*	0.0	21.2	24.3	0.0	0.0
	T2	8.4	20.8	25.3	9.2	21.1	12.6	30.1	20.2	85.9	22.2	336.3	13.2	29.1	24.3	50.3	27.1	0.0	1205
	C	300.9	434.4	251.9	164.5	380.3	106.8	180.2	273.0	380.5	343.8	1184	503.1	372.1	92.2	57.6	84.0	13.8	1522
919N	M	0.0	0.0	0.0	0.0	0.0	0.0	13.1	0.0	0.0	28.9	25.1	0.0	0.0	4.6	29.2	16.7	31.8	77.0
	T2	15.0	16.0	18.2	13.3	16.1	14.0	50.5	24.2	47.9	0.0	881.4	336.5	13.3	34.6	13.3	179.6	16.9	0.0
	C	453.9	331.2	243.2	396.5	309.9	290.4	530.5	364.2	358.6	272.6	1706	437.8	157.4	19.5	185.8	508.3	47.8	19.0
923N	M	16.5	12.8	30.0	19.8	24.4	4.8	46.3	22.2	44.9	17.0	59.3	5.9	0.0	4.4	5.4	5.6	3.0	10.4
	T2	27.8	65.9	31.8	26.1	100.5	165.2	67.0	72.1	259.2	212.0	798.7	22.5	456.2	678.5	15.5	320.3	15.6	1537
	C	331.8	480.5	276.5	130.9	313.5	159.1	277.0	148.8	102.7	267.9	1726	254.2	155.6	341.8	81.3	*	49.8	1484
993N	M	0.0	0.0	52.4	0.0	0.0	0.0	31.5	0.0	0.0	0.0	55.0	0.0	0.0	52.8	7.4	6.5	17.9	0.0
	T2	0.0	16.0	50.2	11.5	13.7	7.0	69.2	36.9	60.1	17.3	83.7	13.3	0.0	4.3	20.6	0.0	11.1	59.3
	C	194.2	372.6	322.3	270.2	281.9	213.3	256.1	353.8	173.2	282.0	628.1	340.5	22.4	27.1	41.2	40.7	103.3	103.8
1031N	M	5.9	0.0	2.2	0.0	0.0	0.0	42.9	8.0	0.0	6.3	79.2	0.0	*	26.1	12.4	35.8	25.4	0.0
	T2	9.9	22.7	58.0	11.2	17.7	10.4	49.2	28.4	68.3	3.2	645.9	15.9	*	17.6	0.0	32.9	18.9	10.6
	C	940.8	1029	928.3	546.0	683.6	433.0	508.4	581.8	645.0	489.4	871.9	177.4	*	63.3	11.9	15.3	13.6	37.2
1034N	M	0.0	24.3	22.8	23.2	22.6	21.9	46.8	30.2	66.7	1.3	133.4	5.7	*	15.6	6.7	96.5	14.5	9.4
	T2	0.0	28.5	76.8	30.1	20.3	4.9	37.3	23.1	69.8	2.5	505.8	7.4	22.4	21.2	0.0	904.2	11.6	0.0
	C	138.0	441.2	399.6	140.4	288.4	120.7	189.6	135.1	79.9	162.4	1237	215.4	*	134.2	34.4	*	126.5	23.0
1117N	M	13.0	14.1	21.1	12.8	21.5	9.0	15.1	12.0	15.0	2.5	53.0	7.6	5.6	26.2	0.0	8.8	38.4	46.3
	T2	32.6	10.0	2.6	23.2	17.2	3.0	18.7	38.1	56.7	0.0	28.1	10.0	38.5	407.2	13.6	7.9	39.3	5.7
	C	217.8	167.1	72.1	71.5	123.3	31.7	116.0	189.9	155.9	191.6	422.4	95.5	175.8	366.0	60.1	60.4	9.6	104.4

T = *in vitro* treatment: M = medium control; **T2** = toxoplasma tachyzoite antigen at 2μg/ml; **C** = Con A

Appendix 5.21 *Interferon gamma production* in vitro *in groups 3 and 4*

Animal	T	Week												Lymph Node					
		0	1	2	3	4	5	6	7	8	9	10	11	RP LN	P-F LN	Med LN	MLN	PT	spleen
		IFNγ (pg/ml)																	
GROUP 3. INTRANASAL IMMUNISATION WITH PARTICULATE TOXOPLASMA ANTIGEN																			
715N	**M**	21.1	40.3	26.1	24.3	24.4	10.5	21.6	21.5	40.6	13.7	0.0	7.5	0.0	1.6	3.5	11.8	0.0	4.8
	T2	69.2	116.4	25.2	56.4	46.8	34.7	27.9	18.7	75.8	54.1	25.8	333.2	0.0	0.0	7.9	14.3	11.5	57.9
	C	492.7	570.9	333.3	186.8	176.6	140.5	212.1	406.9	320.4	461.4	306.5	656.2	144.0	142.7	148.4	133.9	130.1	91.2
739N	**M**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	39.9	0.0	26.8	0.0	0.0	0.0	0.0	552.4	*	0.0
	T2	2.5	17.2	43.0	12.1	56.0	0.6	43.4	156.3	65.2	42.4	51.4	80.4	429.9	205.5	865.3	1179	0.0	1684
	C	665.6	591.6	601.0	260.3	520.0	164.6	572.1	686.5	615.8	583.3	485.4	585.5	109.4	118.6	239.8	380.4	249.1	1034
764N	**M**	18.3	0.0	0.0	0.0	0.0	0.0	4.9	0.0	0.0	0.0	21.9	0.0	0.0	0.0	0.0	10.9	0.0	0.0
	T2	8.9	15.0	19.1	16.5	13.5	8.8	46.2	16.8	42.4	6.9	42.4	256.1	468.3	132.9	904.4	309.4	19.2	835.9
	C	234.5	449.5	354.5	236.0	241.2	432.8	249.8	314.6	255.9	453.2	242.5	425.7	251.5	292.7	1214	256.7	170.4	704.8
1067N	**M**	17.2	20.4	20.6	14.2	22.0	7.7	21.2	32.3	29.7	0.0	0.0	6.5	3.9	0.0	0.0	0.0	21.2	0.0
	T2	0.0	30.2	0.0	24.2	31.1	4.1	28.4	81.5	62.4	16.4	25.8	16.7	0.0	107.9	107.8	70.8	1.9	12.0
	C	399.5	479.8	448.8	206.6	273.3	259.8	171.7	227.1	244.1	201.3	266.5	247.1	39.8	87.8	98.4	64.5	36.2	30.8
GROUP 4. INTRANASAL IMMUNISATION WITH PARTICULATE TOXOPLASMA ANTIGEN + CHOLERA TOXIN																			
770N	**M**	0.0	3.0	21.8	12.9	25.0	10.7	29.6	24.7	53.9	41.8	68.9	6.6	1248	28.3	3.3	47.1	17.8	7.3
	T2	0.0	72.5	37.4	101.0	1307	189.7	517.7	309.3	476.3	1366	65.9	120.9	1485	1017	199.8	1748	54.8	1558
	C	949.9	1066	524.8	808.7	980.4	724.7	609.8	593.3	645.7	774.4	362.4	340.6	665.0	1010	300.4	495.7	149.9	815.5
989N	**M**	12.1	19.6	16.6	23.3	26.7	5.2	38.1	32.2	68.1	23.6	78.0	6.0	7.4	0.0	0.0	4.0	3.7	0.0
	T2	18.9	26.1	35.2	15.9	67.5	12.8	68.7	34.1	69.7	30.5	158.7	152.7	15.7	53.6	0.0	26.1	0.0	149.8
	C	213.5	341.8	131.0	187.0	71.8	106.9	144.0	132.1	138.5	155.7	375.6	611.7	70.7	124.4	64.3	105.8	157.1	298.4
1030N	**M**	5.9	20.0	15.8	12.9	26.3	8.4	37.5	28.1	0.0	1.4	0.3	6.9	2.2	0.6	3.3	6.4	5.4	0.7
	T2	10.3	29.7	45.5	46.6	40.4	89.0	38.5	45.5	143.7	259.2	101.0	54.3	0.0	0.0	16.4	39.5	0.0	62.1
	C	319.6	260.6	88.9	177.2	171.1	152.6	130.7	156.5	146.8	148.3	147.2	171.3	78.8	41.2	38.7	124.7	26.7	28.9
1116N	**M**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.3	0.0	0.0	13.0	0.0	8.7	0.0	11.4	12.8	41.3	42.2
	T2	13.8	15.3	33.5	13.5	23.5	9.0	24.4	11.4	18.5	8.9	29.4	154.3	238.9	1117	5.3	1120	217.7	2.0
	C	593.5	643.7	662.4	398.7	390.1	294.0	296.0	512.6	325.9	425.3	581.7	475.4	195.6	137.3	252.7	390.9	484.4	20.3

T = *in vitro* treatment: M = medium control; **T2** = toxoplasma tachyzoite antigen at 2μg/ml; **C** = Con A

Publications Arising from this Thesis

Stanley, A.C., Huntley, J.F., Jeffrey, M. and Buxton, D. (2001). Characterization of ovine nasal-associated lymphoid tissue and identification of M cells in the overlying follicle-associated epithelium. *J.Comp.Path.* **125**, 262-270.

Proceedings of Meetings

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Novel Approaches to Helminth Control 3, Edinburgh, UK, July 2002. *A novel vaccination strategy for the control of parasitic diseases in sheep.*

BSI Congress, Harrogate, UK, December 2001. *Characterisation of ovine nasal-associated lymphoid tissue (NALT) and the identification of M cells in the overlying FAE.*

6th IVIS, Uppsala, Sweden, July 2001. *Characterisation of ovine nasal-associated lymphoid tissue (NALT) and the identification of M cells in the overlying FAE.*

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While the gross morphological features of ovine respiratory tract-associated lymphoid tissue (RT-ALT) have been described (Chen *et al.*, 1989), the present study investigates the structure and composition of the lymphoid tissue in the ovine nasopharyngeal tract, including the distribution of B- and T-cell subsets and the presence of M cells in the mucosal epithelium, as a prelude to future studies on intranasal vaccination.

Materials and Methods

Animals

Ten Greyface cross Suffolk sheep, aged 0·5–1 year, were housed in a conventional loose box, fed on hay and concentrates, and given free access to fresh water.

Collection and Preparation of Lymphoid Tissue

Sheep were stunned with a captive bolt and exsanguinated, and their spinal cords severed. Each head was removed and sagitally sectioned at the atlanto–occipital joint. The nasal septum was then removed, exposing both halves of the nasopharyngeal cavity. In a preliminary pilot study, lymphoid nodules were located macroscopically in the nasopharyngeal cavity of two sheep with glacial acetic acid (Cornes, 1965; Chauhan and Singh, 1970). The tissue containing nodules was subsequently removed from the 10 experimental sheep. Blocks of tissue (area 1 cm^2) were fixed by immersion in glutaraldehyde 3% v/v in 0·1 M phosphate buffer (PB), pH 7·4 for scanning electron microscopy (SEM) and individual nodules were fixed by immersion in glutaraldehyde 2·5% v/v in 0·1 M PB, pH 7·4 for transmission electron microscopy (TEM). The remaining tissue samples were then placed in a non-formaldehyde zinc salts fixative (Gonzáles *et al.*, 2001); after fixation, they were processed by routine methods and embedded in paraffin wax. Tissue sections (5 µm) were cut, stained with haematoxylin and eosin (HE), and examined for the presence of lymphoid tissue. Appropriate blocks were then sectioned further for immunohistochemical examination.

Immunohistochemistry

The antibody signal was amplified with the Envision Plus HRP System (Dako Ltd, Ely, UK). Briefly, dewaxed slides were incubated in a peroxidase "block" (0·03% hydrogen peroxide) for 5 min at room temperature, and then in 25% normal goat serum for 30 min at room temperature to prevent non-specific labelling of tissue antigens. The slides were then incubated with 100 µl of the appropriate monoclonal antibody (Mab) dilution overnight at 4°C. Controls were provided by replacing the primary antibody with the equivalent concentration of an IgG fraction from normal mouse serum for the same length of time. The secondary antibody (peroxidase-labelled polymer conjugated to goat anti-mouse immunoglobulins) was then applied to tissue sections for 30 min at room temperature. The sections were finally incubated with substrate chromogen for 7·5 min at room temperature, washed in distilled water, counterstained with haematoxylin, dehydrated in graded alcohols, cleared and mounted. Tris-buffered saline (0·05 M Tris HCl, 0·15 M NaCl, pH 7·2–7·6) was used to wash tissue sections between each incubation and to prepare the normal goat serum and antibody dilutions. The monoclonal antibodies employed, and their specificity, source and dilution, are shown in Table 1 (see also Gonzáles *et al.*, 2001).

TEM

After fixation for 48 h, the tissue samples were placed in 0·1 M PB for 15 min. The PB was replaced by 2% osmium tetroxide for 2 h, and the tissue was then dehydrated in graded water-acetone mixtures and embedded in an Epon-Araldite mixture. After polymerization at 60°C for 48 h, ultrathin sections were cut and stained with uranyl acetate and lead citrate and examined by a Jeol JEM 1200EX transmission electron microscope operated at 80 kV.

SEM

After fixation for approximately 48 h the tissue was washed in 0·1 M PB and post-fixed in osmium tetroxide 1% w/v in 0·1 M PB, by a method incorporating the enhancement of osmium penetration with thiocarbohydrazide (Malick and Wilson, 1975). After post-fixation, the specimens were rinsed in several changes of PB, dehydrated through a series of graded ethanols (to 100%), and placed in acetone. The specimens were then subjected to critical point drying with liquid carbon dioxide, attached to aluminium stubs by means of silver conductive paint, sputter coated with gold and examined in a Stereoscan S250 Mark III scanning electron microscope at 10–20 kV.

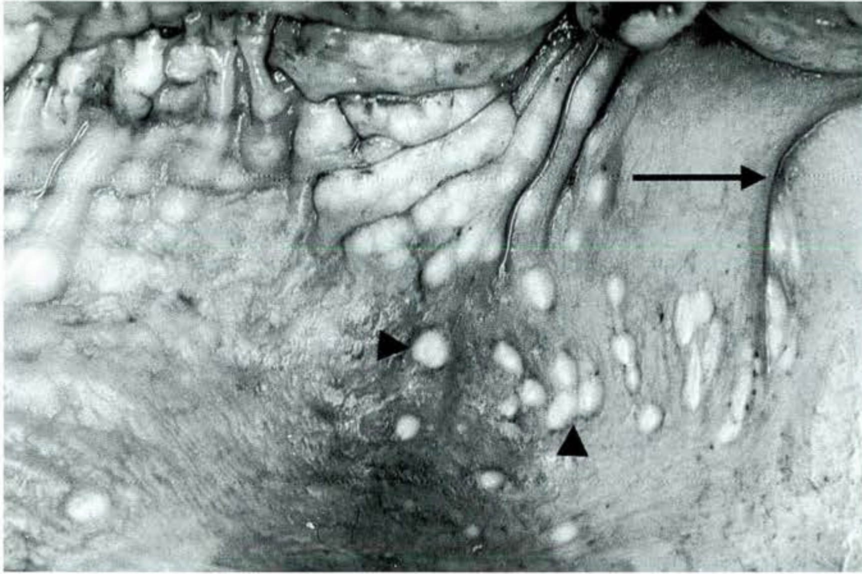

Fig. 1. Appearance of nasal lymphoid nodules after exposure to glacial acetic acid. Nodules appear as opaque white foci (arrowheads) and are clustered posterior to the opening of the Eustachian tube (arrow). ×10.

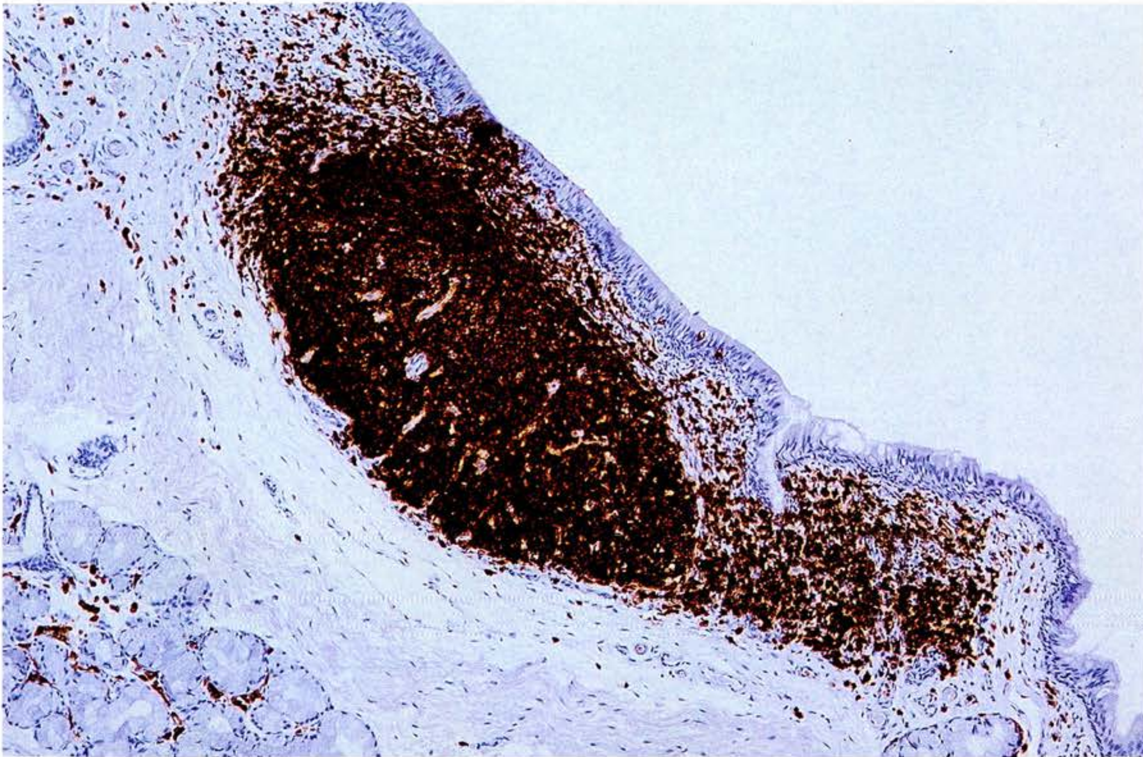

Fig. 2. B cells labelled with Mab VPM13 to surface IgM in a nasal-associated lymphoid follicle. B cells are predominant in the follicle and the immediate parafollicular area. ×107.

IgG_2 was observed throughout the tissue, and for IgA on the surface of the epithelium. Immunohistochemical analysis of serial sections revealed numerous αβ T cells surrounding B-cell areas. CD4+ T cells were typically concentrated around one pole of the follicle and in the immediately surrounding parafollicular area (Fig. 3A), while CD8+ T cells were apparently clustered in smaller

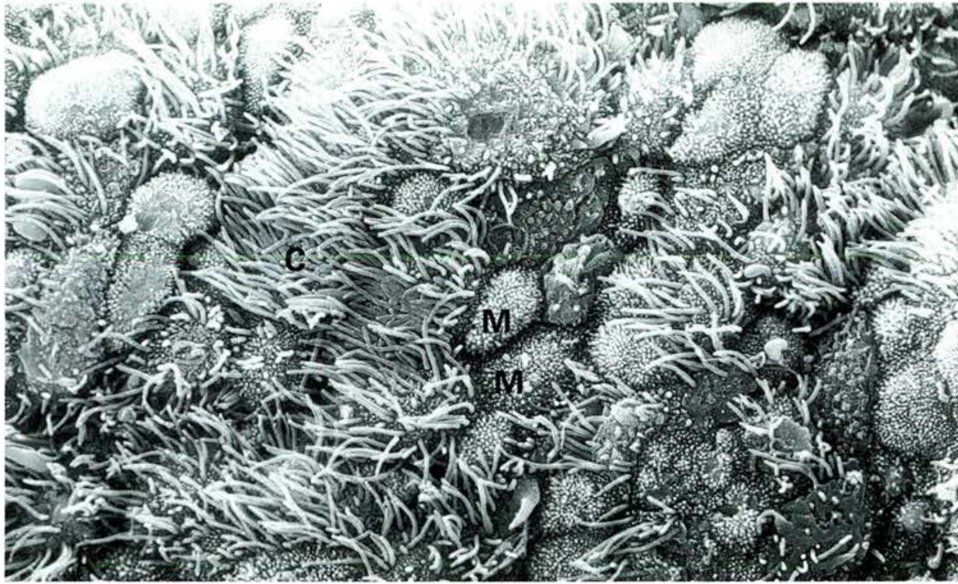

Fig. 4. Epithelium overlying NALT, showing the distribution of ciliated respiratory epithelial cells (C), microvillous cells (M) and mucus-producing cells in the area directly surrounding the flattened area of the dome epithelium. SEM. ×2300.

numbers in the parafollicular area (Fig. 3B). γδ T cells were scattered around the follicles and often seen within the overlying epithelium (Fig. 3C). Dendritic cells (CD1+) were scattered in small numbers around the follicles, and follicular dendritic cells (CD21+) were present in the central region of the follicle. Macrophages were dispersed throughout the follicle.

Ultrastructure of the Lymphoepithelium

SEM showed that both non-ciliated and ciliated cells were present in the follicle-associated dome epithelium. Non-ciliated cells had varying numbers of short, irregular microvilli or microfolds, which were sometimes flattened to form irregular ridge-like microplicae. In the central area of the dome, a region of flattened, relatively smooth epithelium was present; where this had been disrupted, large numbers of lymphocytes were seen, apparently extruding from the surface. Outside this area the epithelium was composed of ciliated cells, microvillous cells and goblet cells in varying proportions (Fig. 4). Ciliated and microvillous cells were approximately equal in number in the region directly surrounding the flattened area, with microvillous cells often clustered together. Towards the edge of the dome epithelium, a higher proportion of ciliated cells was present, with a few isolated microvillous cells scattered amongst them.

TEM showed that the epithelial cells overlying the NALT were ultrastructurally heterogeneous. Respiratory epithelial cells with long, regular cilia were interspersed with mucus-producing cells and epithelial cells displaying stumpy, irregular, fused microvilli. These microvillous cells were more electron-dense and appeared darker than the respiratory epithelial cells, and were frequently seen in close association with lymphocytes in the underlying lymphoid tissue (Fig. 5). Tangential sections showed that desmosomes formed tight junctions between both types of epithelial cell, and that lymphocytes were present within or in close association with the more electron-dense cells. Microvillous cells were absent from non-lymphoid associated areas.

Discussion

The present study confirmed that lymphoid tissue found in the ovine nasopharynx is characteristic of the MALT, and may be referred to as NALT. The concepts of mucosal immune protection can therefore be applied to ovine NALT, which contains all the elements necessary to function as a potent antigen sampling site.

In the sheep, the nasopharyngeal lymphoid nodules were clustered posterior to the opening to the Eustachian tube. Lymphoid tissue has been observed in this location in many species, including the horse (Mair *et al.*, 1988), where it may help guard against infection spreading from the pharynx towards the inner ear. Our results are therefore in accord with the report of Chen *et al.* (1989), which

and demonstration of their functional activity is now required to confirm that these cells are functionally similar to M cells described in other species and in other locations.

On the basis of the composition of the lymphoid tissue and the morphological characteristics and particular localization of the microvillous cells, it seems justified to conclude that this tissue belongs to MALT and has a similar role in mucosal immunity. Ovine NALT is strategically placed and contains the necessary components required to sample nasally administered antigens or vaccines. Microvillous cells will take up particulate antigens from inhaled air and present them to MALT via classic antigen-presentation pathways to generate effective mucosal and systemic immune responses. The induction of mucosal tolerance by soluble proteins has been well described in the respiratory tract in other animal models (reviewed in Lowrey *et al.*, 1998), but it remains to be determined whether the application of particulate proteins via the nasopharyngeal route elicits similar responses in the sheep. It is hoped that by targeting M cells this problem may be overcome. Ovine NALT, together with the pharyngeal tonsil, is the main component of defence in the ovine upper respiratory tract and, due to its strategic location and ability to trap, process and respond to inhaled antigens, may play an important role in the development of successful nasal vaccines.

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