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MUCOSAL VACCINATION WITH A NOVEL MICROPARTICLE DELIVERY SYSTEM

By

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This work is dedicated with much love

to the memory of my mother, Jeanne,

and to my family: Darren, Zoe and Sam.

The reward for a thing well done, is to have it done.

-Ralph Waldo Emerson

ABSTRACT

Despite recent interest in developing novel microparticle (MP)-based antigen delivery systems for mucosal vaccination, existing MP formulations possess a number of liabilities potentially precluding their widespread use. Nevertheless, there is sufficient evidence to suggest that if immunogenically stable vaccine material can be incorporated into biocompatible/biodegradable MPs and delivered to various mucosal surfaces, this may be an effective way to elicit protective systemic and mucosal immunity. To overcome difficulties associated with existing MP formulations, the present work describes the development of a novel polymer-grafted starch MP system which is capable of entrapping a wide variety of soluble antigens under mild conditions and which will elicit efficacious immune responses following intragastric (i.g.) or intranasal (i.n.) administration. The novel MP delivery technology was developed using starch, a well-studied, biologically acceptable and non-toxic material when given orally or parenterally. Antigen-containing starch MP were subsequently grafted with a hydrophobic silicone [3-(triethoxysilyl)-propyl-terminated polydimethylsiloxane, TS-PDMS] which, it was hypothesized, would protect microentrapped antigen from the deleterious environment found in the gastrointestinal (GI) tract, facilitate MP uptake by M cells overlying mucosae-associated lymphoid tissues (MALT) and/or act as an adjuvant or immunopotentiator. Moreover, following selective transport into mucosal inductive

sites, antigen-containing MPs should stimulate the generation of robust antigen-specific disseminating mucosal and circulating humoral immune responses.

In the work reported here, it was demonstrated that antigen-containing TS-PDMS-grafted MPs could be fabricated under mild conditions, allowing for the successful entrapment of a variety of protein and peptide antigens without any demonstrable loss in immunogenicity. Furthermore, it demonstrated that under acidic conditions, protein release from MPs was retarded when MPs were grafted with TS-PDMS. Although protein release from TS-PDMS-grafted MPs compared to ungrafted MPs was hindered, microentrapped protein was still released from TS-PDMS-grafted MPs, thus demonstrating that the MP composition allowed for entrapped proteins to readily leach from the MP matrix. Overall, these observations suggested that TS-PDMS-grafted MPs could serve as an efficacious MP-based mucosal vaccine delivery system.

To be considered an attractive alternative to current mucosal MP vaccine delivery vehicles, TS-PDMS-grafted MPs should incite both local mucosal and systemic humoral immunity following mucosal administration of low doses of microentrapped antigen. The studies in this report clearly demonstrated that oral immunization with very low doses of TS-PDMS-grafted MPs stimulated both systemic and mucosal humoral immune responses. Indeed, the adjuvanticity of TS-PDMS-grafted MPs seems to have arisen via a unique physicochemical relationship occurring between protein antigen and silicone in the starch matrix. This led to a

predominantly T_H2-type immune response following oral MP administration of relatively small amounts of microentrapped antigen. Furthermore, serum antibody titres were augmented after an oral or systemic boost, suggesting that the initial immunization protocol stimulated serum antibody memory capability, an advantage when developing successful mucosal immunization strategies. Surprisingly, systemic antigen challenge failed to boost antigen-specific sera IgA titres following i.g. immunization with microentrapped or soluble antigen. These results suggest Peyer's patch (PP)-stimulated IgA lymphocytes migrate to mucosal lymphoid compartments following i.g. MP administration, while antigen-specific PP-stimulated IgG plasmacytes have the propensity to migrate to both mucosal and systemic lymphoid compartments. In addition to stimulating specific serum antibody responses, i.g. immunization with TS-PDMS-grafted or ungrafted MPs resulted in specific sIgA responses in the gut; this is in contrast to soluble antigen which was incapable of inciting specific intestinal immunity following i.g. administration. Thus, compared to soluble antigen, TS-PDMS-grafted MPs have immunopotentiating activity when delivered orally.

The studies outlined in this work strongly suggest that i.g. administration of TS-PDMS-grafted MPs stimulated mucosal immunity via PP. Following i.g. immunization with a low dose of antigen-containing MP, but not soluble antigen, specific proliferation of PP cells was observed. Lymphocyte proliferation was subsequently observed in mesenteric lymph node (MLN) and splenic tissue. In

contrast, antigen-specific lymphocyte proliferation was not observed in gut lamina propria (LP) lymphocytes following i.g. immunization with MPs, thus suggesting that MP-induced immunity was incited by MP uptake and processing solely by PP.

It was shown also that i.n. immunization with low doses of microentrapped, but not soluble, antigen evoked robust circulating specific IgG responses, indicating that TS-PDMS-grafted MPs could enhance the immunogenicity of an i.n. administered soluble antigen. Indeed, i.n. immunization with microentrapped protein stimulated greater levels of specific sera IgG than was observed after i.g. MP administration with equal amounts of microentrapped antigen. However, unlike i.g. TS-PDMS-grafted MP immunization, antigen specific IgA was not detected in local mucosal secretions or sera following i.n. immunization with microentrapped antigen, thereby suggesting that i.n. immunization expresses unique immune responses compared to those evoked after comparable i.g. immunization protocols.

Although the nasal-associated lymphoid tissue (NALT) is considered to be the equivalent of Waldeyer's ring in humans, the exact process of generating immune responses when NALT is exposed to antigen is not clear. The present work describes the development of a novel, rapid and precise method for isolating NALT from mice to study its immune function and cell populations. Following the development of this precise NALT isolation technique, the pathway of i.n. administered TS-PDMS-grafted MPs was examined. It was demonstrated that i.n. immunization with low doses of microentrapped, but not soluble protein, evoked

robust circulating IgG responses via NALT cell activation. Numerous antigen-specific spot-forming cells (SFCs) were observed in the NALT and later the posterior cervical lymph nodes (pCLN) and spleen (SPL), confirming the selective drainage of NALT cells exclusively to the pCLN following i.n. administration of a particulate antigen. Although B cell activity was observed in the pCLN following i.n. MP administration, no specific IgA was detected in any lymphoid tissue examined or in nasal secretions following i.n. immunization with TS-PDMS-grafted MPs. This suggested that either pCLN in mice are not intermediate in evoking sIgA responses in the nasopharynx or that TS-PDMS-grafted MPs are incapable of stimulating this arm of the murine immune system. However, since it was previously demonstrated that i.g. immunization with comparable doses of TS-PDMS-grafted MPs evoked intestinal IgA responses, the inability to detect antigen-specific IgA in nasal secretions probably reflects differences between murine NALT and PP and not a unique inability of TS-PDMS-grafted MPs to evoke secretory immunity in the nose.

Thus, the present work describes the successful development of a novel polymer-grafted starch MP system which is capable of entrapping a wide variety of soluble antigens under mild conditions and which can elicit efficacious immune responses both orally and nasally, via MALT activation.

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ABBREVIATIONS

AP	alkaline phosphatase
APC	antigen presenting cell
APP	appendix
BALT	bronchus-associated lymphoid tissue
BCG	*Bacillus Calmette-Guérin*
BCIP	5-bromo-4-chloro-3-indolylphosphate
C_H	heavy chain
ConA	conconavalin A
cpm	counts per minute
CT	cholera toxin
CTB	cholera toxin B subunit
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DTH	delayed-type hypersensitivity
ELISA	enzyme-linked immunosorbant assay
ELISPOT	enzyme-linked spot-forming assay
FACS	fluorescence-activated cell sorting
FAE	follicle-associated epithelium
GALT	gut-associated lymphoid tissue

GI	gastrointestinal
GW	gut wash
HBSS	Hanks' balanced salt solution
HEPES	*N*-n-hydroxy-ethylpiperizine-*N'*-2 ethanesulphonic acid
HEV	high endothelial venule
HIV	human immunodeficiency virus
HSA	human serum albumin
HSV-2	herpes simplex virus type-2
[^{3}H]Tdr	[^{3}H]thymidine
i.d.	intraduodenal
IEL	intraepithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
i.g.	intragastric
IL	interleukin
i.n.	intranasal
ISCOM	immune-stimulating complex
LP	lamina propria
LPL	lamina propria T lymphocyte
LPS	lipopolysaccharide
mAb	monoclonal antibody

MadCAM-1	mucosal vascular addressin cell adhesion molecule-1
MALT	mucosae-associated lymphoid tissues
MHC	major histocompatibility complex
mIgA+	membrane IgA positive
mIgM+	membrane IgM positive
MLN	mesenteric lymph nodes
MP	microparticle
NALT	nasal-associated lymphoid tissue
NBT	nitroblue tetrazolium
NC	nitrocellulose
NGW	normal gut wash
NMR	nuclear magnetic resonance
NMS	normal mouse sera
NW	nasal wash
OD	optical density
OVA	ovalbumin
PBL	peripheral blood lymphocyte
PBS	phosphate-buffered saline
pCLN	posterior cervical lymph node
PE	phycoerythrin
PDMS	polydimethylsiloxane

pIgA	polymeric immunoglobulin A
pIgR	poly-Ig receptor
PLG	poly(lactide-co-glycolide)
PLN	peripheral lymph node
PP	Peyer's patch
SC	secretory component
SCID	severe combined immunodeficient
sCLN	superficial cervical lymph node
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEB	staphylococcal enterotoxin B
SEM	scanning electron microscopy
SFC	spot-forming cell
sIgA	secretory immunoglobulin A
sIgM	secretory immunoglobulin M
SLN	solitary lymphoid nodule
SPL	spleen
TBS	tris-buffered saline
TcR	T cell receptor
TD	thoracic duct
TEM	transmission electron microscopy

TGF	transforming growth factor
T_H	T helper cell
TNF	tumour necrosis factor
T_s	suppressor T lymphocyte
TS-PDMS	3-(triethoxysilyl)-propyl-terminated polydimethylsiloxane
T_{sw}	switch T lymphocyte
Tween-80	polyoxyethylene [20] sorbitan monoleate

CHAPTER 1

REVIEW OF THE LITERATURE

1.1 INTRODUCTION

Metazoans exist in a hostile environment and are exposed to a multitude of potentially harmful non-self substances, termed antigens, such as allergens and infectious agents. Although pathogens enter and invade organisms via several anatomic routes, they enter usually via mucosal surfaces. The mucosae, characterized by a secretory epithelia, include the gastrointestinal (GI), respiratory and urogenital tracts, the lactating mammary glands, the oral cavity, the nasopharynx and the middle ear. These sites constitute enormous surface areas which are susceptible to invasion by intruders due to inherent structural and environmental features. Additionally, intruders can also intrude systemically, breaching an organism's external integument by physical trauma, but this is a relatively rare event compared to the influx of intruders at the various mucosae.

To resist invasion and maintain physiological homeostasis, metazoans evolved protective anatomic structures and physiological responses. Metazoans possess several non-specific defences, collectively termed innate immunity. These defences can be structural, such as the integument and mucosal epithelium, or physiological such as haemorrhage, expiration, mucociliary transport, oral and respiratory expulsion, peristalsis and mucous, enzyme and acid secretions. Additionally, when these barriers are breached in mammals and aves, intruders encounter specialized molecules and cells, principally of the natural killer (NK) cell and monocyte/macrophage lineages which, together with elements of the

complement system, are additional effector components of innate immunity capable of ridding the host of intruders. However, despite the fact that these non-specific defences are broad in mechanism and most are present at parturition, they are not antigen-specific and do not improve upon subsequent exposure to a given antigen. Thus, phylogenetically more advanced metazoans, termed vertebrates, evolved sophisticated physiological defence mechanisms, collectively termed adaptive immunity, in addition to innate physiologic and physical defences.

The adaptive immune system is composed of specialized molecules and cells, such as antibodies, lymphocytes and other cell types which, following primary antigen exposure, elicit initial immune reactions termed primary responses in an attempt to eliminate the primary infection and prevent reinfection by the same or very closely related agent. A typical primary humoral immune response is characterized by a delay in its appearance, consisting primarily of immunoglobulin (Ig) M (IgM) molecules, the presence of which wanes as antigen is eliminated. Compared to immune responses following primary antigenic exposure, subsequent adaptive immune reactions, termed secondary immune responses, occur more rapidly, persist for longer periods of time and are greater in magnitude and potency than those responses associated with innate and primary adaptive defence mechanisms. Enhanced secondary immune responses occur via activation of quiescent, antigen-specific immune effector cell populations generated after primary antigenic exposure. Such responses result from the clonal selection and expansion

of specific immune cells, a collection of events operationally termed specific immunological memory. The Ig response of the secondary immune response may be IgG, IgA, or IgE, depending on the location in which the immune response takes place.

Adaptive immune responses can be divided into systemic and mucosal reactions primarily on the basis of the anatomic sites at which these are expressed. Although these reactions utilize some common humoral and cellular immune effector mechanisms, systemic and mucosal immune reactions are characterized by distinct effector cells and molecules performing different functions.

1.2 SYSTEMIC IMMUNITY

Systemic humoral immunity consists of mainly of circulating IgM and IgG. IgG is the most abundant antibody isotype associated with systemic humoral immunity and has the capacity for transplacental transport (Leach *et al.*, 1996) and transudation from the circulation into interstitial spaces, especially as a result of lymph formation, and into some luminal areas during inflammatory processes (Pollara, 1965). Transudation of serum IgG can occur at certain sites interfacing with the portals of the body, termed mucosal surfaces, such as the lungs (Kaltreider, 1984), genital tract (Brandtzaeg *et al.*, 1994), tears (Allansmith, 1973) and breast milk and saliva (Hurlimann, 1971) and represents a mechanism by which systemic immunity can be delivered to the various mucosae.

Although not usually considered to be a major component of the systemic humoral immune system, IgA is also found in serum, existing primarily as a monomer. However, in some species the proportion of polymeric IgA (pIgA) in serum can vary between less than ten percent to sixty percent (Underdown & Mestecky, 1994). In contrast to IgG, which functions primarily by neutralizing and opsonizing blood-borne antigens, the function of IgA within the blood (and lymph) is less well-established. Circulating pIgA can remove antigen via the liver (Russell *et al.*, 1981, 1983; Socken *et al.*, 1981) and serum IgA is capable of neutralizing viruses and agglutinating bacteria. However, the extent to which this antibody isotype interacts with inflammatory cells and the complement system is still under investigation. Evidence even suggests that in some systemic bacterial infections, serum IgA antibodies may attenuate IgG and complement-mediated bacterial destruction (Jarvis & Griffis, 1991).

In many instances, systemic immune responses are hallmarked also by cellular responses, such as delayed-type hypersensitivity (DTH) reactions and cytotoxic T lymphocyte (CTL) effector responses. Despite the proficiency of systemic immune responses in eradicating systemic infections, often these responses are unable to effectively protect organisms from mucosal pathogens since systemic responses are poorly expressed or absent at mucosal surfaces. This suggests that protection against mucosal invasion should utilize immunization protocols which best stimulate the mucosal arm of the immune system.

1.3 MUCOSAL IMMUNITY

Although pathogens can enter organisms through the integument, invasion via the mucosal portals is much more common. Mucosal surfaces constitute large surface areas which, unlike the integument, do not benefit from a protective, keratinized epithelium. Instead, these large surfaces are composed of diverse cell types which function principally in absorbing nutrients, exchanging gases and secreting fluids. Consequently, the local environments found at the mucosae are very conducive to pathogenic colonization and invasion, and likely represent the primary sites of exogenous antigen stimulation of the entire immune system.

Considering the elevated antigen load at mucosal sites, especially the GI and respiratory tracts, it is not surprising that tissues associated with mucosal surfaces are well populated with cells of the immune system, resulting in a highly efficient mucosal immune defence capability.

Mucosal immunity can be separated into humoral and cellular immune responses. The humoral component consists primarily of secretory IgA (sIgA) in glandular and mucosal secretions, with modest levels of IgM (Brandtzaeg, 1985) and IgG (Brandtzaeg *et al.*, 1986) and some IgD (Leslie and Teramura, 1977) and IgE (Durkin *et al.*, 1981; Jonard *et al.*, 1984). sIgA is a dimeric or polymeric IgA molecule associated non-covalently with an additional polypeptide termed secretory component (SC). The SC moiety is essential for IgA transport, via the polymeric Ig

receptor (pIgR) system (Mostov *et al.*, 1984; see section 1.3.1) and contributes to resistance to attack by metabolic and microbial enzymes (Lindh, 1975) resulting in sIgA being particularly stable and well-suited to function in the enzymatically-hostile environment that prevails at some mucosal surfaces. Although secretory Ig, in particular sIgA, provides a major protective element at the mucosae, the presence of unique mucosal T cell populations strongly suggests that they also play an important role in local mucosal defences. Working in conjunction with elements of the innate immune system, mucosally-situated B and T lymphocytes are usually effective in protecting the mucosae from invasion from pathogenic invasion.

1.3.1 Secretory Immunoglobulin A: The Hallmark of Mucosal Immunity

Historically, the presence of sIgA in secretions has been viewed as one of the best hallmarks of mucosal immunity available as its presence correlates with resistance to mucosal infection (Besredka, 1927; Amoss & Taylor, 1917). In contrast to circulating IgA, which is typically monomeric and bone marrow-derived, external secretions mostly contain locally produced (Jonard *et al.*, 1984) dimeric or pIgA (Tomasi & Bienenstock, 1968). Interestingly, serum IgA is thought to not make a significant contribution to the IgA antibodies in secretions and, conversely, IgA antibodies in secretions are not absorbed into the circulation. Dimeric IgA consists usually of two disulphide-linked IgA molecules formed through covalent conjugation of their Fc portions by an additional polypeptide termed the J (i.e. joining) chain

(McCune *et al.*, 1981), added during the antibody polymerization process in plasma cells immediately prior to secretion (Parkhouse & Della Corte, 1973).

In humans, two subclasses of IgA have been identified, IgA1 and IgA2, which are encoded by separate genes expressed in a tissue-specific manner (Mestecky & McGhee, 1987). For example, IgA1-producing plasmacytes predominate over those synthesizing IgA2 in the spleen, peripheral and mesenteric lymph nodes (PLN and MLN, respectively), tonsils, stomach and duodenum. The number of cells producing the two subclasses are more nearly equivalent in the lactating mammary and salivary glands and IgA2-producing plasma cells predominate in the large intestine and female genital tract (Crago *et al.*, 1984; Kett *et al.*, 1986). Although there is a high degree of identity between IgA1 and IgA2, a thirteen amino acid deletion is found in the hinge region of the human α2-chain which renders IgA2 resistant to IgA1-specific proteases secreted by a number of bacteria, including *Haemophilus influenzae, Streptococcus pneumoniae, S. sanguis, Neisseria gonorrhoeae* and *N. Meningitidis* (Kilian & Russell, 1994). Since the protective functions of sIgA depend on the integrity of the Ig molecule, any significant degradation of IgA is likely to influence its functional activity.

Following synthesis and secretion of dimeric IgA by plasmacytes residing beneath the secretory epithelia, interstitial dimeric IgA binds to a membrane receptor on the basolateral surface of mucosae-associated epithelial cells, termed the pIgR. With or without its Ig ligand, the receptor is internalized and transported

across the cell (Mostov *et al.*, 1984) where, at the apical surface, the pIgR is proteolytically cleaved and the large extracellular fragment (SC) is released into secretions with or without the IgA ligand (Apodaca *et al.*, 1991). Indeed, the single most important distinguishing feature of sIgA with respect to serum IgA is the presence of SC. Moreover, SC masks proteolytic cleavage sites in the hinge region of IgA1 molecules (Lindh, 1975), thereby rendering sIgA more resistant than other immunoglobulins to most mucosal proteases.

Once secreted into the lumen of mucosal surfaces, sIgA is believed to function as an effector molecule by several mechanisms. Traditionally, sIgA is thought to prevent contact of most pathogens with epithelial surfaces by complexing and entrapping them in the mucous-rich environment of the intestinal tract, or the respiratory and genital tracts, lactating mammary glands, saliva and tears thereby facilitating clearance by peristalsis in the gut, mucociliary transport at respiratory surfaces and fluid outflow from the genital lumen and other sites (Tomasi, 1983; Underdown & Schiff, 1986). Furthermore, sIgA can directly block epithelial attachment either by binding to specific sites on antigens (Abraham & Beachey, 1985) or by sterically hindering their interaction with receptors on mucosal surfaces (Childers *et al.*, 1989). Interestingly, sIgA appears capable of hindering the intestinal uptake of environmental macromolecules, such as antigenically intact food antigens (Walker & Isselbacher, 1972), which might protect the host from episodes of food-associated allergy or autoimmunity resulting from absorption of intact

antigen capable of stimulating anti-self reactivity (Homcy *et al.*, 1986; Krisher & Cunningham,1985). Recent studies (Kaetzel *et al.*, 1991; Mazanec *et al.*, 1992) suggest that sIgA also exhibits two other modes of protective action in mucous membranes. sIgA might bind with intracellular immunogens in epithelial cells during pIgR-mediated epithelial cell transport. This could result in virus neutralization, via complexing with nascent viral proteins or by binding to lumenal antigens that have breached or have been synthesized in the mucosa (Mazanec *et al.*, 1992). It might also actively transport Ig-bound antigens residing within epithelial cells or in the underlying lamina propria (LP) into the lumenal contents (Kaetzel *et al.*, 1991), thereby ridding mucosal tissues of antigens which have entered enterocytes.

Although all of these functions of sIgA have been proposed to operate concomitantly to optimize an initial line of mucosal defence against pathogenic intrusion, sIgA deficiency correlates with host morbidity but not mortality. Selective IgA deficiency does lead to increased tendencies to develop upper respiratory tract and oral infections, atopy, autoimmunity and neoplasia (Hong & Amman, 1989), yet most IgA deficient subjects compensate for this by producing IgM at their mucosal surfaces (Plebani *et al.*, 1983) and do not frequently suffer a variety of clinical symptoms associated with disease (Plebani *et al.*, 1983). However, IgA deficiency likely has much more serious consequences in populations lacking modern hygiene and public health facilities where mucosal surfaces are exposed to enhanced antigenic and infectious challenges. Nevertheless, the generation of pathogen-

specific sIgA is judged to be one of the most important defence mechanism against foreign invasion.

1.3.2 Other Immunoglobulins in Mucosal Secretions

Although IgA-secreting plasma cells predominate in mucosal tissues, significant yet variable numbers of IgM, IgG, IgD and IgE plasmacytes can be found also. In particular, secretory IgM (sIgM) is most like sIgA in that it results from transport via the pIgR (Brandtzaeg, 1981). The lower concentration of sIgM in mucosal secretions is likely a reflection of the lower proportion of mucosally-situated, IgM-secreting plasmacytes residing beneath the secretory epithelia. Due to molecular weight restrictions in SC-dependant transport (Schiff *et al.*, 1983), mucosal IgM also might not be transcytosed as well as mucosally-produced IgA. Additionally, SC-dependant IgM transport has not been demonstrated in some species such as rodents and rabbits (Underdown *et al.*, 1992). Nonetheless, heightened levels of sIgM are detected in the mucosal secretions of IgA-deficient individuals (Plebani *et al.*, 1983), thus suggesting that it serves a compensatory function in the absence of sIgA.

In most species, mucosal IgG is found also in similar concentrations to sIgM, although most of it is thought to enter secretions via paracellular transport or fluid phase endocytosis as well as transudation from serum (Rodewald & Kraehenbuhl, 1984). IgD-producing cells are located predominately in the mucosal tissues

associated with Waldeyer's Ring, representing 1-3% of the tonsillar immunocyte population (Korsrud & Brandtzaeg, 1980), a percentage that exceeds that observed in the GALT (Leslie and Teramura, 1977). This specific difference likely reflects an adaption to the unique situation of the tonsil as bacteria that inhabit the upper respiratory and alimentary tracts (but not those found in the gut) have receptors for IgD which stimulates IgD-bearing B cells to divide, hence increasing the numbers present in the tonsil (Forgren & Grubb, 1979). IgE has been detected in relatively low concentrations in respiratory & GI secretions and is often associated with allergic responses of the mucosae (Brown *et al.*, 1975; Mygind *et al.*, 1975). Overall, compared to sIgA, these other isotypes play a lesser role in humoral mucosal immunity and probably are crucial mainly in individuals lacking sIgA.

1.3.3 Mucosal Cellular Immunity

Although secretory Ig provides a major protective element at the various mucosal surfaces, the presence of mucosally-situated T lymphocytes suggests that these also play an important role in local mucosal protection. Two major T lymphocyte populations have been identified in mucosally-situated tissues: intraepithelial lymphocytes (IELs) and LP T lymphocytes. Although up to 10% of the intestinal IEL population in healthy individuals utilize the γδ T cell receptor (TcR) (Groh *et al.*, 1989), the majority of IEL in humans use the αβ TcR, express CD8 on their cell membranes (Brandtzaeg *et al.*, 1989) and demonstrate oligoclonal

expansion of a limited number of Vβ genes (Balk *et al.*, 1991). Although, the function(s) of human IEL is unclear, various hypotheses have been proposed to explain their role in mucosal immunity. Since IEL are situated basally above the basement membrane, it has been postulated that IEL ensure epithelial integrity by rapidly eliminating damaged or virus-infected epithelial cells. However, since IEL are not cytotoxic under normal conditions, recognition of the antigenic targets must involve TcR activation, perhaps via classic major histocompatibility complex (MHC) class I receptors following viral infections (Guy-Grand *et al.*, 1991).

In addition to IEL within the LP (particularly in the small intestine), T lymphocytes with cytotoxic potential and/or the ability to produce cytokines are found. Cytotoxic LP T lymphocytes include cells which can be activated by stimulation of their TcR to produce tumour necrosis factor- (TNF-)α or interferon- (IFN-)γ (Elson *et al.*, 1986; James & Strober, 1986), cytokines shown to affect epithelial cell growth and function including MHC class II molecule expression. Upregulation of MHC class II expression by the intestinal epithelia might provide a means of presenting mucosally-derived luminal or intracellular antigens. However, the majority of LP T lymphocytes express membrane CD4, memory cell markers (in humans) and do not proliferate in response to antigenic stimulation but, rather, secrete cytokines that mediate helper function (Kagnoff *et al.*, 1988). Interactions between mucosal T lymphocytes and the structural elements of the intestine are emerging as important functions to examine in understanding the control of the

physiological function of the gut.

1.3.4 Cytokines Influencing Mucosal Immune Responses

The mucosal immune system is distinguished from other peripheral lymphoid tissues by its capability to secrete sIgA into mucosal fluids. Although a portion of the mechanism that is responsible for the preferential generation of an IgA response in mucosal tissues is understood, the identification of cytokines that specifically stimulate IgA expression *in vitro* and *in vivo* raises the possibility that local production of specific cytokines within mucosal tissues is involved in the establishment of a microenvironment conducive to the generation of a sIgA response. Since T lymphocytes manufacture numerous cytokines and IgA responses are highly T cell dependant (Clough *et al.*, 1971; Ebersole *et al.*, 1979), mucosally-situated T lymphocytes are likely critical in orchestrating the production of sIgA in mucosal secretions.

Several cytokines have been identified that affect IgA expression. Interleukin (IL)-5 was first described as an IgA-enhancing factor and has since been demonstrated to dramatically enhance IgA secretion by lipopolysaccharide (LPS)- or T cell-stimulated Peyer's patch (PP) B lymphocytes; IL-5 likely stimulates maturation of IgA-committed precursors and not heavy-chain class switching of plasma cells to IgA production (Lebman & Coffman, 1988b). *In vivo* studies support these findings, demonstrating that IL-5 induces PP membrane IgA positive (mIgA+)

germinal centre cells to elicit a high rate of IgA secretion (Beagley *et al.*, 1988). It is important to remember, however, that although PP are the source of IgA-secreting B cell precursors, maturation of IgA-committed B lymphocytes does not occur at this site (Craig & Cebra, 1971; see section 1.4.1). Thus, if IL-5 acts *in vivo* to stimulate maturation of IgA blasts, it does not do so within PP.

In addition to IL-5, IL-6 has also been demonstrated to be essential for the development of IgA plasma cells. In PP B cell cultures, IL-6 was shown to induce a higher level of IgA secretion than IL-5 (Beagley *et al.*, 1989). Others have shown that IL-5 or IL-6 alone can incite modest levels of IgA secretion in T-cell depleted PP cultures, but that combining the two cytokines greatly enhanced IgA secretion (Kunimoto *et al.*, 1989). Like IL-5, IL-6 did not cause mIgA- PP cells to secrete IgA, suggesting that IL-6 also does not stimulate isotype switching to IgA. Although it is now generally accept that IL-5 and IL-6 are essential cytokines for the development of IgA plasma cells, IL-2 has been shown also to enhance IgA synthesis in LPS-stimulated B cell cultures (Coffman *et al.*, 1987). Thus, although IL-5 and IL-6 tend to play a major role in differentiation of mIgA+ B lymphocytes to IgA plasma cells, other cytokines also influence IgA responses.

Unlike the aforementioned cytokines, transforming growth factor (TGF)-β has been shown to stimulate isotype switching to IgA at the cellular level (Lebman *et al.*, 1990a). Moreover, the combination of TGF-β and IL-2 was shown to induce a transition from mIgA- to mIgA+ to IgA-secreting cells (Lebman *et al.*, 1990a).

Further, the addition of IL-2 to TGF-β-containing cultures increased IgA secretion without increasing the frequency of IgA-secreting cells (Kim & Kagnoff, 1990b). Collectively, these studies indicate that TGF-β induces B cell isotype switching to IgA and IL-2 acts to induce terminal differentiation of mIgA+ B cells.

Analyses of the effects of cytokines on IgA production has provided insights into both the role of cytokines and the specific T helper (T_H) lymphocyte populations involved in the generation of IgA responses at mucosal sites. Specific sIgA inductive and effector sites (e.g., PP and LP, respectively) contain several distinct T lymphocyte subsets that possess unique biological characteristics for the induction and regulation of IgA immune responses. In PP, two major subsets of CD4+ T lymphocytes have been isolated that are capable of regulating IgA isotype switching, proliferation and differentiation of IgA-committed B lymphocytes to become IgA-secreting plasmacytes. One T lymphocyte subset, termed T switch (T_{sw}) lymphocytes has been isolated from PP and, *in vitro*, induce membrane IgM positive (mIgM+) B cells to switch to mIgA expression (Mayer *et al.*, 1985; Kawanishi *et al.*, 1983; Kawanishi *et al.*, 1985), possibly via TGF-β secretion (Lebman *et al.*, 1990a). However, PP T_{sw} lymphocytes do not incite mIgA+ B cells to differentiate into IgA-producing plasma cells; this event is induced by a second population of PP-derived, FcαR expressing T_H cells (Kiyono *et al.*, 1982; Kiyono *et al.*, 1984).

T_H lymphocytes are classified into two subsets, T_H1 and T_H2, depending on

the cytokines they secrete and, although helper activity can be provided to B cells by both T_H subsets, T_H2 cells seem to be much more effective in this function (Lebman & Coffman, 1988a). This difference likely reflects the cytokine profile of T_H1 and T_H2 lymphocytes. Whereas T_H1 clones exclusively produce IL-2, IFN-γ and TNF-β (lymphotoxin), T_H2 cells synthesize IL-4, IL-5, IL-6 and IL-10 (Mosmann *et al.*, 1986; Mosmann & Coffman, 1989). In support of this theory, Xu-Amano *et al.* (1992) and others (Jain *et al.*, 1996b; Xu-Amano *et al.*, 1993) have demonstrated that although mature PP T lymphocytes are multipotent and can become either T_H1 or T_H2 cells, intestinal antigen delivery preferentially stimulates T_H2 cells in PP. Since IL-5 and IL-6 are potent factors for the differentiation of IgA-committed B cells (Lebman & Coffman, 1988b), hypothetically, T_H2 cells might incite mIgA+ B cells to become IgA-producing plasma cells. Indeed, recent evidence shows that the FcαR is always associated with T_H2, but not T_H1 cells (Sandor *et al.*, 1990) and is consistent with the finding that FcαR PP T cells are responsible for the differentiation of IgA plasma cells (Kiyono *et al.*, 1982; Kiyono *et al.*, 1984).

Although the precursors of IgA-secreting cells are generated preferentially in MALT (e.g. PP), an IgA-plasma cell precursor site, final maturation of PP B cells to IgA secretion also occurs in effector sites such as the intestinal LP and salivary glands (Craig & Cebra, 1971; Tomasi, 1983;). Indeed, the only mucosal tissue that appears enriched for IL-5-secreting cells is the LP (Taguchi *et al.*, 1990), demonstrating T_H2 cell cytokine enrichment in an IgA-effector site.

Although it is now generally accept that T_H2-derived IL-5 and IL-6 are essential cytokines for the development of IgA plasma cells, IL-2, a product of stimulated T_H1 cells, has been shown to also enhance IgA synthesis in LPS-stimulated B cell cultures (Coffman *et al.*, 1987), suggesting that T_H1 cells and their cytokines also influence IgA responses. Thus, although no single cytokine appears to be expressed uniquely in mucosal tissues, the optimal balance of cytokine-expressing T_H1 and T_H2 cells at mucosal sites may be critical to maintaining the efficacy of the mucosal immune system via the maintenance of IgA responses.

1.3.5 Mucosal Tolerance

Environmental antigens from food and microbial flora are in constant contact with mucosal surfaces and provide a potential stimulus for the entire immune system. Although a common result of such stimulation is the induction of mucosal and systemic immunity, an alternative outcome is a state of systemic unresponsiveness or tolerance to specific antigens. The term oral tolerance refers to a state of systemic unresponsiveness to parenteral immunization that is induced by previous enteric antigen exposure. Oral tolerance may represent an important immunoregulatory process that limits immune responses to commonly encountered, non-threatening, environmental antigens. This phenomena has been best described in laboratory rodents (Thomas & Parrott, 1974; Hanson *et al.*, 1977; Kagnoff, 1978; Challacombe & Tomasi, 1980; Kagnoff, 1982), but its presence has

been implied in humans (Korenblat *et al.*, 1968; Lowney, 1968; Walker, 1987). Indeed, North American Indians reportedly ingested poison ivy in an attempt to prevent what is now understood to be contact sensitivity to a plant-derived immunogen (Dakin, 1829).

Multiple mechanisms have been implicated in being responsible for the induction and maintenance of tolerance to mucosally-administered antigens including active suppression, clonal anergy and clonal deletion (Kroemer & Martinez, 1992; Miller & Moraham, 1992); low does of orally administered soluble antigen favour active suppression whereas higher doses favour clonal anergy (Weiner *et al.*, 1994). Local and systemic suppressor T (T_s) cell circuits may be of particular importance with regard to orally-induced tolerance. Antigen feeding can generate antigen-specific T_s cells in murine PP which later populate systemic lymphoid tissues such as the spleen (SPL) (Mattingly & Waksman, 1978). These findings raise many intriguing issues concerning the possible role of mucosal lymphocytes in regulating immunity to dietary antigens. Additionally, in several experimental autoimmune diseases, such as experimental allergic encephalomyelitis (Higgins & Weiner, 1988; Lider *et al.*, 1989; Nussenblatt *et al.*, 1990; Whitacre *et al.*, 1991), experimental autoimmune uveoretinitis (Nussenblatt *et al.*, 1990), diabetes (Zhang *et al.*, 1991), experimental myasthenia gravis (Wang *et al.*, 1993) and collagen-induced arthritis (Thompson & Staines, 1986; Nagler-Anderson *et al.*, 1986), autoantigen feeding has blocked the induction or

ameliorated established disease, suggesting that the feeding of autoantigens might be an effective therapy for human autoimmune diseases. However, while oral administration of soluble antigen will down regulate autoimmune disease states, relatively large antigen doses are required and gastric and intestinal degradation might the limit the effectiveness of this route.

Recently, several laboratories have also reported the amelioration of a variety of rodent autoimmune disorders following intranasal (i.n.) antigen administration (Harrison *et al.*, 1996; Ma *et al.*, 1996; Myers *et al.*, 1997). I.n. administration of collagen peptides (Myers *et al.*, 1997), insulin (Harrison *et al.*, 1996) or acetylcholine receptor (Ma *et al.*, 1996) suppressed collagen-induced arthritis, insulin-dependant diabetes mellitus and myasthenia gravis, respectively. Delivery of protein via the upper respiratory tract has a theoretical advantage as this route might allow for the use of smaller quantities of antigen which would otherwise be rapidly degraded in the intestinal tract and, thus, might represent a more efficacious approach to treating autoimmune diseases. However, the mechanism(s) of mucosal tolerance also must be clearly understood in order to effectively develop new mucosal vaccination protocols. Such vaccines clearly will be unsuccessful unless the induction of mucosal tolerance can be avoided and/or overcome.

1.4 MUCOSAL LYMPHOID TISSUES

The various mucosal surfaces including the middle ear, nasopharynx,

conjunctiva, lactating mammary and salivary glands, and the GI, respiratory and urogenital tracts (and the lamina propria associated with each site) contain organized and/or unorganized accumulations of lymphoid cells and, collectively, are referred to as mucosae-associated lymphoid tissues (MALT). In addition to sharing many structural similarities, several MALT (namely, the gut- and bronchus-associated lymphoid tissues, GALT and BALT, respectively) are characterized by local Ig production and the finding that activated lymphocytes derived from either mucosal site can circulate and localize selectively at other mucosal sites. Thus, the various MALT play important and interactive roles in the generation, differentiation and dissemination of mucosal lymphoid cells.

Lymphoid tissue in the GI tract, termed GALT, has been studied best due to its accessibility and ease of experimental manipulation. Consequently, the structural and functional relationships of the GALT have often been extrapolated to other mucosal sites. In most studies, this extrapolation has proven to be appropriate although structural and functional differences exist in the various MALT, especially amongst species.

1.4.1 The Gut-associated Lymphoid Tissue (GALT)

The total mucosal surface of the human GI tract is 200-300 m^2 making it the largest area of the body in contact with the external environment. However, despite its large surface area, this mucosal surface is protected physically only by a

monolayer of epithelium making it extremely vunerable to invasion. Although digestion and absorption of nutrients is the primary function of the GI tract, this structure also contains several innate physiological defences, such as bile, gastric acid, pancreatic enzymes, mucous and commensal bacteria which act in concert to protect the intestine from invasion by various harmful agents. Additionally, the GI mucosa contains a highly evolved adaptive immune defence system, termed the GALT, which is comprised of sites of organized (PP) and unorganized (the solitary lymphoid nodules, SLN) lymphoid tissue, associated MLN and LP cells with the complex task of protecting this mucosal surface.

1.4.1.1 Organization of the GALT

The GALT is principally comprised of organized lymphoid follicles (i.e. PP) but includes also SLN, MLN and, in some species, the appendix (APP). GALT tissue is also found in an unorganized manner throughout the gut and includes IEL and the LP. Thus, the GALT is the major site of antigen sampling and generation of specific effector and immunologic memory cells and, collectively, forms the largest lymphoid organ in the body.

Mucosal follicles in the GI tract can occur singly (SLN) but are mainly collected together to form large aggregates, termed PP. PP in rodents and humans consist of several to one hundred lymphoid follicles, each with a peripheral zone and a germinal centre (Owen & Nemanic, 1978); however, they lack both a defined

capsule and afferent lymphatic vessels. These follicles contain antigen presenting cells (APCs) such as B lymphocytes, dendritic cells (DCs) and macrophages, whereas T lymphocytes predominate in inter- and parafollicular regions (Spencer *et al.*, 1986). Following antigenic stimulation, most B lymphocytes in the periphery of the follicles are mIgM+ (Spencer *et al.*, 1986), whereas the majority of germinal centre B cells rearrange their Ig genes to express mIgA (Murray *et al.*, 1987). This is in contrast to PLN germinal centre B lymphoblasts which bear mainly IgM during primary immune responses and IgG during a secondary response (Kraal *et al.*, 1982). Between the follicles, thymus-dependant areas are found which contain numerous small T lymphocytes and conventional high endothelial venules (HEVs), through which circulating lymphocytes gain access to PP. The follicles are separated from the mucosal epithelium by a dome-shaped, CD4-bearing T_H cell region termed the corona (Ermak & Owen, 1986). The mucosal surface overlying the corona and underlying follicle, termed the follicle-associated epithelium (FAE), is devoid of villi and is composed of specialized differentiated M-cells, columnar epithelial cells, IEL and goblet cells. It is this specialized lymphoepithelium which facilitates transmission of antigen from the gut lumen to the underlying lymphoid tissue and is thought to be critical for initiation of intestinal immunity.

M cells occupy approximately fifty percent of FAE surface area in rabbit and ten percent of that in human and mouse (Pappo *et al.*, 1988). The key structural features of M cells include (1) fewer, shorter, and more irregular microvilli than are

found on adjacent absorptive cells; (2) close spatial association with immunocompetent cells that reside in pocket-like extensions of the intercellular space between M cells and adjacent epithelial cells; (3) a thin apical cytoplasmic rim created by these intrusive lymphocytes and macrophages, which is then the only epithelial barrier between intestinal lumen and immunocompetant cells; and (4) numerous endocytic vesicles that are especially abundant in this rim of cytoplasm (Bockman & Cooper, 1973; Bye *et al.*, 1984; Owen & Jones, 1974; Trier, 1991).

Although M cells represent an exceeding small minority in the intestinal epithelium, their functional significance is amplified greatly by their unique position over the GALT. M-cells endocytose and transport a wide variety of soluble intestinal antigens and microorganisms including particulates (Bockman & Cooper, 1973; Neutra *et al.*, 1987; Owen, 1977; Pappo & Ermak, 1989), viruses (Amerongen *et al.*, 1991; Sicinski *et al.*, 1990; Wolf *et al.*, 1983), bacteria (Inman & Cantey, 1984; Kohbata *et al.*, 1986; Owen *et al.*, 1986) and parasites (Marcial & Mandara, 1986) from the lumen of the small intestine into a specialized invaginated pocket in their basolateral surface which enfolds lymphocytes and macrophages (Trier, 1991). Thus, luminal antigens are likely taken up through M cells and then transported into the underlying lymphoid follicle, where antigen priming occurs and immune responses are then initiated. M cells also have binding sites for Ig on their apical surfaces (Rudzik *et al.*, 1975). Although the role of M cell Ig binding is unclear, one possibility is that it promotes re-uptake of small amounts of antibody-bound lumenal

antigen into inductive sites. Whether M cells can function also as APCs remains controversial because of the conflicting observations on the expression of class II MHC molecules and the existence of endolysosomal compartments by M cells (Allan *et al.*, 1993; Bjerke & Brandtzaeg, 1988). Nonetheless, since PP lack afferent lymphatics, M cells are likely a crucial component of the afferent limb of the intestinal immune system. However, M cells may also contribute to disease pathogenesis by providing a site at which potential pathogens, such as reovirus, can breach the epithelial barrier (Trier, 1991; Wick *et al.*, 1991; Wolf *et al.*, 1983). Consequently, factors predicted to influence M-cell activities both positively and negatively should be of interest for rational design of mucosal immunization strategies.

In humans and rodents, *in vitro* studies revealed that enterocytes express MHC class I and II molecules on their basolateral surface, thereby also potentially serving as APCs (Bland & Warren, 1986a; Mayer *et al.*, 1991). *In vitro* functional studies demonstrated that these class-II-MHC-expressing epithelial cells can present soluble peptide antigens (Bland & Warren, 1986a; Hoyne *et al.*, 1993) and activate primed T cells. However, in the intestine the frequency of interaction of enterocytes bearing basolateral MHC II with CD4+ T lymphocytes is unclear since the latter cell types generally are found in the LP and not within the epithelium (Bland & Warren, 1986a). Additionally, unlike PP-associated T cells, the LP T cell response is suppressive (Bland & Warren, 1986b), suggesting that such a

mechanism may play a role in mucosal immunologic unresponsiveness or tolerance (Weiner *et al.*, 1994). These observations suggest that oral vaccines should be formulated and delivered in such a manner to avoid uptake and presentation by gut enterocytes, as this might hinder the generation of mucosal immune responses.

1.4.1.2 GALT-derived Immune Responses

PP are considered the major initiation site of both mucosal and systemic humoral and cellular immune responses following intestinal antigen exposure. In a series of elegant studies by Craig and Cebra (Craig & Cebra, 1971, 1975), cells from a variety of lymphoid structures were adoptively transferred from donor rabbits into lethally-irradiated, allogeneic recipients. Only adoptively transferred GALT lymphocytes repopulated both the SPL and gut LP of recipient animals with a preponderance of IgA plasma cells. The importance of PP in the induction of humoral mucosal immunity was later described by Robertson and Cebra (Robertson & Cebra, 1976), using externalized intestinal loops. They demonstrated that introduction of antigens into the lumen of intestinal loops resulted in specific intestinal sIgA only if the immunized loop contained a PP. Additionally Pierce and Gowans (1975) demonstrated that intraduodenal (i.d.) cholera toxin (CT) immunization resulted in the appearance of specific IgA plasmablasts in thoracic duct (TD) lymph followed by their accumulation in the gut LP. Using this model with extirpation of either PP or MLN, it was demonstrated that the presence of intact PP,

but not MLN, were required for the appearance of IgA plasmablasts in TD lymph (Husband & Gowans, 1978). These observations are consistent with the importance of PP in antigen uptake from the gut lumen and as sites where antigen-specific B lymphocytes committed to IgA synthesis are generated. Moreover, adoptive transfer of subsets of PP B cells, purified by fluorescence-activated cell sorting (FACS), to irradiated rabbit recipients indicated that the richest source of precursors for IgA plasma cells contained sIgM- sIgA+ B cells that bore allotype markers for IgA (Craig & Cebra, 1975; Jones & Cebra, 1974). Together, these studies demonstrated that PP contain the precursors of IgA-secreting plasma cells and represent the major inductive site for intestinal IgA responses.

Once antigen is transported into PP via M cell transcytosis, it interacts first with the APCs and lymphocytes present in the intraepithelial pocket of M cells. Whether an immune response is generated at this sequestered intraepithelial site is not known. However, in the dome region below the FAE, IgM+ B cells, CD4+ T cells, DCs and macrophages form a cellular meshwork in which the antigens are likely to be taken up, processed and presented to lymphocytes prior to their activation (Ermak & Owen, 1986).

Following activation, PP B cells undergo a process of maturation and differentiation that differs markedly from that observed in peripheral lymphoid tissue and it is clear that there is a complex interplay among different lymphoid cells and cytokines (see section 1.3.4) which is responsible for the commitment and

differentiation of mucosal B lymphocytes. Three steps in the process have been proposed (McGhee *et al.*, 1989). The first step is antigenic activation of naive, resting B cells by antigens which causes them to enter the S phase of the cell cycle. Activation is parallelled by increased expression of class II molecules and IL-5 receptors. IL-5 promotes cell division and contributes to the progression of the lymphocytes through the cell cycle. This first step is likely to take place in PP germinal centres. The second step is Ig heavy-chain (C_H) isotype switching. This requires the interaction of CD4+ T cells, DCs and several cytokines, including TGF-β (Arnaud-Battandier *et al.*, 1980; Spalding & Griffin, 1986; Spalding *et al.*, 1984). The resultant germinal centre mIgA+ B cells are considered the immediate precursors of the IgA-committed effector B lymphoblasts that leave the PP, enter the mesenteric lymphatics and the blood, migrating to remote mucosal and glandular sites where they differentiate into IgA-secreting plasma cells. This third step requires the development of a well-developed secretory apparatus in the cells, the splicing of the α-C_H mRNA by removing an exon encoding membrane insertion, and induction of J chain expression, a polypeptide with one Ig-like domain that triggers IgA oligomerization (Matsuuchi *et al.*, 1986).

In addition to containing IgA-precursor cells, early studies (Kagnoff & Campbell, 1974) also demonstrated that the GALT contained CTL, although whether mucosal application of antigen was a necessary prerequisite for the generation of mucosal CTL was unclear. Experimental intestinal infection with

vaccinia virus (Isselkutz, 1984), reovirus (London *et al.*, 1986) and rotavirus (Offit *et al.*, 1988, 1989), has now demonstrated virus-specific CTL activity in various GALT tissues. Indeed, the presence specific CTL precursors (London *et al.*, 1986) and the ability of enteric virus to generate specific, long-lasting CTL responses in PP, demonstrated that adaptive cytotoxic reactions exist in the immunological repertoire in PP. For viruses that impinge on the GI mucosa, the presence of local specific CTL activity might result in the local containment, limitation and resolution of infectious agents.

1.4.2 The Bronchus-associated Lymphoid Tissue (BALT)

Lymphoid tissue situated at the bifurcations of the bronchi consists of both loosely distributed lymphocytes and those that are well organized into follicles and lymphoid aggregates, termed the BALT. Since its initial discovery in 1867 (Burden-Sanderson, 1867), the BALT has been identified in rabbits (Bienenstock *et al.*, 1973a; Bienenstock *et al.*, 1973b; Racz *et al.*, 1977; Tenner-Racz *et al.*, 1979), mice (Milne *et al.*, 1975) and rats (Bienenstock *et al.*, 1973a; Chamberlain *et al.*, 1973; Fournier *et al.*, 1977). However, unlike the GALT which is readily identifiable in humans and most experimental animals, several species do not have a well-developed BALT, the most notable being humans (Jeffrey & Corrin, 1984; Pabst & Gehrke, 1990). However, most species (including humans), can develop BALT structures under conditions of increased antigen load (Bienenstock *et al.*, 1973a;

Bienenstock *et al.*, 1973b; Weisz-Carrington *et al.*, 1987) suggesting that differences in the natural occurrence of BALT (and GALT) might be explained in terms of environmental stimulation. Indeed, in contrast to the GI tract, which is exposed continuously to hundred of grams of foreign antigens daily, the respiratory system is exposed to only a few micrograms of airborne antigens annually (Marsh, 1975). Additionally, most inhaled antigens are removed from the lung and delivered to the GI tract as either free antigen, via the action of the mucociliary escalator, or as material consumed and delivered to the gut by alveolar macrophages (Sedgwick & Holt, 1983; Spritzer *et al.*, 1968). It is only those airborne antigens not cleared by the aforementioned mechanisms with which the respiratory lymphoid apparatus must contend.

1.4.2.1 Organization of the BALT

The epithelial barrier of the bronchus consists of large areas of ciliated columnar epithelium with discrete areas of specialized lymphoepithelium overlying the lymphoid follicles of the BALT (Bienenstock & Johnson, 1976). Here, although antigen absorption is not as well understood as in the gut, the lymphoepithelium overlying BALT appears to be the major site of airborne antigen absorption in some animals (Fournier *et al.*, 1977; Racz *et al.*, 1977; Richardson *et al.*, 1976). Obvious comparisons have been made with the FAE of PP and BALT antigen-absorbing cells have been called M cells by analogy, yet their similarities appear more

functional than anatomical (Sminia *et al.*, 1989). First, although the FAE overlying BALT contains M cells (Bienenstock *et al.*, 1973a; Bockman & Cooper, 1973), the epithelium is not as specialized as that observed overlying PP, as light and electron microscope studies have revealed numerous differences between M cells and the covering cells of BALT (Pabst, 1992; Sminia *et al.*, 1989). Additionally, although the FAE overlying BALT can absorb antigens via pinocytosis (Fournier *et al.*, 1977; Tenner-Racz *et al.*, 1979) (as established for PP M cells), there is evidence that antigen may be taken up and transported across the respiratory epithelium and, presumably, into lymphatics (Braley *et al.*, 1978; Richardson *et al.*, 1976).

Under normal conditions there is relatively low antigen load in the respiratory tract and in the mammalian BALT classical germinal centres do not develop (Bienenstock *et al.*, 1973a). In humans they are observed only after antigenic stimulation (Bienenstock *et al.*, 1973a). Like PP, BALT follicles appear to contain primarily mIgA+ or mIgM+ B cells, in addition to follicular DCs (Sminia *et al.*, 1989). It has been suggested that the parafollicular areas lateral to the follicles (Racz *et al.*, 1977) are T-dependent due to the presence of interdigitating macrophages and reticular cells in these areas. Overall, these observations suggest that the architecture of BALT is very similar to PP and that the necessary lymphoid elements exist within BALT to initiate primary immune responses.

1.4.2.2 BALT-derived Immune Responses

Due to its strategic location at the bifurcations of the bronchi, the BALT is exposed to inhaled antigens. The lymphoepithelium overlying the BALT appears to be important in the delivery of antigen to the abundant numbers of lymphocytes situated in the underlying follicle. For example, *Bacillus Calmette-Guérin* (BCG) organisms introduced into the respiratory tract of rabbits have been found in the FAE and in underlying BALT follicles (Braley *et al.*, 1978; McDermott *et al.*, 1982). However, compared to PP, it is relatively difficult to obtain BALT and, thus, little is known about BALT-derived B and T lymphocytes. In view of its close physical resemblance to PP, it has been proposed that BALT lymphocytes might functionally resemble those of PP (Bienenstock *et al.*, 1973a; Bienenstock *et al.*, 1973b). Since the BALT, like the PP, has been shown to contain IgA plasma cell progenitors (Craig & Cebra, 1971; McDermott *et al.*, 1982; Rudzik *et al.*, 1975a), a portion of the sIgA appearing in respiratory secretions following antigenic stimulation of mucosal surfaces of the bronchi (McDermott *et al.*, 1982; Tomasi, 1982) and/or gut is likely BALT-derived. Indeed, the repopulation studies of Rudzik *et al.* (1975a, 1975b), in which bronchial and intestinal LP cells were transferred to lethally irradiated rabbits, demonstrated IgA-containing plasma cells from both sites repopulated both the gut and bronchial mucosa.

In comparison to local sIgA synthesis, little is known about the site(s) of elaboration of specific antibody of the IgM or IgG classes which are found often in bronchial secretions. For example, infection of respiratory surfaces with adenovirus,

poliomyelitis or measles virus, all of which penetrate the mucosa and disseminate systemically (viraemia), results in the appearance of Ig in serum as well as in respiratory secretions (Tomasi, 1982). In many of these situations it is difficult to define the relative extent to which local generation of specific Ig, as opposed to transudation or exudation from the serum, contributes to mucosal immunity.

Cell-mediated immunity is important also in defence of lung tissue against foreign materials, performing the functions of DTH and resistance to intracellular microbes and viruses. Indeed, the majority of lymphocytes retrieved from lungs of humans and experimental animals are T lymphocytes (McDermott *et al.*, 1982). In addition, it has been demonstrated experimentally that both cytokine-producing T lymphocytes and CTL can be specifically induced to appear in the lung parenchyma after appropriate immunization. However, although BALT plays a major role in lung immunology of several mammalian species, there are doubts concerning its importance in the human lung (Pabst & Gehrke, 1990; Pabst, 1992). Since there appears to be no major differences in mucosal handling of antigen, nor development of local immunity noted between species containing a well defined BALT and those in which BALT has less structure or is absent (Bienenstock & Clancy, 1994), it is unclear whether BALT plays an essential role in human lung immunology.

1.4.3 Waldeyer's Ring

Since normal human lung, unlike other species examined, usually lacks an organized BALT, the major source of B and T lymphocyte precursors in the upper respiratory tract of humans is thought to be the lymphoid tissue of Waldeyer's ring, a collection of oronasopharyngeal lymphoid tissues including the nasopharyngeal tonsil (adenoid), the pharyngeal lymphoid bands and the palatine and lingual tonsils (Goering & Vidić *et al.*, 1987). Tissues equivalent to Waldeyer's ring have been identified also in several other species (Kuper *et al.*, 1992) and are similarly and strategically located at the gateway of the respiratory and alimentary tracts where they are continually directly bombarded by antigen. Thus, Waldeyer's ring likely participates in respiratory and GI defence (Hameleers *et al.*, 1990, 1991; Nedrud *et al.*, 1987; Tamura *et al.*, 1989; Wu *et al.*, 1993), having been shown to display structural and functional similarities with other MALT (Kuper *et al.*, 1992).

1.4.3.1 Organization of Waldeyer's Ring

Waldeyer's ring displays characteristic lymphoid architecture and contains well-developed follicles, mantle zones, extrafollicular regions and reticular crypt epithelia (Koornstra *et al.*, 1991). The deep, partly branched crypts function to both increase tonsillar surface area and trap environmental materials. Additionally, M cells located in the base of palatine tonsil crypts transport entrapped antigens into underlying lymphoid follicles (Junqueira & Cameiro, 1980). Many HLA-DR+ cells also exist deep in the crypt that might also be important in antigen uptake

(Brandtzaeg, 1984; Tsunoda *et al.*, 1980).

A variety of immunocompetant cells have been identified in specific Waldeyer's ring tissue compartments including T and B lymphocytes as well as various types of APCs. Human tonsillar germinal centre cells reveal IgM as the predominant Ig isotype on the surface of B cells, followed by IgA, IgG and IgD (Tsunoda *et al.*, 1980). T lymphocytes characteristically occupy extrafollicular areas, but are present also in germinal centres (Yakanaka *et al.*, 1983). Thus, Waldeyer's ring harbours the requisite immunocompetant cells required to incite local immune responses.

1.4.3.2 Waldeyer's Ring-derived Immune Responses

Microbial and food antigens have been observed to be absorbed selectively by macrophages, HLA+ cells and M cells in the crypts of tonsillar tissues (Berstein *et al.*, 1994), suggesting that antigens transported in such a manner might incite local and disseminating immunity in much the same way as occurs in PP. Supporting this premise, tonsillar- and adenoid-derived lymphocytes have been shown to exhibit specific activity against respiratory syncytial viruses well as *Streptococcus pneumoniae*, *Haemophilus influenzae* and *S. Group A* (Berstein *et al.*, 1994), suggesting that following appropriate stimulation tonsillar B lymphocytes might mature into memory cells or terminally-differentiated IgM- or IgA-secreting plasma cells. Furthermore, palatine and nasopharyngeal tonsil-derived lymphocytes

have been proposed to contribute to the population of cells that supply local mucosal sites with IgA precursors, particularly the nasal mucosa, middle ear and parotid gland (Brandtzaeg, 1987).

The patterns of Ig secretion in Waldeyer's ring, however, differ from those typically found in MALT. IgG-producing cells predominate in palatine and nasopharyngeal tonsils (Brandtzaeg *et al.*, 1978), with IgA plasmacytes representing about 30-35% of the plasma cell population. Unlike the adenoid, there is no tonsillar production of SC (Scadding, 1990), and both IgA and IgG pass out directly into the pharyngeal secretions by transudation between epithelial cells, a process possibly enhanced during episodes of antigen-mediated inflammation. Unlike the GALT, IgD-producing cells are plentiful in Waldeyer's ring, representing 1-3% of the tonsillar immunocyte population (Korsrud & Brandtzaeg, 1980). This likely reflects an adaption to the unique situation of the tonsil. Bacteria that inhabit the upper respiratory tract (but not those in the gut) have receptors for IgD, which probably stimulate IgD-bearing B cells to divide, thereby increasing the numbers present in the tonsils (Forgren & Grubb, 1979). The observation that the nasal and bronchial mucosae, as well as salivary and lacrimal glands, contain an IgA and IgD plasmacyte distribution similar to that of tonsils (Brandtzaeg & Haneberg, 1997) suggests that these secretory sites are populated mainly by B-cell blasts generated in tonsillar tissue and perhaps in BALT. In support of this hypothesis, Ogra (1971) reported that combined tonsillectomy and adenoidectomy in children resulted in

diminished polio virus-specific antibody levels in nasopharyngeal secretions. Furthermore, the preferential appearance of IgA antibodies in parotid secretions was recently shown in rabbits after tonsillar antigen exposure (Brandtzaeg *et al.*, 1996) and activated human tonsillar B cells, transferred to severe combined immunodeficient (SCID) mice, were shown to migrate selectively to the lung and not to the gut mucosa (Brandtzaeg & Haneberg, 1997). Together, these observations suggest that Waldeyer's ring plays an important role in local immunity, likely suppling secretory sites in the upper aerodigestive tract. However, the precise role of this tissue in local and systemic immunity is not known as studies of Waldeyer's ring have been largely restricted to humans as investigations of structural and, until recently, functional equivalents had not been described in suitable experimental animal models.

1.4.4 The Nasal-associated Lymphoid Tissue (NALT)

Since the nasal mucosa is the first site of contact with inhaled antigens, it is possible that a nasal analogue of Waldeyer's ring exists in non-primate mammals lacking classic oropharyngeal lymphoid structures. Recently, a putative Waldeyer's ring equivalent has been described in the nasopharynx of the rat (Spit *et al.*, 1989), hamster (Kuper *et al.*, 1992) and mouse (Asanuma *et al.*, 1995; Belai *et al.*, 1977; Ichimiya *et al.*, 1991; Reuman *et al.*, 1989) appearing as paired lymphoid cell accumulations. This nasal-associated lymphoid tissue (NALT) might compensate

for the lack of tonsils as NALT is similar in structure to the pharyngeal tonsil, being covered also by ciliated epithelium. Although there are relatively few studies of rodent NALT, it is clear that NALT is an immunocompetant lymphoid inductive site, although whether it is an analogue of human BALT, GALT or Waldeyer's ring remains unclear.

1.4.4.1 Organization of the NALT

Immunohistological descriptions of NALT, particularly in the rat, have identified rodent NALT as a member of the MALT family (Kuper *et al.*, 1990; Spit *et al.*, 1989). In the rat, each member of a NALT pair appears as a cylindrical lymphoid accumulation (1 mm x 7 mm) oriented parallel to the nasal septum at the entrance of the nasopharyngeal duct (Koornstra *et al.*, 1991; Spit *et al.*, 1989). In the mouse, NALT has a similar shape, measuring 0.5 mm x 3 mm (McDermott & Snider, 1997).

Rat NALT contains lymphoid cell types found in classical mucosal inductive sites. Considerable numbers of reticulated cells and macrophages are present, as well as clearly-defined T and B lymphocyte areas (Reuman *et al.*, 1989), which are about equal in size; in this respect, NALT resembles BALT (Van der Brugge-Gamelkoorn & Sminia, 1985). Furthermore, rat NALT B cell areas are well-populated with mIgM+ and mIgG+ B lymphocytes, with surface IgA or IgE expressing cells being rare (Kuper *et al.*, 1990). This is in contrast to both BALT

and PP, where far more B lymphocytes carry mIgA (Plesch, 1982; Butcher *et al.*, 1982).

Rat NALT also contains lymphoid follicles, germinal centres, epithelial crypts and non-ciliated, cuboidal cells in the FAE which show morphological similarities to M cells (Richardson *et al.*, 1976; Koornstra *et al.*, 1991; Spit *et al.*, 1989). Rat NALT appears highly vascularized with many HEVs (Koornstra *et al.*, 1991) needed for lymphocyte migration into this tissue. Indeed, cell traffic studies in rats showed that NALT-derived lymphocytes migrated back to the NALT and its draining lymph nodes in far greater numbers that did PP-derived cells (Koornstra *et al.*, 1991). Moreover, T lymphocytes have been demonstrated to adhere better than B lymphocytes to NALT HEVs (Koornstra *et al.*, 1991), implying that more T than B cells enter NALT. In contrast, more B than T cells enter PP. The strength of T cell adhesion to NALT HEVs supports the suggestion, from these migration studies, that NALT is an important station in lymphocyte recirculation. Overall, these observations suggest that NALT might have properties distinct than PP and BALT in the mucosal immune system.

The degree of lymphoid structural definition observed in rat NALT has not yet been reported in mice, although preliminary evidence indicates that mouse NALT is organized with follicular areas (McDermott & Snider, 1997). No analogous lymphoid structure has been reported in humans. It cannot be ignored that rodents may have developed these specific NALT tissues, an evolutionary variation,

reflecting their nature as breathing and prone animals.

Recently, somewhat onerous dissection and/or enzymatic methods have been used to isolate mouse NALT cells (Asanuma *et al.*, 1995; Reuman *et al.*, 1989). Both studies reported approximately equal numbers of T and B lymphocytes (Asanuma *et al.*, 1995; Reuman *et al.*, 1989) and a predominance of CD4+ T cells as measured by FACS analyses (Asanuma *et al.*, 1995). However, in both studies, the crude isolation techniques resulted in cell preparations which likely contained lymphocytes from other sources in addition to the NALT, including IEL and LP. Since these lymphocyte preparation contained cells from both inductive and effector sites, it is difficult from these studies to determine the true lymphoid architecture and cellular composition of murine NALT and examine its role as a mucosae-associated inductive site. Clearly, novel NALT isolation techniques must be developed in order to examine the role of the NALT as a inductive site for nasally-situated immune responses.

1.4.4.2 NALT-derived Immune Responses

Non-invasive i.n. immunization has recently attracted increased attention because of its apparent greater efficacy in inducing both mucosal and systemic immune responses compared to systemic or enteric immunization (Abraham, 1992; Langermann *et al.*, 1994; Quiding-Järbrink *et al.*, 1995; Tamara *et al.*, 1989; Russell & Wu, 1991; Wu & Russell, 1993). Recently, several

investigators found that mice immunized i.n. with a variety of antigens including bacterial protein antigens coupled to the CT B subunit (CTB) (Russell *et al.*, 1996; Wu & Russell, 1993) and liposome-encapsulated measles virus (deHaan *et al.*, 1995) respond more strongly, and to lower antigen doses, than when the same inoculant is administered i.g. Specifically, with regard to inducing immunity in the oronasopharynx, i.n. immunization appears to induce higher levels of secretory immunity than that incited following i.g. or systemic immunization (deHaan *et al.*, 1995; Wu & Russell, 1993). For example, Wu & Russell (1993) demonstrated that, following i.n. immunization of mice with a protein antigen from *Streptococcus mutans* plus CTB, specific antibody responses were induced predominantly in the saliva and trachea, whereas i.g. antigen immunization with the same antigen induced the strongest IgA response in the gut. I.n. immunization also has been repeatedly demonstrated to induce better protection from trachea and lung infection, compared to i.g. immunization, after challenge with a variety of antigens including *Bordetella pertussis* (Shahin *et al.*, 1992) and respiratory syncytial virus (RSV) (Kanesaki *et al.*, 1991). Unexpectedly, i.n. immunization induces strong sIgA responses not only in the respiratory tract, saliva and circulation, but also in the female genital tract (Gallichan *et al.*, 1995; Hordnes *et al.*, 1997; Russell *et al.*, 1996; Wu & Russell, 1993). In fact, a recent study showed that vaginal responses induced by i.n. immunization could establish long-term protection against *Chlamydia trachomatis* challenge in the mouse vagina (Pal *et al.*, 1996).

The nature of antigens administered to the nasal mucosa and NALT seems to influence the resulting specific immune responses. Although inhaled particulates impacting into the mucous layer of the nasal mucosa (Proctor *et al.*, 1973) might be cleared rapidly by ciliary action, particulates are also selectively delivered into NALT via M cell transcytosis (Kuper *et al.*, 1992). Particulate antigens given i.n. initiate the migration of NALT-derived lymphocytes to mucosal and systemic sites via the posterior cervical lymph nodes (pCLN) (Koornstra *et al.*, 1991). In contrast, soluble antigens penetrate the entire nasal epithelium (Kuper *et al.*, 1992), reaching the superficial cervical lymph nodes (sCLN) which, in turn, drain to the pCLN (Koornstra *et al.*, 1991; Tilney, 1971). Unlike antigens directly stimulating the pCLN, those stimulating the sCLN, given i.n., have not been shown to stimulate secretory immunity (Kuper *et al.*, 1992). Thus, particulate antigens are the most efficacious in provoking mucosal and systemic immunity following i.n. administration. Although less conventional than the oral route, i.n. immunization offers several advantages for mucosal vaccine delivery. Smaller doses of immunogen are effective, probably largely due to avoidance of exposure to dilution and digestive and peristaltic processes in the GI tract. I.n. antigen administration is also best able to generate local secretory immunity in the oronasopharynx than immunization via other mucosal routes. Although the mechanism is unknown, i.n. antigen administration is able also to generate responses in the female genital tract (whether the male genital tract can respond similarly remains to be established). In contrast i.g.

immunization seems particularly adept at generating responses that are preferentially expressed in the intestinal mucosa. Thus, despite a paucity of studies, it is clear that rodent NALT has clear potential as an immunocompetent inductive site as i.n. antigen administration is seems more effective in inducing protective immunity in the oronasopharynx than immunization by other mucosal routes.

1.5 MIGRATION AND HOMING OF LYMPHOCYTES

Lymphocyte migration and homing are essential for an efficient and adequate immune defence of mucosal surfaces. This process allows for both the entry of naive cells into antigen-sampling sites (such as PP) and also provides for widespread dissemination of effector and memory cells to distant mucosal surfaces (e.g., gut LP). Cell-cell interactions and cell-substrate interactions mediated by receptors and ligands expressed on circulating lymphocytes and resident endothelial cells, govern both phenomena.

Specialized endothelial cells lining postcapillary venules, termed HEVs, display organ-specific surface recognition molecules called "vascular addressins" that are recognized by specific cell adhesion molecules, the "homing receptors", on lymphocytes (Springer, 1995). Organ-selective lymphocyte migration was first described by Gowans and Knight (1964). Radiolabelled lymphoblasts recovered from the TD and injected intravenously (i.v.) selectively homed to the GALT, such

as the PP and APP, as well as to the intestinal LP. Accordingly, in the GALT, HEVs are present in the T cell areas between B cell follicles and lymphocytes also extravasate through these vessels into the intestinal LP, the interstitial space of the respiratory or genital mucosa and glandular tissues.

Endothelial vascular addressins in the GALT (and other mucosal tissues) and lymphocyte homing receptors are structurally and functionally distinct from those found in PLN. One of the best characterized lymphocyte-HEV recognition systems in mucosal tissues consists of the α4β7 integrin homing receptor, the primary homing receptor found on murine PP-derived lymphocytes (Hu *et al.*, 1992), and its corresponding mucosal vascular addressin cell adhesion molecule-1 (MadCAM-1), which is present on HEVs in the GALT (Berlin *et al.*, 1993). MadCAM-1 is also expressed, albeit to a lesser extent, by HEVs in MLNs and by ordinary venules in the gut LP and lactating mammary gland (Streeter *et al.,* 1988); MadCAM-1 is involved in lymphocyte binding to PP as antibodies to MadCAM-1 block lymphocyte interactions with HEVs *in vitro* and lymphocyte homing to PP HEVs *in vivo* (Briskin *et al.*, 1993; Streeter *et al.,* 1988).

The β7 chain can also associate with another α chain, designated αE. This integrin is expressed predominantly on lymphocytes residing in intestinal sites and seems to be involved in the interaction with epithelial cells (Cepek *et al.*, 1993). αEβ7 is expressed on the surface of more than 95% of intestinal IEL, but less than 2% of peripheral blood T cells (Cerf-Bensussan *et al.*, 1987). The receptor is also

expressed on IEL in mammary glands.

L-selectin (human Leu-8 or LECAM-1; mouse MEL-14) was initially defined as the major homing receptor for PLN (Gallitin *et al.*, 1983). However, it also seems to participate in lymphocyte homing to PP, as suggested by experiments *in vivo* (Hamann *et al.*, 1990, 1994) and *in vitro* (Berg *et al.*, 1993). L-selectin interacts with several different ligands, all of which may be expressed by HEVs in PP. However, L-selectin is mainly expressed by circulating B and naive T lymphocytes. Thus, according to the current gut lymphocyte homing model, L-selectin and α4β7 operate in concert to recruit primarily naive cells to the GALT whereas only the latter contributes significantly to extravasation in the LP (Hamann *et al.*, 1994; Berg *et al.*, 1993). This targeted, region-specific trafficking of lymphocytes should enhance their opportunities to encounter and respond to intestinal antigens and to participate in the regulation of mucosal immune responses.

1.6 THE MUCOSAL IMMUNE SYSTEM

In many experimental animal models, local antigenic stimulation of mucosal surfaces and secretory glands has been demonstrated to result in the production of specific antibodies, not only at the site of stimulation but also in the circulation and in remote external secretions. For example, Heremans and Bazin (1971) showed that IgA-producing cells in orally immunized germ-free mice were found in the intestine as well as extraintestinal organs, including the MLN and SPL. Weisz-

Carrington *et al.* (1979) demonstrated also that ferritin-fed mice had anti-ferritin IgA plasmacytes of MLN origin in their lungs and gut, as well as in mammary and salivary glands. Similarly, Goldblum *et al.* (1975) showed that after infecting the intestinal tract of lactating mothers with live *Escherichia coli*, specific IgA antibodies were detectable in colostrum and milk to LPS from the infecting bacterium. Thus, clearly IgA plasmacytes derived from one mucosal site had the capability of localizing to other remote mucosal regions.

As early as 1961, Jacobson *et al.* noted the ability of PP to repopulate other tissues and restore their immunological capacities: mice subjected to total body irradiation, but with shielded PP, retained their immune functions. This finding was interpreted as evidence of migration of lymphocytes from PP to peripheral organs. However, Craig and Cebra (1971) clearly demonstrated for the first time that PP are also an enriched source of mucosal IgA plasma cells. Adoptive transfer of lymphocytes from PP but not PLN from a donor rabbit into an irradiated recipient resulted in repopulation of the recipient intestine by IgA plasma cells of donor allotype. When PLN and SPL were used as sources of lymphocytes in these adoptive transfer experiments, however, few IgA-producing cells were seen in these mucosal tissues and glands. These studies were extended by Rudzik *et al.* (1975a,b), who also investigated organized BALT and demonstrated similarities between BALT and GALT: lymphoid accumulations of the bronchus were a rich source of IgA precursors and transfer of such cells into a recipient animal resulted

in the repopulation of LP of both the gut and bronchi with IgA plasma cells of donor origin (Rudzik *et al.*,. 1975a). In addition to respiratory and intestinal tracts, the IgA precursor cells from GALT and BALT also populated remote secretory sites such as the intestine (Guy-Grand *et al.*, 1974; McDermott & Bienenstock, 1979; McWilliams *et al.*, 1975, 1977), respiratory and female genital tracts (McDermott & Bienenstock, 1979) and the salivary (Montgomery *et al.*, 1983) and lactating mammary glands (McDermott & Bienenstock, 1979; Weisz-Carrington *et al.*, 1979) (see section 1.5.1). Despite their preference to selectively localize in mucosal sites, however, IgA plasmacyte precursors do not preferentially travel to systemic sites such as the SPL and PLN, where systemically-stimulated IgG plasmacyte precursors predominate. Conversely, although a small number of plasma cell progenitors isolated from PLN migrate to mucosae, precursors from systemic sites primarily contain cells destined to become IgG-secreting plasmacytes that selectively migrate to systemic locations. The concept of a common mucosal immune system arose from these early observations demonstrating that the natural pathway for stimulation of the mucosal immune system occurs through GALT (or BALT) where environmental or artificially introduced antigens penetrating through M cells covering GALT and BALT, interact with resident APCs. Following antigen stimulation, B cells that contain a large number of IgA precursors leave GALT and BALT, enter regional lymph nodes (e.g., mesenteric), and enter the general circulation through the TD. They then migrate and, via homing receptors (see

section 1.5), localize to the LP of intestinal and respiratory tracts, salivary, lacrimal, mammary and cervical uterine glands where they differentiate into IgA plasma cells producing IgA antibodies specific for ingested or inhaled antigen.

Since the original description of the common mucosal immune system, a certain regional preference has been shown to exist. For example, MLN cells localize much better to the small intestine than in the lungs and the reverse is true of cells from the mediastinal lymph nodes (McDermott & Bienenstock, 1979). Moreover, despite the potential of BALT-derived B lymphocytes to populate the intestinal mucosa (Scicchitano *et al.*, 1984), respiratory tract immunization appears to have little influence on the intestinal immune responses (Bice & Shopp, 1988; Scicchitano *et al.*, 1984); the predominant flow of mucosally-primed lymphocytes seems to be from the GI to respiratory tracts (Scicchitano *et al.*, 1984; Van der Brugge-Gamelkoorn *et al.*, 1986). Additionally, antigen-stimulated tonsillar lymphocytes have been proposed to preferentially supply the upper respiratory and digestive tracts with specific IgA-precursors (Tsunoda *et al.*, 1980), although evidence for this view is circumstantial. NALT has been proposed as a rodent Waldeyer's ring equivalent, yet i.n. immunization of rats or mice did not result in disseminated antibody responses in other mucosal sites despite the detection of specific immune responses in the draining pCLN (Brandtzaeg & Haneberg, 1997). Moreover, in rats, NALT-derived lymphocytes were shown to migrate back to the NALT and its draining lymph nodes in far greater numbers that did PP-derived cells

(Koornstra *et al.*, 1991). These data suggest that specific responses induced by i.n. immunization are restricted primarily to the upper respiratory tract. Overall, however, current knowledge of the origins and pathways of mucosally-derived B and T lymphocytes indicates that the development and application of vaccines to should exploit this feature of the mucosal immune system to achieve protection of mucosal membranes.

1.7 VACCINATION

Vaccination is a deliberate manoeuver intended to safely elicit adaptive immune responses in the host that participate in resistance to potentially harmful intruders. Historically, the systemic compartment of the immune system has been the focus of vaccine design resulting in the development of many efficacious systemic vaccination protocols. However, there is a paucity of efficacious mucosal vaccination protocols. This largely reflects a more rapid advancement of an understanding of mechanisms responsible for systemic immunity than that concerning mucosal immune responses. However, since the mucosae are major sites of entry for pathogens, it should be possible to develop vaccination protocols which mimic the naturally-occurring induction of immunity against foreign invasion at mucosal surfaces.

1.7.1 Mucosal Route of Immunization

New approaches to mucosal vaccination have received attention recently, possibly due to an intense effort to develop a vaccine against human immunodeficiency virus (HIV). Mucosal immunization is particularly attractive because it might reduce the cost and risks associated with the use (and reuse) of needles and syringes and represents an inexpensive and simple means to administer vaccines to large rural populations where health care delivery can be suboptimal. More importantly, the dissemination of mucosal immune responses to distant mucosal sites provides further support for mucosal immunization as a possible means of inducing protective responses at remote mucosae, preferably accompanied by cellular and systemic immune responses.

1.7.2 Conventional Vaccine Design

Conventional vaccine protocols consist of attenuated viable organisms, killed whole organisms, subunits prepared from organisms or inactivated toxoids produced by pathogens. Vaccines containing viable replicating organisms are generally inexpensive to manufacture, are efficient at stimulating protective immunity and are regarded as safe. However, viable vaccines also have disadvantages including the need for refrigerated storage, their association with adverse reactions, especially in immunocompromised individuals, and occasional reversion to virulence with subsequent induction of disease (Bloom, 1989; Brown, 1990). Given these difficulties, it has been attractive to create vaccines composed of killed organisms,

their subunits, or relevant inactivated toxins, nearly all of which have proven safe and efficacious. In particular, using defined products in subunit vaccines as opposed to whole microorganisms has the advantage of including only those antigens required for protective immune responses while excluding extraneous and toxic cellular or nuclear components. Peptides and recombinant proteins are particularly attractive, since large quantities can be inexpensively synthesized or purified. The development of successful subunit vaccines, however, has been hampered by their insufficient immunogenicity when administered without delivery vehicles and/or adjuvants designed to facilitate effective and efficient uptake, presentation and processing of antigen. This is particularly striking in attempting to develop subunit vaccines for mucosal delivery in order to elicit secretory as well as systemic immunity. Indeed, even antigens that are adequately immunogenic when injected, together with alum, frequently are ineffective when administered via mucosal routes, even when excessive doses are administered (Mestecky, 1987). Thus, there is an urgent need to develop improved vaccine formulations to overcome these deficiencies associated with current mucosal immunization strategies and to develop new vaccines capable of providing protection against mucosal diseases.

1.8 PARTICULATE ANTIGEN DELIVERY SYSTEMS

An alternative to conventional vaccines is immunization with antigens

prepared by biosynthesis or by chemical synthesis of peptides. Such non-living immunogens reduce the risk of adverse reactions. However, compared with currently available vaccines, these antigens are poorly immunogenic for systemic or mucosal administration and might require vaccine delivery systems to enhance their immunogenicity. Particulate antigen delivery methods offer an approach to enhancing the immunogenicity of these soluble immunogens as they might temporarily protect mucosally-administered soluble antigen from degradation and/or dilution, facilitate M-cell uptake and transport into PP and establish antigen depots at mucosal sites.

1.8.1 ISCOMs

Immune-stimulating complexes (ISCOMs) (Morein *et al.*, 1984) are cagelike micelles, 30 to 40 nm in size, comprised of a matrix consisting of Quil A (a saponin extract from *Quillaja saponaria* Molina bark), cholesterol, phospholipids and various amphipathic antigens (Lovgren & Morein, 1988). Multiple copies of an antigen can be incorporated into ISCOMs via hydrophobic interaction with the matrix, allowing for its appropriate presentation to cells of the immune system. Antigens incorporated into ISCOMs and delivered systemically have been shown to stimulate enhanced humoral immunity (Howard *et al.*, 1987; Morein *et al.*, 1984) and can elicit CTL and DTH reactions (Mowat & Donachie, 1991). Mucosal immunization with ISCOMs has been reported also to stimulate cell-mediated immune responses

including DTH (Howard *et al.*, 1987; Lunden *et al.*, 1993; Mowat *et al.*, 1991) and to prime for antigen-specific, MHC-restricted CTL responses (Takahashi *et al.*, 1990). For example, in a study of murine responses to influenza virus, protective immunity was achieved with only one i.n. immunization with virus-containing ISCOMs (Lovgren *et al.*, 1990). However, other studies with ISCOMs showed that nasal IgA levels induced via the i.n. route were not very efficacious (Ahmeida *et al.*, 1993). ISCOMs have been demonstrated to resist acid and bile salts (Howard *et al.*, 1987) and have been shown to be capable of stimulating local and circulating antibody responses and cell-mediated immunity to soluble antigens when delivered i.g. (Mowat *et al.*, 1991, 1993). Although the potential of ISCOMs to elicit effective levels of secretory and circulating humoral immune responses and the induction of cell-mediated immunity is promising, several potential problems with this delivery system need to be addressed. First, repeated doses of ISCOMs need to be administered i.g. to elicit a strong immune response (Mowat *et al.*, 1993). There is concern also regarding saponin toxicity observed following systemic administration of large doses of ISCOMs, although these effects might be reduced with oral vaccination regimens. Finally, oral vaccination strategies would require a more prolonged and intense immunization response not afforded by current ISCOMs formulations, a problem that could probably be overcome by the inclusion of immunomodulators within the ISCOM matrix.

1.8.2 Liposomes

Liposomes are synthetic microscopic vesicles, composed of unilamellar or concentric phospholipid bilayer membranes surrounding an aqueous phase. Bioactive materials can be trapped inside the vesicle or incorporated into the liposomal membrane, allowing for the accommodation of virtually any substance, regardless of its shape, size, solubility and charge. Liposomes have been utilized for the successful delivery of hormones, enzymes, drugs and genetic material (Gregoriadis, 1990). The adjuvanticity of liposomes appears to depend on a number of variables, including vesicle size and structure, targeting to APCs, route of immunization and inclusion of immunomodulatory molecules. Antigen-loaded liposomes have been demonstrated to be a useful antigen delivery and adjuvant system, capable of stimulating protective immunity following systemic immunization (Brynstad *et al.*, 1990; Bulow & Boothroyd, 1991; Desiderio & Campbell, 1985). Promising results have also been obtained following mucosal immunization with liposome-associated antigens via the i.n. (Abraham & Shaw, 1992; Aramaki *et al.*, 1994; deHaan *et al.*, 1995; Wong *et al.*, 1994) and i.g. (Jackson *et al.*, 1990; Michalek *et al.*, 1989; Pierce & Sacci, 1984) routes. However, evidence also suggests that liposomes may not be as efficacious as other mucosal delivery/adjuvant systems (Ahmeida *et al.*, 1993) as are notoriously difficult to prepare reproducibly an are incapable of inducing optimum antibody responses, at least via the i.n. route, unless anaesthesia is used (deHaan *et al.*, 1995).

1.8.3 Protein Cochleates

A novel type of particulate vaccine delivery technology has been described recently, termed protein cochleates (Gould-Fogerite & Mannino, 1993; Mannino & Gould-Fogerite, 1995). These structures consist of protein-phospholipid-calcium precipitates that, unlike liposomes, are stable and have no internal aqueous space. During fabrication, the structure begins as a sheet and rolls into a scroll upon the addition of calcium. Cochleates survive in the GI environment and can be taken up by PP (Gould-Fogerite & Mannino, 1993; Mannino & Gould-Fogerite, 1995). As little as one i.g. immunization with cochleate-containing influenza viral glycoproteins was reported to facilitate the gradual appearance of specific circulating and secretory antibodies as well as systemic cytotoxic responses and protection following i.n. influenza challenge (Gould-Fogerite & Mannino, 1993; Mannino & Gould-Fogerite, 1995). Although study of cochleates as an antigen delivery technology is in its infancy, these structures may be a suitable particle-based technology to elicit immune responses at mucosal and systemic sites.

1.8.4 Proteosomes

Proteosomes have received attention as a delivery vehicle with the potential to enhance the immunogenicity of a variety of vaccine antigens including LPS, proteins and peptides. Proteosome is the term that has been used to describe the outer membrane proteins of *Meningococci* (Lowell *et al.*, 1988a, 1988b), which have

been used to create hydrophobic multimolecular, membranous 60-100 nm vesicles (Frasch & Peppler, 1982; Lowell *et al.*, 1988a, 1988b; Lynch *et al.*, 1984). These vesicles can complex with a wide variety of antigens, in the absence of detergent and situate complexed antigens in an optimal orientation for enhanced recognition by APCs or M cells. Indeed, any antigen with a hydrophobic moiety is instantly suitable to utilize the proteosome system without modification; this is especially practical since important epitopes are usually highly hydrophilic (Hopp & Woods, 1981). The practicality of proteosome vaccine formulation for commercial vaccine development is substantiated by the proven safety of meningococcol outer membrane antigens for human use (Lowell, 1997), the simplicity of large-scale proteosome production (Frasch & Peppler, 1982) and the observation that proteosome vaccines can by lyophilized and later reconstituted without any loss of potency (Lowell, 1997).

Proteosomes have been demonstrated to enhance both mucosal and systemic immunity following mucosal administration of a variety of complexed antigens, including polysaccharides, proteins and peptides. I.n. or i.g. immunization of mice with proteosome- complexed *Shigella* LPS has been shown to induce specific IgG and IgA in sera as well as bronchial and intestinal secretions (Orr *et al.*, 1993; Mallett *et al.*, 1995). In addition, long-term immunogenicity studies in mice indicate that serum anti-*Shigella* antibody levels were still strongly elevated one year after two i.n. immunizations with low doses of proteosome vaccine (Lowell *et al.*,

1995b). Furthermore, experimental infection of guinea pig eyes with *Shigella* was substantially or completely prevented by i.n. or i.g. immunization with *Shigella*-containing proteosomes (Orr *et al., 1993*). Proteins such as staphylococcal enterotoxin B (SEB) (Lowell *et al.*, 1995a) and HIV gp 160 (Lowell *et al.*, 1997) have also been successfully incorporated in proteosomes and studies have demonstrated that following i.n. immunization with entrapped, but not soluble antigen, high levels of specific serum IgG and IgA are observed (Lowell *et al.*, 1995a, 1997). Furthermore, proteosome-peptide vaccines have also been shown to stimulate anti-peptide IgA antibodies in bronchial and intestinal lavage fluids as well as sera IgG (Aboud-Pirak *et al.*, 1993; Kaminski *et al.*, 1993). Thus, although the cellular and immunological basis of proteosome vaccine success has not yet been fully explored, proteosomes should prove to be a productive approach to the generation of subunit vaccines against several disease targets.

1.8.5 Microparticles

Microparticles (MPs) offer an attractive alternative to current vaccine delivery strategies and studies suggest that antigen-containing MP delivery systems can enhance both mucosal and systemic immune responses following mucosal administration (Challacombe *et al.*, 1989; Eldridge *et al.*, 1989, 1990; O'Hagan *et al.*, 1993; Marx *et al.*, 1993; Moldoveanu *et al.*, 1993). In particular, MPs have several advantages for oral vaccine delivery. In addition to enhancing M cell uptake

and transport into the GALT, MPs might temporarily protect vaccine material from acid hydrolysis and enzyme proteolysis and establish an antigen depot allowing for controlled or pulsatile release of vaccine material that locally triggers mucosal and systemic immunity. MP delivery systems could also include immunomodulators, especially for use with poorly immunogenic subunit vaccines. Lastly, MPs might stabilize antigens against changes in temperature, thereby precluding the need for refrigeration.

1.8.5.1 Mechanism of Action

Early studies demonstrated that intestinal uptake of particulates such as starch granules and pollen occurs (Volkheimer, 1968). These particulates were found in blood, urine and cerebrospinal fluid after enteric administration, presumably after passing between enterocytes. These findings were subsequently challenged in a series of acute and chronic feeding studies (LeFevre *et al.*, 1980). Following i.g. administration of 15.8 and 5.7 μm diameter particles to mice, investigators failed to identify any particles in the aforementioned sites. However, 5.7 μm particles accumulated in the PP. Additional studies showed that chronically administered 2 μm diameter latex particles were largely found in the PP, suggesting that this might be a site where enterically-administered particulates selectively accumulate (LeFevre *et al.*, 1984). Latex particles were also found to adsorb, in high concentrations, to the lumenal surface of the intestinal epithelium around the domes

of PP (Jepson *et al.*, 1993; Scherer *et al.*, 1993). Since PP are an important site containing IgA plasma cell precursors (see section 4.1.1), delivery of antigens in MPs to the PP could be critical in the induction of mucosal and systemic antibody responses.

Studies suggest that M-cells overlying MALT (PP, BALT and NALT) are involved in the uptake and transport of particles (Pappo & Ermak, 1989). Uptake and transport of intestinally-administered fluorescent latex particles (600-750 nm) occurred solely by M-cells in the FAE of rabbit PP (Pappo & Ermak, 1989). Additionally, although inhaled MPs impacting into the mucous layer of the nasal mucosa (Proctor *et al.*, 1973) might be cleared by ciliary action, particulates given i.n. are also selectively delivered into NALT via M cell transcytosis (Kuper *et al.*, 1992). Present studies suggest also that, at least via the oral route, MPs are preferentially delivered to macrophages in PP (Eldridge *et al.*, 1989) and that MP are almost exclusively delivered to the mesenteric lymph (and MLN) within these migrating macrophages which can facilitate the subsequent dissemination of MPs to the systemic compartment (Eldridge *et al.*, 1989; Jenkins *et al.*, 1994; LeFevre & Joel, 1984; Wells *et al.*, 1988). Together, these results indicate that if particulate carriers are to be used successfully as either drug or antigen delivery systems, selective uptake by M-cells overlying MALT might be very important.

The extent and detail of MP uptake appears to vary considerably depending on the nature of the MP and possibly the animal model used. Species differences

are apparent in the uptake of MP materials from mucosal surfaces. Pappo and Ermak (1989) found the uptake of polystyrene MPs was at least an order of magnitude lower in murine PP than in rabbits. Rabbit FAE is rich in M cells so the capacity for MP uptake may be greater in this species. MP uptake from the human gut awaits investigation but the numbers of M cells in the human PP FAE appears to be less than that observed in rabbits (Pappo *et al.*, 1988; Trier, 1991).

MP uptake has been reported to depend on size (Eldridge *et al.*, 1990; Jani *et al.*, 1992; LeFevre *et al.*, 1980), hydrophobicity (Eldridge *et al.*, 1990; LeFevre *et al.*, 1985) and charge (Jani *et al.*, 1989, 1992). Notably, MP size appears to be a critical determinant of the fate of orally administered MP; MPs of less than 10 μm in diameter are absorbed by M cells and translocated into PP T and B cells areas (Eldridge *et al.*, 1989, 1990, 1991). Studies using poly(lactide-co-glycolide) (PLG) MPs demonstrated that those of less than 5 μm in diameter were enodcytosed and transported by macrophages (MAC 1+) cells through efferent lymphatics to systemic lymphoid tissues while those MP greater than 5 μm in diameter remained in PP (Eldridge *et al.*, 1990).

Differences are notable also in the ability of different types of particulates of comparable size to accumulate in the PP. In chronic feeding studies with five different particulates (asbestos, quartz, carmin, carbon and iron oxide), only carbon and iron oxide were demonstrable in PP tissue in appreciable amounts, despite exposure to high concentrations of all the particulates for extended periods of time

(LeFevre *et al.*, 1985). Iron oxide and carbon were the smallest and the most hydrophobic of the particulates examined. Similar findings have been reported in other studies (Eldridge *et al.*, 1990; Frey *et al.*, 1997), where the ability of MPs to accumulate in PP was found also to be dependent on the hydrophobicity of the polymeric material. Thus, particle size appears to be an important factor in particle uptake, with hydrophobic particulates less than 10 μm in diameter accumulating preferentially in PP.

1.8.5.2 Microparticle-induced Immune Responses

MPs have been constructed from a variety of biodegradable/biocompatible materials including PLG (Cowsar *et al.*, 1985), derivitized starch (Artursson *et al.*, 1984, 1986), albumin (Longo *et al.*, 1982;Yapel, 1985), thermally-condensed polyaminoacids (Santiago *et al.*, 1993), alginate-spermine (Offit *et al.*, 1994; Periwal *et al.*, 1997), polymethacrylate-coated sugar cores (Jain *et al.*, 1996a, 1996b), polyacrylamide (O'Hagan *et al.*, 1989) and phosphazenes (Payne *et al.*, 1995) and offer an alternative to current systemic and mucosal vaccine delivery strategies.

Several studies indicate that antigens entrapped in various types of MPs are immunogenic following systemic (Artursson *et al.*, 1984, 1986; Offit *et al.*, 1994; Payne *et al.*, 1995) or mucosal administration (Jain *et al.*, 1996a, 1996b; Offit *et al.*, 1994; Periwal *et al.*, 1997). However, it is PLG MPs which have received the most attention recently as mucosal vaccine candidates. Studies administering a variety

of PLG MP-entrapped antigens, both by mucosal and systemic routes, have demonstrated the ability of PLG MPs to elicit high levels of antigen-specific humoral and cellular immunity compared to administering soluble antigen alone. Notably, administration of PLG MPs via the mucosae is an effective way to incite specific humoral immunity in circulation (Allaoui-Attarki *et al.*,1997; Challacombe *et al.*, 1992; Eldridge *et al.*, 1989; Jones *et al.,1996*; Marx *et al.*, 1993; Shahin *et al.*, 1995) and, in some cases, mucosal secretions (Allaoui-Attarki *et al.*,1997; Challacombe *et al.*, 1992; Eldridge *et al.*, 1989; Jones *et al.*,1996; Shahin *et al.*, 1995). However, although antigen-specific IgA has been demonstrated in intestinal and/or salivary fluids following i.g. (Allaoui-Attarki *et al.*,1997; Challacombe *et al.*, 1992; Eldridge *et al.*, 1989; Jones *et al.*,1996; O'Hagan *et al.*, 1993) or i.n. (Shahin *et al.*, 1995) immunization with microencapsulated antigens, others have failed to demonstrated significant mucosal humoral responses following i.g. (Edelman *et al.*, 1993; Marx *et al.*, 1993; Moldoveanu *et al.*, 1993) or i.n. MP administration (Ray *et al.*,1993). Additionally, some researchers have failed to observe enhanced immunity following mucosal MP administration, compared to that observed following administration of soluble antigen (Cahill *et al.*,1995; Ray *et al.*,1993).

In addition to enhanced humoral immunity, studies have demonstrated that parenterally-administered PLG MP-entrapped, but not soluble antigen, can also prime animals for specific CTL responses both *in vitro* (Kovacsovics-Bandowski *et al.*, 1993) and *in vivo* (O'Hagan *et al.*, 1993; Partidos *et al.*, 1997). However, i.n.-

administered PLG MP-entrapped measles virus peptides, although demonstrated to stimulate specific CTL responses in splenocyte preparations, were not as efficacious as CTL responses stimulated when peptide was administered i.n. in saline or using CT as an adjuvant (Partidos *et al.*, 1996). Thus, although PLG MP have promise as a mucosal vaccine delivery system, it is not clear whether this MP formulation can reproducibly elicit mucosal humoral (Cahill *et al.*, 1995; Ray *et al.*,1993) or systemic cellular immunity (Partidos *et al.*, 1996) following mucosal immunization. Additionally, it is uncertain whether PLG MPs will provide efficient mucosal immunization for antigens other than those which are highly immunogenic and stable throughout the MP formation process (Alonso *et al.*, 1994). Nevertheless, there is good evidence to suggest that if immunogenically stable vaccine material can be incorporated into biocompatible/biodegradable MPs and delivered to various mucosal surfaces, this may be an effective way to elicit protective systemic and mucosal immunity.

1.9 Conclusion

Although there are numerous problems associated with current MP formulations, there is good evidence to suggest that if MPs can be created which are capable of entrapping a wide variety of antigens under mild conditions, mucosally-administered MPs would make an attractive vaccine carrier. To incite optimal mucosally-induced immune responses, future MP formulations should be

hydrophobic, have a diameter less than 10 μm and protect encapsulated antigens from dilution or the deleterious effects of the acidic and enzymatically hostile environments found in the respiratory and GI tracts. By creating MPs with these aforementioned characteristics, mucosally-administered soluble proteins and/or peptides should be selectively transported into mucosal inductive sites, resulting in the generation of antigen-specific disseminating mucosal and circulating immunity.

CHAPTER 2

RATIONALE, HYPOTHESIS AND OBJECTIVES OF THE EXPERIMENTAL WORK

2.1 RATIONALE OF THE EXPERIMENTAL WORK

It is now well-accepted that plasmacyte progenitors responsible for inciting mucosally-derived immune responses arise in mucosal inductive sites, especially the GALT (Befus *et al.*, 1978; McDermott and Bienenstock, 1979; McDermott *et al.*, 1980, 1986; McWilliams *et al.*, 1977; Rudzik *et al.*, 1975b) and BALT (McDermott *et al.*, 1982). These mucosally-situated precursors are believed to migrate to draining lymph nodes (mesenteric and bronchial, respectively) where they become committed to Ig synthesis (McDermott *et al.*, 1980; McDermott and Bienenstock, 1979; McWilliams *et al.*, 1977; Weisz-Carrington *et al.*, 1979). Subsequently, these precursor cells travel via the lymphatics and blood, ultimately localizing in a selective manner at various mucosae, including the GI, respiratory and female genital tracts and the salivary, lacrimal and lactating mammary glands (McDermott & Bienenstock, 1979; Weisz-Carrington *et al.*, 1979). Such cells do not appear to significantly localize in systemic lymph nodes and SPL. Indeed systemic lymph node and splenic cell populations contain far fewer mucosal IgA and IgG plasmacyte precursors, and cells from these sites do not localize at mucosae but, instead, return to their sites of origin where they synthesize IgG.

On the basis of the selective migration and localization of IgA and IgG plasma cell precursors, it was postulated that the various mucosae are linked together in a common mucosal immunologic system that is relatively distinct from systemic immunity (McDermott and Bienenstock, 1979; Weisz-Carrington *et al.*,

1979). This concept suggests that mucosal presentation of antigens may be one of the best ways to stimulate locally situated, immunocompetant lymphocytes that will migrate ultimately to various mucosal sites. On the basis of knowledge with respect to the origin of mucosal plasmacytes, oral or nasal antigen administration are the routes of choice for mucosal vaccination since antigen should reach the relevant lymphocyte populations.

Several lines of investigation must be pursued to understand the control of immunity by vaccinating at mucosal surfaces, as such information could aid researchers in determining how to manipulate mucosally-incited immunity. First, the types and magnitude of immune responses expressed at the mucosae must be ascertained in order to determine which of these will be relevant in resisting one or more pathogens. Second, the biology and attendant pathology ascribed to a given pathogen must be appreciated in order to stimulate and direct protective immune effector mechanisms against a pathogen at an appropriate phase of the infectious process. Additionally, the antigenic nature of the pathogen must be studied and the critical epitopes identified. Suitable immunologic adjuvants must also be identified to enhance selected immune responses. Finally, safe and effective vehicles for the delivery of critical epitopes and adjuvants to mucosal inductive sites must be developed. Taking all of these factors into consideration, essential criteria can be developed for safe and effective vaccines for use against mucosal pathogens.

Although mucosal surfaces are the optimal sites of vaccine delivery for

protection against mucosal pathogens, many viable vaccines may not be suitable for vaccination against mucosal infections because of safety concerns. Thus, antigens prepared by biosynthesis or by chemical synthesis of peptides are attractive as future candidates for mucosal vaccine material, since such non-living immunogens reduce the risk of adverse reactions. However, compared with currently available vaccines, these antigens are poorly immunogenic for systemic or mucosal administration and might require vaccine delivery systems to enhance their immunogenicity.

MP carrier systems offer an approach to enhancing the immunogenicity of mucosally-administered soluble immunogens. In addition to acting as an antigen depot, MPs can promote mucosal immunity as their particulate properties seem to facilitate interaction with mucosal lymphoid tissue. Furthermore, MPs can be fabricated to resist rapid degradation in the GI lumen, yet undergo controlled degradation and antigen release after uptake into mucosal inductive sites. Also, MPs have the potential to contain immunomodulators and to display surface hydrophobic moieties that promote their adherence to mucosal epithelia. Lastly, MPs might stabilize antigens against changes in temperature, thereby precluding a need for refrigeration. Taken together, these properties should allow MPs to mimic many of the physical characteristics of live antigens (Mestecky and Eldridge, 1991; Morris *et al.*, 1994).

MPs have been constructed from a variety of biodegradable/biocompatible

materials including PLG (Cowsar *et al.*, 1985), derivitized starch (Artursson *et al.*, 1984, 1986), albumin (Longo *et al.*, 1982; Yapel, 1985), thermally-condensed polyaminoacids (Santiago *et al.*, 1993), alginate-spermine (Offit *et al.*, 1994; Periwal *et al.*, 1997), polymethacrylate-coated sugar cores (Jain *et al.*, 1996a, 1996b), polyacrylamide (O'Hagan *et al.*, 1989) and phosphazenes (Payne *et al.*, 1995) and offer an alternative to current systemic and mucosal vaccine delivery strategies. In particular PLG MPs have been successfully used to induce potent humoral immune responses following mucosal administration (Challacombe *et al.*, 1992; Eldridge *et al.*, 1989, 1990; O'Hagan *et al.*, 1993; Marx *et al.*, 1993; Moldoveanu *et al.*, 1993). Presumably, MP-entrapped vaccine material is protected from the acidic and enzymatically hostile environment of the GI tract. Investigation of orally administered MPs has revealed that MPs are preferentially engulfed and transported by M cells into PP (Eldridge *et al.*, 1990; Jani *et al.*, 1992; Joel *et al.*, 1978; LeFevre *et al.*, 1985; Pappo & Ermak, 1989), the efficiency of which is facilitated by MP hydrophobicity and a diameter of less than 10 μm (Eldridge *et al.*, 1990; LeFevre *et al.*, 1985; Pappo & Ermak, 1989). However, despite the growing body of work on the efficacy of utilizing MP as a mucosal vaccine delivery system, it is not clear whether existing MP formulations (particularly PLG MP) will provide efficient mucosal immunization for antigens other than those that are highly immunogenic and stable throughout the MP formation process. Thus, novel MP formulations should be developed which have the capability of acting as a

controlled-release, vaccine delivery system with immunopotentiating activity with the potential for broad application in stimulating mucosal and systemic immunity.

2.2 HYPOTHESIS

To overcome difficulties associated with existing MP formulations, a novel hydrophobic biocompatible/biodegradable MP vaccine delivery vehicle should be created, capable of entrapping a wide variety of antigens under mild conditions. It was postulated that this MP delivery system would protect mucosally administered, encapsulated antigens from dilution or the hostile environment found in the respiratory and GI tracts, respectively. Moreover, it was postulated that antigen-containing, hydrophobic MP less than 10 μm in diameter should be selectively transported into mucosal inductive sites, resulting in the generation of robust antigen-specific disseminating mucosal and circulating humoral immune responses.

A novel MP delivery technology using starch, a well-studied, biologically acceptable and non-toxic material when given orally or parenterally (Laccourreye *et al.*, 1993; Whistler *et al.*, 1984) should be efficacious. The hydroxyl groups of starch facilitate compatibility with a wide range of protein antigens, making it attractive as a vaccine carrier. However, despite these advantages, starch is susceptible to degradation in the gut. Thus, to deliver biologically-active components, antigen-containing starch MP might be grafted with a hydrophobic coating which would protect microentrapped antigen from the deleterious

environment found in the GI tract, facilitate MP uptake by M cells overlying MALT and/or act as an adjuvant or immunopotentiator. Thus, whether starch MPs could be grafted with the silicone polymer 3-(triethoxysilyl)-propyl-terminated polydimethylsiloxane (TS-PDMS), a siloxane polymer similar to Simethicone®, a pharmaceutically acceptable excipient used as an anti-foaming agent in antacids (The United States Pharmacopoeia, 1994) was examined. Utilizing TS-PDMS-grafted MPs, studies were undertaken to determine whether a possible new particulate delivery system could be developed and to examine its mechanism of action in order to help develop efficacious mucosal vaccine formulations.

2.3 OBJECTIVES OF THE EXPERIMENTAL WORK

The primary objectives of the studies reported here are outlined in the following subsections.

2.3.1 Creation of a Novel Microparticle Vaccine Delivery Vehicle

To develop a novel MP delivery system for mucosal antigen administration, it was determined that a carrier system would need to fulfill a number of important criteria. First, a novel MP vaccine delivery vehicle should be chemically inert in biological systems, biologically well-characterized and be non-toxic and non-immunogenic. It should also be able to entrap sufficient quantities of antigen and release it without any demonstrable loss in antigen immunogenicity. Additionally,

i.g. administered MP should protect entrapped antigenic material from degradative intestinal fluids and be sufficiently hydrophobic so as to ensure uptake by M cells associated with various mucosal inductive sites. MP interaction with mucosal inductive sites would be facilitated further by developing MP to have a mean diameter of less than 10 μm. Lastly, novel MP vaccine delivery vehicles should be porous or fragile enough to allow the entrapped antigen to eventually present itself to the mucosal immune system and elicit and immune response. These criteria are addressed in Chapter 3 of the studies reported here.

2.3.2 Demonstration of Immunity Following Intragastric Microparticle Administration

Oral delivery of vaccine material has been widely studied mainly due to the ease of vaccinating via this route. Thus, the studies described in this work will determine whether i.g. administration of antigen-containing TS-PDMS-grafted MPs can stimulate a magnitude of specific immunity not observable following i.g. immunization with soluble antigen alone. Furthermore, whether low doses of orally-administered TS-PDMS-grafted MPs can incite both systemic and local and disseminating mucosal immune responses will be evaluate since this is a prerequisite of novel oral vaccine formulations. Studies discussing this objective are addressed in Chapters 3 and 4 of the work reported here.

2.3.3 Determining the Pathway of Microparticle-induced Immunity Following Intragastric Administration

In order to manipulate MP-induced immunity at mucosal surfaces, the mechanism(s) responsible for inciting antigen-specific local and systemic immunity, following mucosal MP administration, must be ascertained. Thus, following i.g. immunization with TS-PDMS-grafted MP, the site(s) of MP accumulation should be determined to verify that MPs are being transported into the GALT. Additionally, the site(s) of initial MP-induced lymphocyte activation should be determined in order to assess whether GALT inductive sites are being stimulated following i.g. administration of the novel antigen-containing MP delivery vehicle. Finally, the pathway of MP-stimulated lymphocyte migration should be determined to evaluate whether the novel delivery vehicle activates components of the common mucosal immune system. These objectives are examined in Chapter 4.

2.3.4 Determining the Pathway of Microparticle-induced Immunity Following Intranasal Administration

Unlike i.g. antigen administration, few studies have addressed the structure and/or function of mucosal inductive sites in the mouse nasal mucosa. Indeed, although preliminary studies suggested that murine NALT in an organized lymphoid tissue, cumbersome and crude isolation techniques have precluded detailed examination of murine NALT. Thus, before addressing the mechanisms of MP-

induced i.n. immunity, and the potential role of murine NALT in nasally-derived immunity, a novel dissection technique must first be developed to isolate and characterize mouse NALT. Studies addressing this objective are described in Chapter 5 of this report.

Following identification of murine NALT as a mucosal inductive site, studies should be conducted to evaluate the pathway of MP-induced immunity following i.n. immunization, particularly with TS-PDMS-grafted MP. By defining the role of murine NALT and its draining lymph nodes in MP-induced immunity, this route of mucosal vaccine delivery can be exploited in the future. Studies addressing the mechanisms responsible for inciting antigen-specific local and systemic immunity following i.n. antigen administration are detailed in Chapters 5 and 6 of the work reported here.

2.3.5 Demonstration of Immunity Following Intranasal Microparticle Administration

I.n. immunization has received increased attention as a route of variolation. Although i.n. antigen administration apparently stimulates more robust mucosal and systemic immunity than that observed following i.g. antigen administration, whether MP delivery vehicles can serve as an efficacious i.n. vaccine delivery vehicle has not been fully explored. Therefore, it will be determined whether i.n. administration of soluble antigens entrapped in TS-PDMS-grafted MPs can incite specific mucosal and systemic immunity. Furthermore, the relative effectiveness of i.n. MP delivery

will be examined by comparing the magnitude of MP-induced immune responses following i.n. MP administration to those observed following i.n. immunization with soluble antigen or i.g. administration of comparable doses of microentrapped antigens. These objectives will be addressed in Chapter 6.

CHAPTER 3

NOVEL POLYMER-GRAFTED STARCH MICROPARTICLES FOR MUCOSAL DELIVERY OF VACCINES

PREFACE

The synthesis and characterization of TS-PDMS and other silicones used in these studies was conducted by Dr. M. Brook, Department of Chemistry, McMaster University. Flow cytometric analyses of MPs was conducted by Dr. L. Loomes, Department of Pathology, McMaster University.

A formal communication of the work was published as Heritage P.L., Loomes L.M., Jianxiong J., Brook M.A., Underdown B.J. & McDermott M.R. (1996) Novel polymer-grafted starch microparticles for mucosal delivery of vaccines. *Immunology* **88,** 162-168.

3.1 ABSTRACT

Recent reports demonstrate that systemic and mucosal administration of soluble antigens in biodegradable MPs can potentiate antigen-specific humoral and cellular immune responses. However, current MP formulations are not be adequate for all vaccine antigens, necessitating the further development of MP carrier development. In this study, a novel MP fabrication technique was developed in which HSA was entrapped in starch MPs grafted with TS-PDMS, a biocompatible silicone polymer. The immunogenicity of HSA was preserved during the MP fabrication process. Following i.p. immunization of mice, TS-PDMS-grafted MPs dramatically enhanced serum IgG responses, compared with ungrafted MPs or soluble HSA alone ($p < 0.001$). When delivered orally, both TS-PDMS-grafted and ungrafted MP elicited HSA-specific IgA responses in gut secretions, in contrast to orally-administered soluble antigen. Indeed, TS-PDMS-grafted MPs stimulated significantly stronger serum IgG ($p < 0.005$) and IgA ($p < 0.001$) responses compared to those elicited by ungrafted MPs. These findings indicate that TS-PDMS-grafted starch MPs have potential as systemic and mucosal vaccine delivery vehicles.

3.2 INTRODUCTION

Parenteral immunization is highly effective in protecting against systemic infection, in part due to the production of circulating IgG and its transudation into

many extravascular sites, especially during inflammatory processes (Fazekas De St. Groth, 1951). However, parenteral immunization can fail to protect against invasion of mucosae. This failure is attributable to insufficient IgG transudation into mucosal secretions and the inability of systemic immunogen presentation to induce mucosal IgA production, the principal isotype present in external secretions. In contrast, mucosal immunization elicits both mucosal IgA and circulating IgG (Kenrick & Cooper, 1978). These observations support the concept of a mucosal immune system in which mucosally-situated IgA and IgG plasma cell progenitors are stimulated selectively by mucosal immunization (McDermott & Bienenstock, 1979; Weisz-Carrington *et al.*, 1979) and predicts that mucosal immunization can provoke both mucosal and circulating antibody responses better than parenteral immunization.

Oral vaccination protocols, using attenuated or non-viable organisms, their subunits or toxoids, have achieved varying degrees of success (Dudding *et al.*, 1972; Melnick, 1978). Nevertheless, safety concerns indicate that non-viable or subunit vaccines should be used whenever possible, yet these are often poorly immunogenic, thus necessitating large, repeated vaccine doses and the inclusion of immunomodulators such as CT (Elson & Dertzbaugh, 1994). Particulate antigen delivery methods offer an approach to enhancing the immunogenicity of orally-administered soluble immunogens. Such delivery systems include liposomes (Guzman *et al.*, 1993; Jackson *et al.*, 1990; Michalek *et al.*, 1989), ISCOMs (Mowat

et al., 1991, 1993), protein cochleates (Gould-Fogerite & Mannino, 1993) and biodegradable MPs constructed, for example, of PLG (Cowsar *et al.*, 1985; Challacombe *et al.*, 1992; Eldridge *et al.*, 1989, 1990; O'Hagan *et al.*, 1993; Alonso *et al.*, 1994), thermally-condensed polyaminoacids (Santiago *et al.*, 1993), phosphazenes (Payne *et al.*, 1995), albumin (Mora & Pato, 1993; Yapel, 1985) and derivitized starch (Artursson *et al.*, 1984). Notably MPs can enhance and prolong mucosal and systemic antibody responses following oral immunization (Challacombe *et al.*, 1992; Eldridge *et al.*, 1989, 1990; Marx *et al.*, 1993; Moldoveanu *et al.*, 1993; O'Hagan *et al.*, 1993). Evidently, MPs temporarily protect antigen from degradation, facilitate M-cell uptake and transport into PP and establish antigen depots.

In this study, a method for entrapping protein antigen in MPs constructed of soluble starch grafted with the silicone polymer TS-PDMS was developed. Following parenteral immunization of mice, antigen-containing silicone-grafted MPs stimulated a robust, antigen-specific circulating antigen-specific antibody response, while oral immunization with TS-PDMS-grafted MPs stimulated both secretory and circulating specific immunity. Such responses were achieved with relatively low amounts of antigen. These results demonstrate that TS-PDMS-grafted MPs can serve as a systemic and mucosal antigen delivery system, capable of eliciting both systemic and local mucosal immune responses.

3.3 MATERIALS AND METHODS

3.3.1 Synthesis of TS-PDMS

A mixture consisting of 17 mL of hydrogen-terminated polydimethylsiloxane (PDMS, viscosity 1000 cs, MW ca. 28,000; United Chemical Technologies) and 0.8 mL (3.5 mM) allyltriethoxysilane (molar ratio of SiH functional groups on PDMS to H_2C=CH, 1:3) was stirred with 0.05 mL of 1.0 M H_2PtCl_6 in *I*-propanol under nitrogen at 0°C and allowed to reach room temperature overnight. The isopropanol and unreacted allyltriethoxysilane were removed at 140°C at reduced pressure until gas ceased to form in the viscous fluid (approximately 6h). The residue was washed 4 times with H_2O to remove impurities and filtered through a bed of silica gel (2 mM). The product was characterized by ^{1}H-nuclear magnetic resonance (NMR) and infrared spectroscopy, both of which demonstrated the absence of SiH groups and the presence of the vinyl group (Smith, 1991).

3.3.2 Microparticle Fabrication

Using an emulsion-based process, HSA (Fraction V; Sigma, St. Louis, MO) was entrapped in ungrafted starch MPs or starch MPs grafted with a functionalized PDMS. To prepare HSA-containing starch MPs, 1.0 g of soluble potato starch (BDH) was dissolved in 2.0 mL dimethylsulphoxide at 85°C, cooled to 22°C and 1.0 mL of a 10% w/v aqueous solution of HSA was added. In some experiments, HSA was replaced by using ovalbumin (OVA, Sigma). This solution was finely emulsified

in vegetable oil (Crisco®; Proctor and Gamble Inc., Toronto, Canada) by vigorous stirring and sonication (low power setting 10; Bronwill model B10-IV; VWR Scientific, San Francisco, CA). To form MPs, the emulsion was added dropwise to 400 mL of acetone containing 0.5 mL polyoxyethylene [20] sorbitan monoleate (Tween-80; Sigma) with rapid stirring. The precipitated MPs were collected by filtration, washed twice with 1.0 L acetone, air dried and stored under desiccation at 4°C. MPs were used within 2 days of fabrication. Replacing the Tween-80 with 0.5 mL of TS-PDMS resulted in grafting of the silicone polymer to the starch.

3.3.3 Microparticle Characterization

MP size and shape characteristics were determined by flow cytometry using polystyrene microsphere size standards (Bangs Laboratories, Carmel, IN). The MP protein content was determined initially by including an ^{125}I-HSA tracer, prepared by the Iodogen method (Markwell & Fox, 1978), of known specific activity in the MP fabrication process and routinely thereafter by the Bradford protein assay (Bio-Rad Laboratories, Richmond, CA).

3.3.4 Assessment of HSA Contained in Microparticles

To extract protein entrapped within MPs, 10 mg of TS-PDMS-grafted or ungrafted MPs was incubated in 2.5 mL tris(hydroxymethyl)aminomethane (Tris Base)-buffered saline (TBS; pH 7.4) at 37°C. The buffer supernatents were

removed after various time periods and additional buffer was added to re-establish the initial volume. Protein released into the recovered buffer was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using Phastsystem 1-D gel electrophoresis (Pharmacia-LKB, Uppsala, Sweden) under reducing conditions. Samples were electrophoresed using 4-15% Phastsystem PAGE gels (Pharmacia-LKB) and SDS-buffer strips [200 mM N-tris[Hydroxymethyl]methyl glycine, 200 mM Tris Base, 0.55% SDS, 2.8% agarose]. After transferring onto nitrocellulose (NC) membranes (Gelman Sciences, Ann Arber, MI) and blocking NC with 50 mL 5% skim milk powder in TBS for 2h at room temperature, the blots were incubated with 50 mL of alkaline phosphatase (AP)-conjugated goat anti-HSA (O.E.M. Concepts, Toms River, NJ) for an additional 2h. The blots were washed with TBS and protein was visualized by incubating the membrane in 30 mL of developing buffer (100 mM Tris Base, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) containing 200 µl of 50 mg/mL nitroblue tetrazolium (NBT) and 100 µl of 50 mg/mL 5-bromo-4-chloro-3-indolylphosphate (BCIP). Colour development was halted by thorough rinsing with water.

3.3.5 Immunizations

Female BALB/c mice, age 6-8 weeks (Charles River Laboratories, Inc., Montreal, Canada) were used and allowed food and water *ad libitum*. Mice were inoculated i.p. or i.g. on days 0, 7, 14 and 70. Animals received antigen i.p. in 250

µl of phosphate-buffered saline (PBS; pH 7.4) or i.g. in 500 µl of 0.2 M $NaHCO_3$ using PE50 tubing (Becton Dickinson Co., Mountainview, CA).

3.3.6 Collection of Serum and Gut Washes

Individual blood samples were obtained via the retro-orbital plexus. Insoluble material was removed by centrifugation and sera were stored at -70°C until used.

To detect and quantify anti-HSA IgA in the intestinal lumen, mice were exanguinated and their small intestines removed and everted over capillary tubes. The everted intestines were incubated for 4 h in 5 mL of ice cold enzyme inhibitor solution containing 150 mM NaCl, 10 mM Na_2HPO_4, 5 mM ethylenediaminetetraacetic acid, 2mM phenylmethylsulphonylfluoride, 0.05 U/mL Aprotinin® (Sigma) and 0.02% NaN_3. Intestines were removed and the remaining solution (operationally termed gut washes; GWs) were clarified by centrifugation ($2.0x10^4$xg, 15 min), filtered through 0.45 µm syringe filters (Millipore, Mississauga, Canada) and stored at -70°C until used.

3.3.7 Measurement of HSA-specific Antibody Responses

An enzyme-linked immunosorbant assay (ELISA) was used to detect and quantify HSA-specific antibodies in individual sera and GWs. Duplicate serial dilutions of sera and GWs were examined using microtiter plate wells (Costar, Toronto, Canada) incubated with 100 µl HSA (10 µg/mL in TBS) followed by

incubation with 150 µl of 0.1 % gelatin in TBS (blocking buffer). Anti-HSA antibodies were quantitated by incubating wells with 100 µl with AP-conjugated goat anti-mouse IgG or IgA (heavy chain specific; Southern Biotechnology Associates, Birmingham, AL) diluted in blocking buffer. After washing, 100 µl of 1.0 M diethanolamine buffer, pH 9.8, containing 50 mM $MgCl_2$ and 1.0 mg/mL *p*-nitrophenylphosphate (5 mg phosphatase substrate tablets; Sigma) were added to each well and optical densities (ODs) were determined spectrophotometrically at 405 nm (Titerteck Multiskan Plus; ICN Biomedicals, Montreal, Canada). Normal mouse sera (NMS) or normal gut wash (NGW) pools, prepared from untreated animals, were used to establish baseline mean OD values. The results were expressed as reciprocal end-point titres representing the greatest serum or GW dilutions giving OD values exceeding 3 standard deviations (SD) above NMS or NGW mean values.

3.3.8 Statistical Analyses

Statistical analyses of data were done using Minitab® Release 7.1 (Minitab Inc., State College, PA). Rank transformation and parametric analyses were used. One-way ANOVA, Tukey test pair-wise multiple comparisons, Student's *t*-tests and Kruskal-Wallis tests were used to detect and compare mean differences between treatment groups. A level of significance of 95% was chosen for all tests.

3.4 RESULTS

3.4.1 Entrapment of HSA

Preliminary studies indicated that since protein antigen was rapidly degraded in the murine gut (Moldoveanu *et al.*, 1993), TS-PDMS-grafted starch MPs might protect antigen from the deleterious environment found in the GI tract. Thus, HSA was incorporated into TS-PDMS-grafted or ungrafted starch MPs. Figure 1 shows that both TS-PDMS-grafted and ungrafted MPs were approximately spherical in shape and both had a rough and very irregular surface appearance. MP diameter distribution was non-Gaussian, had a range of 1 to 100 μm and an arithmetic mean value (± SD) of 4.2 ± 3.0 μm (data not shown). Occasionally, fractured MPs were found in the preparation and these showed that the interior of both TS-PDMS-grafted and ungrafted MPs was spongelike in appearance (data not shown). TS-PDMS was associated with grafted MPs as judged by NMR spectroscopy. The silicone signal in the $CDCl_3$ extracts could clearly be seen at 0.08 ppm in ^1H NMR (data not shown). MPs fabricated with 100 mg of protein were determined repeatedly to contain 5-6 % w/w protein (HSA), measured initially by incorporation of an ^{125}I-HSA tracer into the MP fabrication process and routinely thereafter by the Bradford protein assay.

3.4.2 Retained Antigenicity of Microentrapped HSA

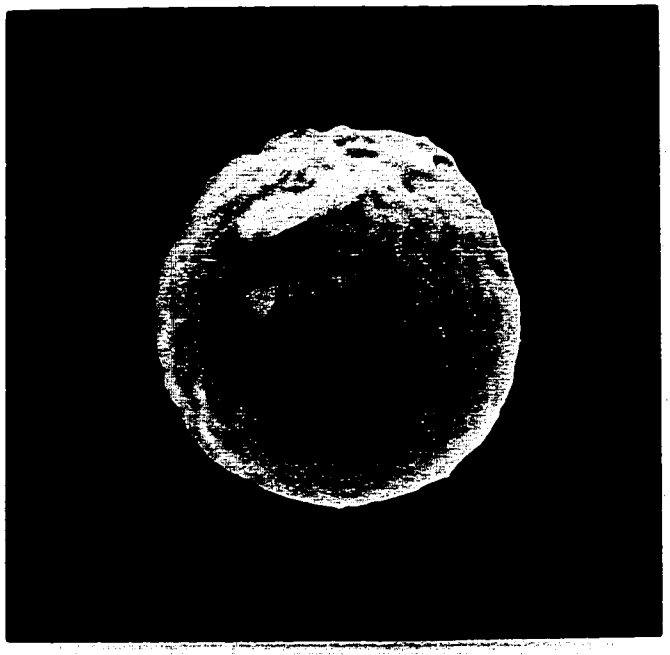
SDS-PAGE analysis of HSA extracted from TS-PDMS-grafted and ungrafted

MPs demonstrated that HSA was not noticeably fragmented by the MP fabrication process compared with the migration characteristics of soluble HSA. Figure 2 shows that HSA extracted from TS-PDMS-grafted or ungrafted MPs had electrophoretic migration characteristics that were virtually identical to those seen with soluble HSA. In all preparations, HSA appeared to contain high and low molecular weight fragments or contaminants. Further, HSA incorporated into either MP type was not altered structurally in such a way as to preclude detection by HSA-specific polyclonal antibodies. These results indicated that both MP types contained HSA in an antigenic form.

Figure 1.

Scanning electron micrographs (SEMs) of human serum albumin (HSA)-containing TS-PDMS-grafted (a) or ungrafted (b) microparticles.

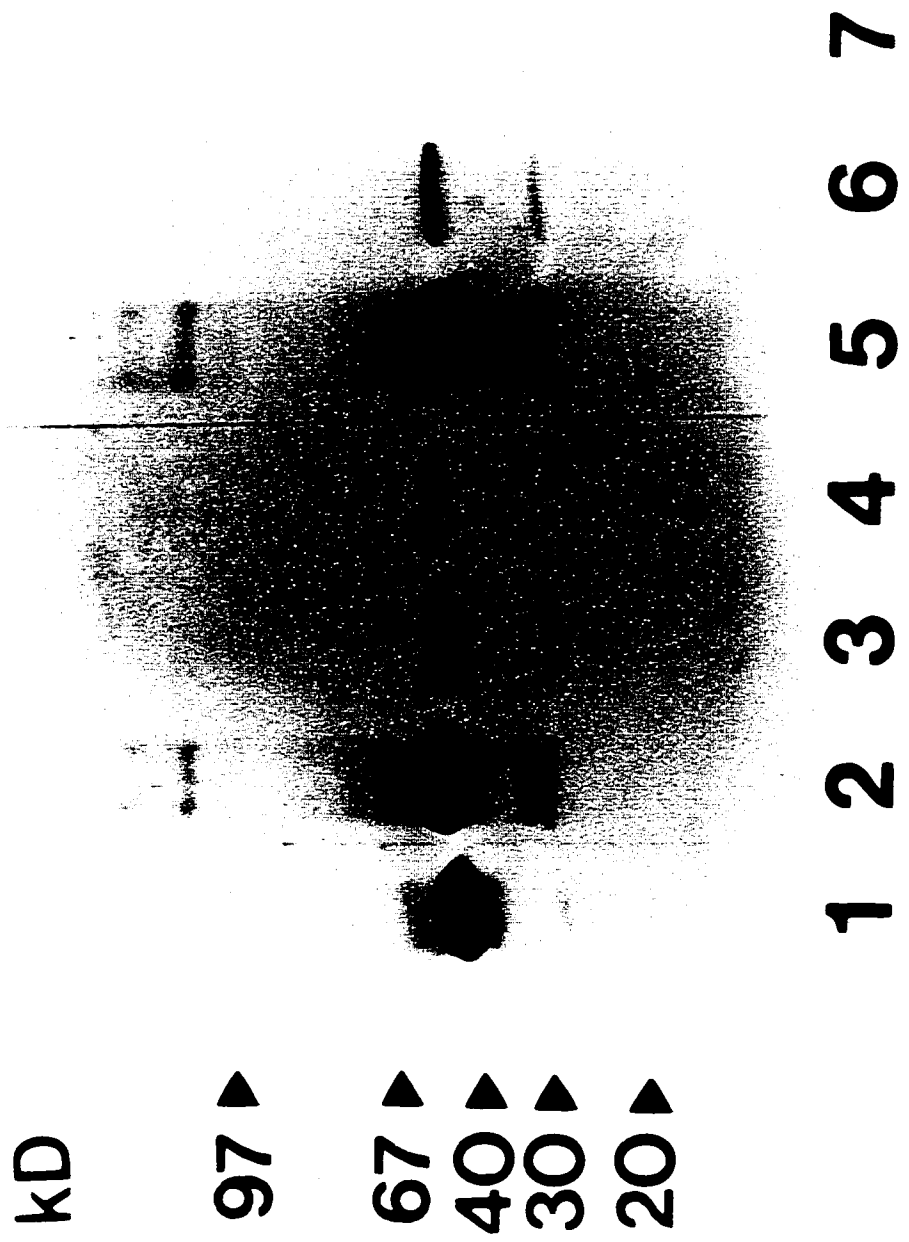
A



B

Figure 2.

Western blot analyses of human serum albumin (HSA) released from TS-PDMS-grafted or ungrafted microparticles (MPs). HSA was extracted into fresh saline (37°C) at each time-point, and visualized using alkaline phosphatase-conjugated, HSA-specific IgG and NBT/BCIP. Lane 1; HSA standard, Lanes 2, 3 & 4; HSA extracted from TS-PDMS-grafted MPs incubated in buffer for 30 min, 1 hr or 3 hr, respectively. Lanes 5, 6 & 7; HSA extracted from ungrafted MPs for 30 min, 1 hr and 3 hr, respectively. Molecular size (kD) was that determined using molecular size markers in the original coomassie blue-stained polyacrylamide gel.


1 2 3 4 5 6 7
kD
97
67
40
30
20

3.4.3 Immunogenicity of Systemically-administered Microentrapped HSA

To examine the immunogenicity of HSA entrapped in MPs, groups of mice were immunized i.p. with TS-PDMS-grafted or ungrafted HSA-containing MPs. Control mice were immunized with the same dose of HSA mixed with TS-PDMS-grafted MPs fabricated to contain the experimentally-irrelevant protein OVA or soluble HSA alone. Animals immunized with TS-PDMS-grafted or ungrafted MPs showed no gross pathologies throughout the experiments. Figure 3 shows that relative to immunization with HSA alone or HSA mixed with OVA-containing MPs, anti-HSA sera IgG antibody titres induced by HSA delivered in TS-PDMS-grafted or ungrafted MPs were significantly greater ($p < 0.001$). Additionally, TS-PDMS-grafted MPs elicited serum IgG responses that were significantly greater than those measured after immunization with ungrafted MPs ($p < 0.001$). These results indicated that MPs can enhance the immunogenicity of entrapped antigen and that this adjuvant effect can be significantly amplified by using MPs grafted with TS-PDMS.

3.4.4 Immunogenicity of Intragastrically-administered Microentrapped HSA

The finding that i.p. inoculation of mice with MP-entrapped HSA induced a strong circulating HSA-specific IgG antibody response suggested that i.g. administration of TS-PDMS-grafted HSA-containing MPs might induce circulating and mucosal antibody responses against HSA. This hypothesis was based on the

notion that MPs grafted with hydrophobic TS-PDMS might protect HSA from acidic hydrolysis and proteolysis in the gut (Moldoveanu *et al.*, 1993) and/or act as an adjuvant or immunopotentiator.

Figure 4 shows that at various time points after tertiary i.g. immunization, anti-HSA IgG sera titres induced by i.g. administration with 10 µg or 50 µg of HSA-containing TS-PDMS-grafted MPs were consistently higher than HSA-specific IgG responses elicited by ungrafted MPs and those responses were clearly augmented after i.g. boosting on day 70. (figs. 4a and 4c, respectively). Indeed, sera IgG antibody responses elicited following i.g. immunization with 50 µg HSA contained in TS-PDMS-grafted MPs were significantly higher compared with IgG responses elicited following immunization with ungrafted MPs ($p<0.005$). Serum IgG antibodies induced by i.g. immunization were almost exclusively IgG1; very little IgG2a or IgG2b and no IgG3 antibodies were present (data not shown). In addition to stimulating sera IgG , i.g. administration of 10 µg or 50 µg of HSA in TS-PDMS-grafted MPs, compared with ungrafted MPs, stimulated stronger anti-HSA serum IgA responses prior to day 63 (figs. 4b and 4d, respectively). IgA and IgG responses were amplified by boosting on day 70, with IgA responses being significantly higher after immunization with HSA in TS-PDMS-grafted MPs compared to ungrafted MPs ($p<0.001$). At all time points, animals immunized i.g. with soluble HSA failed to produce any detectable anti-HSA sera IgG or IgA (data not shown).

In contrast to the failure of soluble HSA to provoke an appreciable IgA response in intestinal secretions when administered i.g., the delivery of equal amounts of HSA entrapped in TS-PDMS-grafted or ungrafted MPs resulted in HSA-specific IgA responses in gut secretions ($p < 0.001$) independent of antigen dose (Table 1). Further, only antigen entrapped in MPs stimulated such responses in virtually every animal. Unexpectedly, TS-PDMS-grafted and ungrafted MPs elicited similar results in terms of gut IgA and the proportion of animals responding.

Figure 3.

Sera IgG antibody responses to human serum albumin (HSA) following intraperitoneal (i.p.) Immunization with various forms of HSA. Groups of six mice were immunized i.p. on days 0, 7 and 14 with HSA incorporated into TS-PDMS-grafted (solid bars) or ungrafted (hatched bars) microparticles (MPs), HSA in phosphate-buffered saline (PBS, open bars) or HSA mixed with ovalbumin (OVA)-containing MP (cross-hatched bars). Sera obtained on days 21, 35, 49 and 63 were evaluated for the presence of anti-HSA IgG antibodies using an ELISA. Figures represent mean ± SEM.

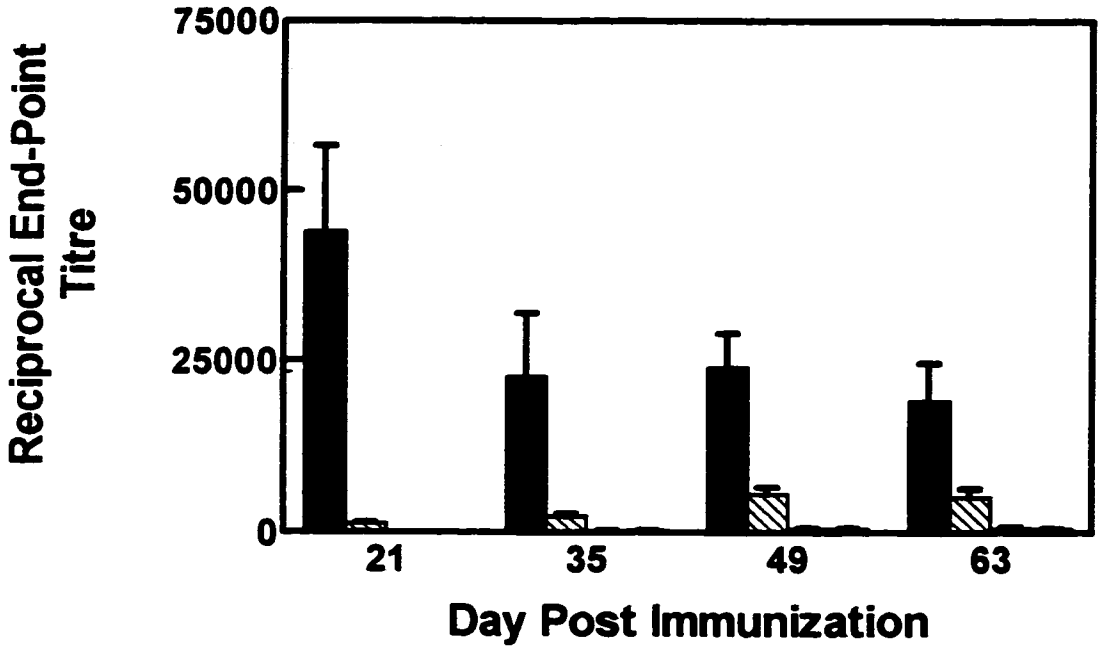

Reciprocal End-Point Titre
75000
50000
25000
0
21
35
49
63
Day Post Immunization

Figure 4.

Sera antibody responses following intragastric (i.g.) immunization with various forms of human serum albumin (HSA). Groups of 15 mice were immunized i.g. on days 0, 7, 14 and 70 with 50 μg (a, b) or 10 μg (c, d) of HSA incorporated into TS-PDMS-grafted (solid bars) or ungrafted (hatched bars) microparticles (MPs). Sera obtained on days 35, 49, 63 and 77 were evaluated for the presence of sera anti-HSA IgG (a, c) or IgA (b, d) using an ELISA. Figures represent mean ± SEM.

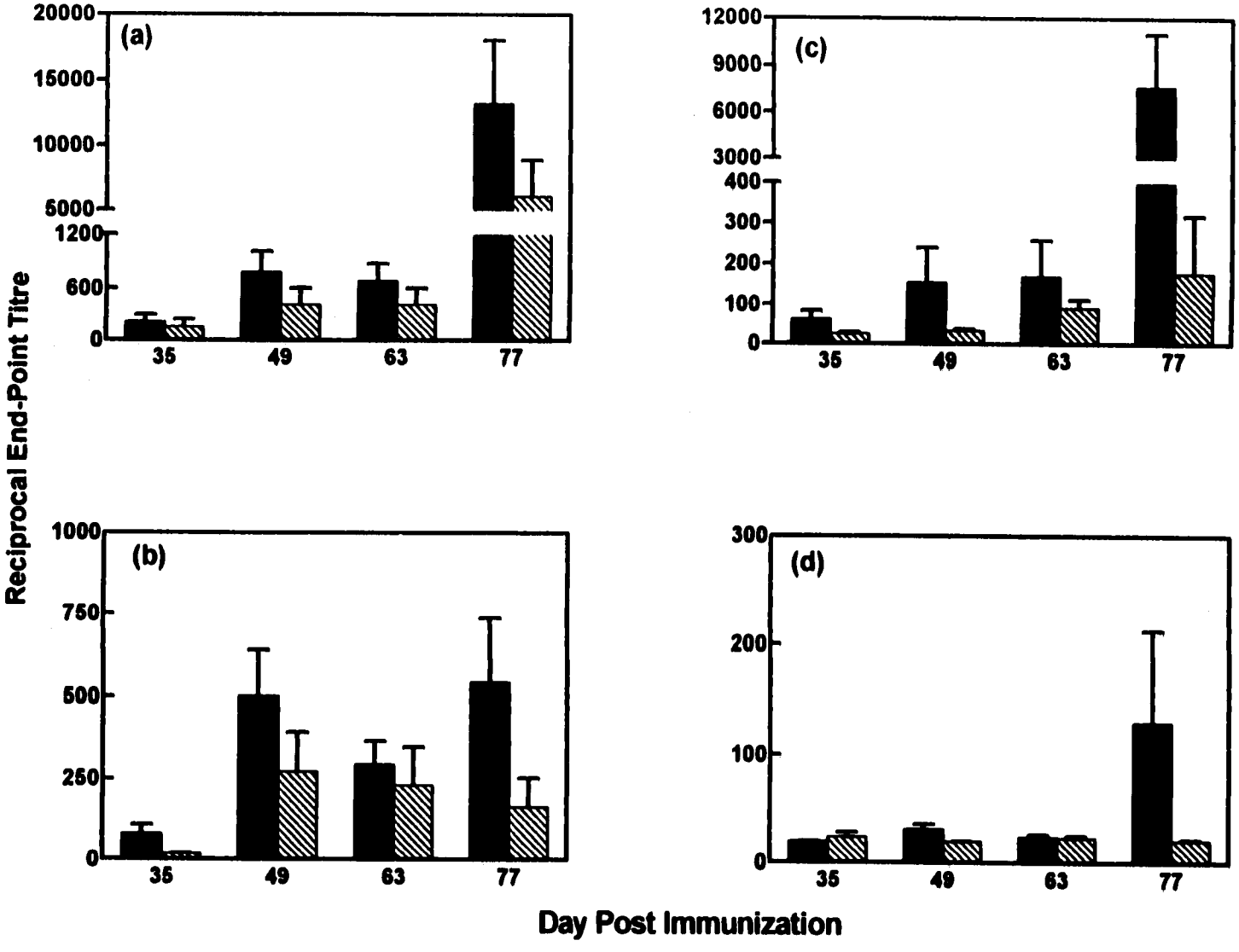

(a)
(b)
(c)
(d)
Reciprocal End-Point Titre
Day Post Immunization
20000
15000
10000
5000
1200
600
0
1000
750
500
250
12000
9000
6000
3000
400
300
200
100
35
49
63
77

Table 1. Intestinal anti-human serum albumin (HSA) IgA antibody responses elicited by intragastric (i.g). immunization with various forms of HSA

Form	Dose (µg HSA)	Intestinal IgA titre (mean ± SEM)	% animals responding
TS-PDMS-grafted MPs	50	3.33 ± 0.57	87
	10	3.14 ± 0.46	100
	5	3.20 ± 0.26	100
Ungrafted MPs	50	3.46 ± 0.61	87
	10	3.71 ± 0.19	100
	5	3.08 ± 0.37	92
Soluble	50	ND	0
	10	NE	NE
	5	ND	0

Animals (n=10) were immunized on days 0, 7, 14 and 70 with various doses of HSA incorporated in TS-PDMS-grafted or ungrafted microparticles (MPs) or HSA in 0.2 M $NaHCO_3$. Intestinal gut washes obtained on day 77 were evaluated for the presence of anti-HSA IgA using an ELISA.

ND, not detectable. The limit of detection is no dilution.

NE, not examined.

3.5 DISCUSSION

Biodegradable MP are a useful addition to mucosal and systemic vaccine delivery strategies, with several studies demonstrating that MP delivery vehicles can enhance both mucosal and systemic immune responses following oral administration (Challacombe *et al.*, 1992; Eldridge *et al.*, 1989; Eldridge *et al.*, 1990; Marx *et al.*, 1993; Moldoveanu *et al.*, 1993; O'Hagan *et al.*, 1993). Vaccine material entrapped in MPs is presumably protected from the acidic and enzymatically hostile environment of the GI tract. MPs are believed to be preferentially engulfed and transported by M cells into PP and subsequently to the MLN (Eldridge *et al.*, 1990; Jani *et al.*, 1992; Joel *et al.*, 1978; LeFevre *et al.*, 1985; Pappo & Ermak, 1989), the efficiency of which is facilitated by MP hydrophobicity and a diameter of less than 10 μm (Eldridge *et al.*, 1990; LeFevre *et al.*, 1985; Pappo & Ermak, 1989). In PP and MLN, MPs are thought to establish a depot allowing controlled or pulsatile release of antigen that locally triggers mucosal and systemic immunity.

A novel MP delivery technology was developed using starch, a well-studied, biologically acceptable and non-toxic material when given orally or parenterally (Whistler *et al.*, 1984; Laccourreye *et al.*, 1993). The hydroxyl groups of starch facilitate compatibility with a wide range of protein antigens, making it attractive as a vaccine carrier. Despite these advantages, starch is susceptible to degradation in the gut. Thus, to deliver biologically active components, starch MPs were grafted

with a hydrophobic coating. Fabrication of antigen-containing starch MPs grafted with TS-PDMS, a siloxane polymer similar to Simethicone®, a pharmaceutically acceptable excipient used as an anti-foaming agent in antacids (The United States Pharmacopoeia, 1994), was accomplished under mild conditions. Unlike Simethicone®, which is stable and nonreactive in aqueous environment, TS-PDMS possess reactive triethoxysilyl end groups that are capable of spontaneously coupling the abundant hydroxyl groups on starch. Evidently, the grafting of TS-PDMS to starch is necessary to achieve enhanced oral immunogenicity of MPs, as MPs exposed to a number of non-functionalized silicone polymers, which cannot graft to starch, failed to provoke enhanced local or systemic humoral immune responses (data not shown).

The results demonstrate the successful entrapment of HSA into TS-PDMS-grafted and ungrafted MPs at a core loading that is consistent with that found by others using different MP fabrication materials (Challacombe *et al.*, 1992; Eldridge *et al.*, 1991; O'Hagan *et al.*, 1991). MPs had a diameter of 4.2 ± 3.0 μm, a size which is advantageous for M-cell transport into PP and potentially to MLN (Eldridge *et al.*, 1990). HSA released from MPs was not fragmented or altered structurally by the fabrication process so as to preclude detection by HSA-specific polyclonal antibodies (Fig. 2), and was immunogenic (Figs 3 and 4). More recently, other protein and peptide antigens have been entrapped, including CT, tetanus toxoid, herpes simplex virus type 2 (HSV-2)-infected cell lysate and HSV-2 glycoprotein

peptides without any demonstrable loss in immunogenicity (P.L. Heritage, B.J. Underdown & M.R. McDermott, unpublished observations). These results indicate that this MP fabrication process is suitable for several antigens.

Animals immunized parenterally with TS-PDMS-grafted or ungrafted MPs elicited significantly higher anti-HSA serum antibody titres than those induced by immunizing with soluble HSA or HSA mixed with TS-PDMS-grafted OVA-containing MPs (Fig. 3), thus demonstrating the ability of starch MPs to enhance immune responses. These results are in concordance with previous PLG MP studies, demonstrating the necessity for soluble antigen to be associated physically with MPs in order to potentiate a humoral immune response (Eldridge *et al.*, 1991; O'Hagan *et al.*, 1991). Of greater significance, HSA entrapped within TS-PDMS-grafted MPs induced HSA-specific systemic antibody responses that were greater than those elicited following parenteral immunization with ungrafted MPs, thus suggesting that the silicone polymer is an important component of the delivery vehicle.

This study clearly demonstrated that TS-PDMS-grafted MPs have potential as a mucosal vaccine delivery vehicle, due to the fact that oral immunization stimulated both systemic (Fig. 4) and mucosal (Table 1) humoral immune responses. Maximal serum antibody titres occurred after an oral boost, suggesting that the initial immunization protocol stimulated serum antibody memory, an advantage when developing successful mucosal vaccination strategies. The ability

of TS-PDMS-grafted MPs to stimulate reproducibly elevated levels of antigen-specific serum antibody responses following oral immunization with very low doses of microentrapped antigen was unanticipated. This demonstrated the efficacy of i.g. administered TS-PDMS-grafted MPs in stimulating robust humoral immune responses.

A major obstacle to oral immunization with soluble antigens has been the instability of such antigens to stimulated local secretory immune responses. Here, it was demonstrated that in contrast to soluble HSA or HSA mixed with TS-PDMS-grafted OVA MPs (data not shown), the delivery of equal amounts of MP-entrapped HSA resulted in specific sIgA responses in gut secretions. Although the titres of HSA-specific sIgA in gut secretions initially might appear low, these results are comparable with humoral immune responses reported in other studies (Challacombe *et al.*, 1992; O'Hagan *et al.*, 1993) and as intestinal GWs were diluted to a total volume of 5 mL/mouse, the IgA concentration was probably substantially more than revealed in Table 1. Although antigen-specific IgA has been demonstrated in intestinal and salivary fluids following i.g. immunization with microencapsulated staphylococcal enterotoxin B (SEB, Eldridge *et al.*, 1989), others have failed to demonstrated significant mucosal humoral responses following i.g. administration of microencapsulated viral antigens (Marx *et al.*, 1993; Moldoveanu *et al.*, 1993). Furthermore, i.g. immunization with microencapsulated OVA stimulated low titres of specific salivary IgA only after i.g. delivery of six doses of 20-

100 times more antigen than was used in the present studies (Challacombe *et al.*, 1992; O'Hagan *et al.*, 1993). In many of these cases, widely differing immunization regimens do not allow legitimate comparison of the antigen delivery systems. Nevertheless, the intestinal anti-HSA IgA antibody responses that were observed after immunization with microentrapped HSA are comparable with or exceeded those responses demonstrated by several other laboratories. The mode of action of orally administered TS-PDMS-grafted or ungrafted MPs is presently unknown, although previous investigations suggest particle uptake and transport by M cells in the PP (Pappo & Ermak, 1989). The investigations in this study suggest that TS-PDMS-grafted MPs probably do not gain access to the GALT by disrupting the intestinal epithelium in a pathological manner, as MPs fabricated without the silicone polymer elicited detectable serum and mucosal immune responses. Irrespective of the manner of entry, the present findings suggest that TS-PDMS-grafted MPs have immunopotentiating activity when delivered via systemic or mucosal routes, and have considerable potential as both parenteral and mucosal vaccine delivery vehicles.

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CHAPTER 4

ORAL ADMINISTRATION OF POLYMER-GRAFTED STARCH MICROPARTICLES ACTIVATES GUT-ASSOCIATED LYMPHOCYTES AND PRIMES MICE FOR A SUBSEQUENT SYSTEMIC ANTIGEN CHALLENGE

PREFACE

The synthesis and characterization of TS-PDMS and other silicones was conducted by Dr. M. Brook, Department of Chemistry, McMaster University.

A formal communication of the work has been accepted for publication as: Heritage P.L., Underdown B.J., Brook M.A. & McDermott M.R. (1998) Oral administration of polymer-grafted starch microparticles activates gut-associated lymphocytes and primes mice for a subsequent systemic antigen challenge. *Vaccine* (in press).

4.1 ABSTRACT

The mucosal and systemic humoral immune systems can function essentially independent of each other, responding to mucosal and parenteral antigens, respectively. Nevertheless, antigen administered by one route can modify responsiveness to subsequent immunization by an alternate route. Here, it was demonstrated, in mice, that in addition to stimulating rapid and robust sera antibody responses, i.g. immunization with HSA-containing starch MPs grafted with TS-PDMS primed for enhanced specific sera IgG following a parenteral antigen boost. After as little as one i.g. immunization with microentrapped, but not soluble, HSA antigen-specific proliferation and antibody secretion was detected in PP; this activity peaked after 3 i.g. MP immunizations. A progressive dissemination of antigen-specific lymphocyte reactivity from PP to splenic tissue was observed following oral MP immunization. Similarly, a shift in HSA-specific antibody-secreting cells from PP and MLN to splenic tissue following i.g. MP immunization was also observed. It was also demonstrated that oral immunization with microentrapped, but not with soluble HSA, resulted in enhanced numbers of spontaneous T_H2-cytokine secreting lymphocytes which disseminated from mucosal to systemic lymphoid compartments. This observation coincided with findings that HSA-specific sera IgG1 responses in animals given HSA in MPs were significantly higher than those detected in the sera of mice given soluble HSA i.g., both before and after parenteral antigen challenge. These findings suggest that orally-administered TS-PDMS-

grafted MPs, by stimulating elements of the mucosal immune system, are a valuable addition to mucosal and systemic vaccine delivery vehicles.

4.2 INTRODUCTION

Presently, most vaccines are administered via systemic routes and although parenteral immunization is effective in stimulating systemic immunity, specific mucosal immunity is not often induced. In contrast, mucosal immunization provokes both mucosal and systemic immunity via the stimulation of mucosally-situated IgA and IgG plasmacyte precursors (McDermott & Bienenstock, 1979; Weisz-Carrington *et al.*, 1979). These observations predict that mucosal, but not parenteral, immunization induces immune responses which are capable of both protecting the host from mucosal invasion and eliminating intruders from systemic tissues if the mucosae are breached. In concert, these mucosae-derived responses prevent or reduce the morbidity and mortality resulting from both mucosal and systemic infections (Kilian & Russell, 1994).

It was previously demonstrated that HSA-containing, biocompatible starch MPs grafted with the silicone polymer TS-PDMS stimulated both secretory and circulating antigen-specific humoral immunity following i.g. immunization (Chapter 3, Heritage *et al.*, 1996). Maximal sera HSA-specific responses occurred following oral boosting (Chapter 3, Heritage *et al.*, 1996). This response was manifested in the systemic compartment of the immune system and suggested that the initial

immunization protocol could induce systemic humoral memory. Indeed, some reports suggest that feeding soluble antigens can sensitize animals for systemic DTH responses (Lamont *et al.*, 1989; Perrotto *et al.*, 1974; Stokes *et al.*, 1983). Additionally, since orally-induced memory cells have been repeatedly demonstrated to be activated following subsequent antigen challenge with cholera toxoid or its subunits (Elson & Ealding, 1984; Lycke *et al.*, 1985; Svennerholm *et al.*, 1984; Pierce & Gowans, 1975) a potent mucosal and systemic antigen, i.g. priming of mucosal tissues with TS-PDMS-grafted MPs might also lead to the generation of systemic memory capability, a phenomena not consistently seen with other particulate delivery systems (Lamont *et al.*, 1989).

In this study, it was investigated whether orally-administered HSA-containing TS-PDMS-grafted MPs primed mice for a systemic challenge with soluble antigen. The results showed that i.g. immunization with low doses of microentrapped, but not soluble, HSA activated lymphocytes in the GALT and primed animals for significantly enhanced HSA-specific humoral immune responses following i.p. HSA challenge. The results further support the use of TS-PDMS-grafted MPs as a delivery vehicle for mucosal and systemic vaccines.

4.3 MATERIALS AND METHODS

4.3.1 Microparticle Fabrication and Characterization

HSA (Fraction V, Sigma; St. Louis, MO) was entrapped into TS-PDMS-

grafted MPs using an emulsion-based process and characterized as previously described (Chapter 3, Heritage *et al.*, 1996). Microparticles fabricated by this process routinely contained 5-6% w/w protein.

4.3.2 Immunizations

Female BALB/c mice, age 6-8 weeks (Harlen Sprague Dawley Inc., Indianapolis, IN) were immunized i.g. on days 0, 7 and 14 with 50 µg of soluble or microentrapped HSA in 500 µl of 0.2 M $NaHCO_3$, or vehicle alone using PE50 tubing (Becton Dickinson Co; Mountain View, CA). In some experiments, animals were subsequently immunized i.p. on day 19 with 100 µg HSA solubilized in PBS (pH 7.4).

4.3.3 Collection and Preparation of Sera and Cells

Individual blood samples were obtained via the retro-orbital plexus. For sera evaluations, insoluble material was removed by centrifugation and sera were stored at -70°C until used.

Pooled SPL, MLN and PP were isolated into ice cold Hanks' balanced salt solution (HBSS; pH 7.4). Single cell suspensions of SPL and MLN were prepared by crushing the tissues between the frosted ends of 2 microscope slides. Cell suspensions of PP were prepared as previously described (Chapter 5, Heritage *et al.*, 1997). Single cell SPL, MLN and PP suspensions were washed twice with

HBSS by centrifugation, and the erythrocytes and dead cells were removed using Ficoll-Paque (Fotino *et al.*, 1971) (Pharmacia, Uppsala, Sweden). The remaining cells were resuspended in RPMI-1640 media supplemented with 5% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine and 1% *N*-n-hydroxy-ethylpiperizine-*N'*-2 ethanesulphonic acid (HEPES), pH 7.4 (operationally termed RPMI-5). The viability of cell preparations routinely exceeded 90% as judged by ethidium bromide/fluorescein diacetate staining (Edidin, 1970; Mohr & Trouson, 1980).

4.3.4 Measurement of HSA-specific Antibody Responses

An ELISA was used to measure HSA-specific antibody responses in individual sera as previously described (Chapter 3, Heritage *et al.*, 1996). Briefly, duplicate serial dilutions of sera were incubated on HSA-coated microtitre plate wells. HSA-specific antibodies were quantitated by incubating wells with heavy chain-specific, AP-conjugated goat anti-mouse IgG, IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL). Responses were quantitated by measurement of ODs at 405 nm following incubation of wells with 1.0 M diethanolamine buffer, pH 9.8, containing 50 mM $MgCl_2$ and 1.0 mg/mL *p*-nitrophenylphosphate (5 mg phosphatase substrate tablets; Sigma). Sera prepared from buffer-treated animals were used to establish baseline OD values. The results were expressed as reciprocal end-point sera titres representing the greatest sera

dilutions giving OD values exceeding 2 times buffer alone mean values.

4.3.5 Lymphocyte Proliferative Assay

Quadruplicate cultures of PP, MLN and SPL lymphocytes (5 x 10^5) were prepared in 96-well round-bottomed, sterile plates in the presence or absence of various amounts of HSA (250-1000 µg/mL) for 96 hours at 37°C with 5% CO_2 in air. One µCi of [^{3}H]thymidine ([^{3}H]Tdr, 740.0 GBq/mmol; Dupont/NEN, Mississauga, ON) was added to each well for the final 24 h of culture. Cells were harvested with a PhD Harvester (Cambridge, MA) and [^{3}H]Tdr incorporation was measured by standard liquid scintillation counting methods. The results were expressed as mean counts per minute (cpm) from quadruplicate cultures.

4.3.6 Enumerating of HSA-specific Spot-forming Cells

A modified version of a previously described enzyme-linked spot-forming assay (ELISPOT) was used to detect HSA-specific spot-forming cells (SFCs) in pooled PP, MLN and SPL preparations (Chapter 5, Heritage *et al.*, 1997). Briefly, duplicate serial dilutions of single cell suspensions (beginning at 1 x 10^5 cells/100 µl/well) were examined using NC plates incubated with 100 µl/well of HSA (100 µg/mL in PBS). After incubating the wells with AP-conjugated goat anti-mouse IgG1 (heavy chain-specific; Southern Biotechnology Associates), SFCs were visualized. The results represent the mean number of HSA-specific SFCs per 1 x 10^6 cells (±

SEM) in duplicate wells containing at least 2-fold more SFCs than wells incubated with cells from buffer-treated animals.

4.3.7 Enumeration of IFN-γ and IL-4-producing Cells

An ELISPOT assay was also used to quantitate spontaneous cytokine spontaneously released by MLN and SPL cells *in vitro*. Spontaneous cytokine secretion was examined since it was concluded that by examining cytokine production immediately following lymphocyte isolation, and not after extensive *in vitro* manipulation, one would have a closer approximation of the *in vivo* cytokine microenvironment following i.g. immunization with microentrapped or soluble antigen. To detect IFN-γ-secreting cells, duplicate serial dilutions of single cell suspensions (beginning at 1 x 10^5 cells/100 µl/well) were examined using NC microtitre plates (Millititer HA; Millipore Corp) previously incubated overnight at 4°C with 100 µl/well of a rat anti-mouse IFN-γ monoclonal antibody (mAb) (clone R46A2; Pharmingen; SanDiego, CA) at 10 µg/mL in a solution of 0.1M $NaHCO_3$, pH 8.2 (coating buffer) and then washed with PBS and treated with 250 µl/well of 0.1% gelatin in PBS. After an 8 h incubation at 37°C, the wells were washed 3 times each with PBS and PBS containing 0.05% Tween-20 (PBS/Tween). The wells were then incubated for 2 h with 100 µl of biotinylated rat anti-mouse IFN-γ mAb (clone XMG1.2; Pharmingen) diluted to 4 µg/mL in PBS/Tween containing 0.1% gelatin (dilution buffer). The wells were washed 3 times each with PBS and PBS/Tween

and incubated for 30 min at 37°C with AP-conjugated streptavidin (Southern Biotechnology Associates) in dilution buffer. After washing the wells 4 times each with PBS and PBS/Tween, spots were visualized as previously described (Heritage *et al.*, 1997). The results represent the mean number of spots per 1 x 10^6 cells in duplicate wells containing at least 2-fold more spots than wells incubated with cells from PBS-treated mice. To detect IL-4-specific SFCs, rat anti-mouse IL-4 (clone 11B11; Pharmingen) (2 µg/mL) and biotinylated mAb anti-mouse IL-4 (clone BVD6-24G2; Pharmingen) (2 µg/mL) were used in coating and detection, respectively. IFN-γ and IL-4 secretion was examined since these cytokines are usually produced by T_H1 cells and T_H2 cells respectively and reciprocally antagonize the effector function of the other T_H subset.

4.3.8 Statistical Analyses

Statistical analyses of the data were done using GraphPad Prizm® Version 2.00 (GraphPad Software, SanDiego , CA). One-way ANOVA, Tukey test pair-wise multiple comparisons and unpaired Student's *t*-tests were used to detect and compare mean differences between treatment groups. A level of significance of 95% was chosen for all tests.

4.4 RESULTS

4.4.1 Intragastric Administration of Microentrapped HSA Primes Mice For

Subsequent Systemic Challenge

It was previously shown that i.g. immunization with low doses of HSA encapsulated TS-PDMS-grafted MPs induced antigen-specific sera IgG titres which were significantly higher than those elicited by comparable amounts of soluble HSA (Chapter 3, Heritage *et al.*, 1996). Figure 5a confirms these findings by showing that i.g. delivery of low doses of microentrapped HSA (50µg/mouse) rapidly elicited levels of specific sera IgG which were greater than those measured in animals given equivalent doses of soluble antigen orally ($p<0.005$). The earlier work also demonstrated that following i.g. immunization with HSA-containing TS-PDMS grafted MPs, maximal serum antibody titres occurred after oral boosting, on day 70, with microentrapped HSA (Chapter 3, Heritage *et al.*, 1996). Thus, it was explored whether i.g. immunization with microentrapped HSA could also prime mice for enhanced specific sera antibody responses after systemic challenge at an earlier time with HSA. Figure 5b shows that following i.p. boosting with soluble HSA, mice previously given microentrapped HSA i.g. elicited higher levels of anti-HSA sera IgG as compared to those given soluble antigen orally ($p<0.05$) or buffer alone ($p<0.0001$). Furthermore, even when compared to mice immunized i.g. with 50-fold greater doses of soluble HSA (25 mg/mouse; data not shown), i.g. administration of microentrapped HSA stimulated significantly greater HSA-specific sera antibody responses following i.p. antigen challenge ($p<0.01$). Surprisingly, systemic challenge failed to boost HSA-specific sera IgA titres following i.g. immunization with

microentrapped or soluble antigen (data not shown). This was in stark contrast with the previous findings where it was demonstrated that i.g. immunization with microentrapped, but not soluble HSA primed mice for both enhanced sera IgA and IgG responses following an oral MP boost (Chapter 3, Heritage *et al.*, 1996). Taken together, these results demonstrated that, unlike soluble antigen, i.g. administration of TS-PDMS-grafted MPs evoked rapid and robust humoral immune responses (Fig. 5a) and also primed mice for enhanced sera IgG responses following systemic antigen challenge.

4.4.2 Characterization of HSA-specific Lymphocyte Proliferative Responses

Oral immunization with soluble and particulate antigens can elicit systemic antibody responses due to the dissemination of cells from the GALT (Heremans, 1974; André *et al.*, 1973). Since particulate antigens are selectively taken up from the intestinal lumen by PP following i.g. immunization (Eldridge *et al.*, 1990; Ermak *et al.*, 1995; Frey *et al.*, 1997; Jani *et al.*, 1992; LeFevre *et al.*, 1985; Pappo & Ermak, 1989), it was explored whether i.g. administration of TS-PDMS-grafted MPs might prime mice for enhanced systemic humoral responses following parenteral antigen challenge via activation and subsequent emigration of primed PP cells. Within 4 days after i.g. administration of MPs, antigen-specific PP cell proliferation was detectable following *in vitro* restimulation with a high dose (1000 µg/mL) of HSA (Table 2). This response rose to a maximum by day 16 post-immunization as

judged by the efficacy of lower *in vitro* restimulatory doses of HSA and higher levels of ^{3}[H]Tdr incorporation. No significant SPL cell proliferation was detected following *in vitro* HSA restimulation until 10 days after i.g. immunization with HSA-containing MPs, although substantial proliferation was measured at day 16. Moreover, no significant PP or SPL proliferation was observed in mice immunized i.g. with soluble HSA or buffer alone (data not shown). These results indicated that a single i.g. immunization with microentrapped, but not soluble, HSA resulted in the induction of antigen-specific PP cells and that multiple i.g. MP administrations resulted in a shift of HSA-specific lymphocyte responsiveness from the mucosal (PP) towards the systemic (SPL) compartment of the immune system.

4.4.3 Kinetics of HSA-specific B Cell Responses Following Intragastric Immunization with Microentrapped HSA

I.g. immunization with HSA-containing TS-PDMS-grafted MPs activated PP lymphocytes (Table 2) and elicited antigen-specific serum IgG (Fig. 5a), suggesting that antigen-specific B cells were stimulated initially in the GALT. Thus, it was examined whether HSA-specific antibody secretion by gut-associated and peripheral lymphocytes could be detected at various times after i.g. immunization with encapsulated or soluble HSA (Fig. 6). Following primary i.g. immunization with HSA-containing TS-PDMS-grafted MPs, appreciable numbers of HSA-specific IgM-secreting SFCs were detected only in PP cell isolates (Fig. 6a). The numbers of

IgM-secreting cells in PP and MLN (Fig. 6b) increased substantially until day 21 post-immunization, after which time no specific IgM SFCs were detectable in either tissue. HSA-specific IgM-secreting SFCs were undetectable in SPL cell isolates until 3 weeks post-immunization (Fig. 6c). As certain types of MPs have been shown to accumulate primarily in the GALT following i.g. administration (Eldridge *et al.*, 1990), these results suggest that i.g. administration of microentrapped HSA activated antigen-specific B cells in PP and these, or MP-containing PP-derived phagocytes, subsequently migrated to the SPL via the MLN (Clancy *et al.*, 1983; Mestecky *et al.*, 1978). Antigen-specific IgG-secreting SFCs in PP and MLN (Figs 6d and 6e, respectively) were not detected until day 21, thus implying that isotype switching of activated, HSA-specific B cells had occurred by this time. The greatest numbers of IgG-secreting SFCs in PP and MLN appeared 3 wk after primary immunization (Fig. 6d and 6e, respectively) while maximal splenic IgG production was not observed until day 28 (Fig. 2f), which suggested that IgG plasmacyte precursors might have migrated also from PP through MLN. In contrast, no appreciable numbers of IgM- or IgG-secreting SFCs were observed in the GALT following i.g. immunization with soluble HSA; this confirmed the previous finding that soluble HSA failed to activate PP lymphocytes (Table 2). These results demonstrate that orally delivered, microentrapped HSA activated gut-associated B cells; such activation with microentrapped, but not soluble, HSA might be responsible for priming GALT-derived cells for a recall response to i.p. challenge.

Figure 5.

Sera anti-human serum albumin (HSA) IgG antibody responses following intragastric (i.g.) immunization with various forms of HSA. Groups of 10 mice were immunized i.g. on days 0, 7 and 14 with 50 µg of HSA incorporated in TS-PDMS-grafted microparticles (MPs, solid bars), HSA in 0.2 M $NaHCO_3$ (hatched bars) or vehicle alone (open bars). On day 19 animals were boosted i.p. with 100 µg of HSA solubilized in phosphate-buffered saline (PBS). Sera obtained on days 15 (a) and 33 (b) were evaluated for the presence of HSA-specific IgG using an ELISA. Results are expressed as mean reciprocal end-point titres representing the greatest serum dilutions giving OD values exceeding twice the buffer-treated mean values ± SEM.

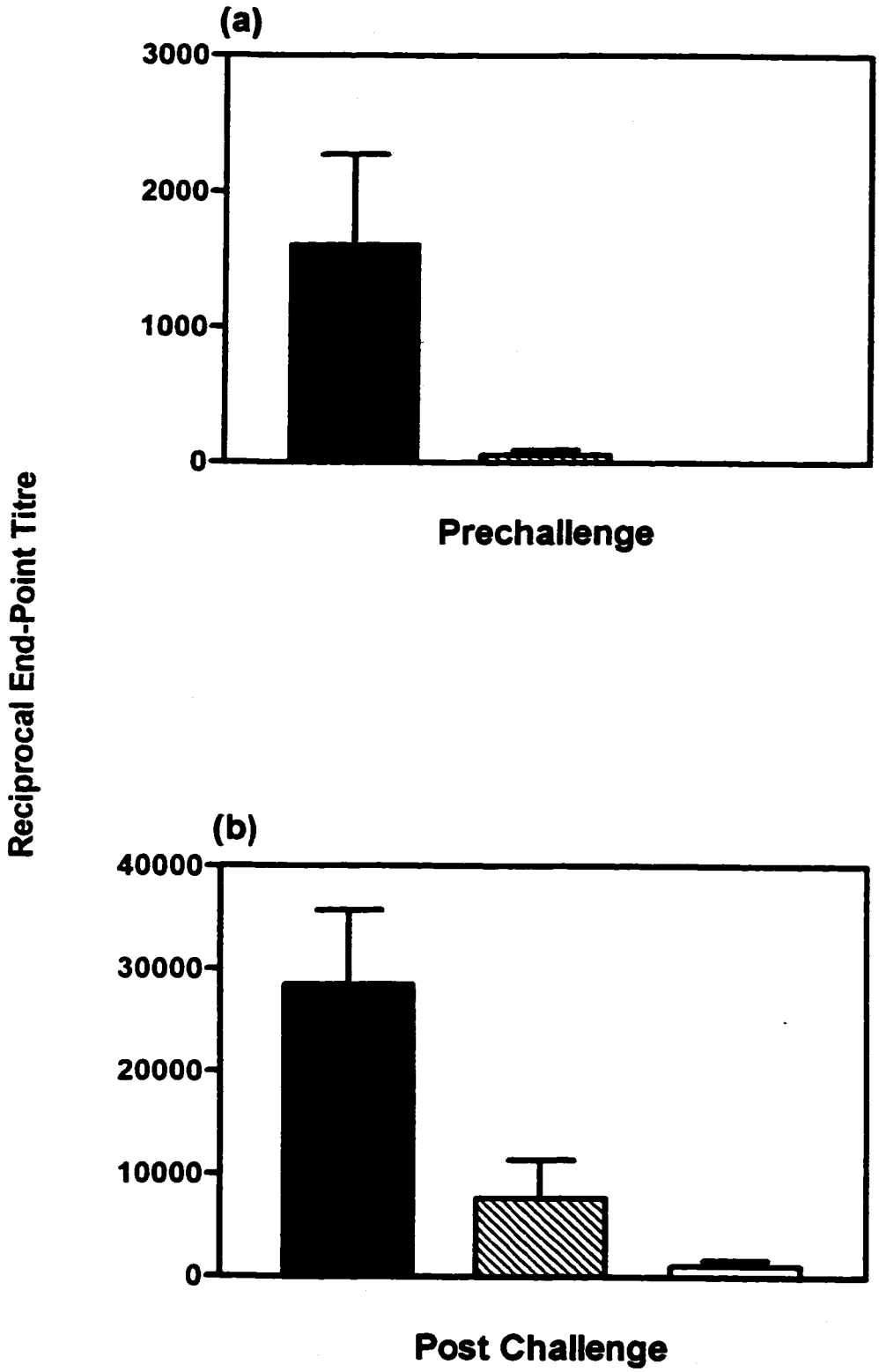

(a)
3000
2000
1000
0
Prechallenge
Reciprocal End-Point Titre
(b)
40000
30000
20000
10000
0
Post Challenge

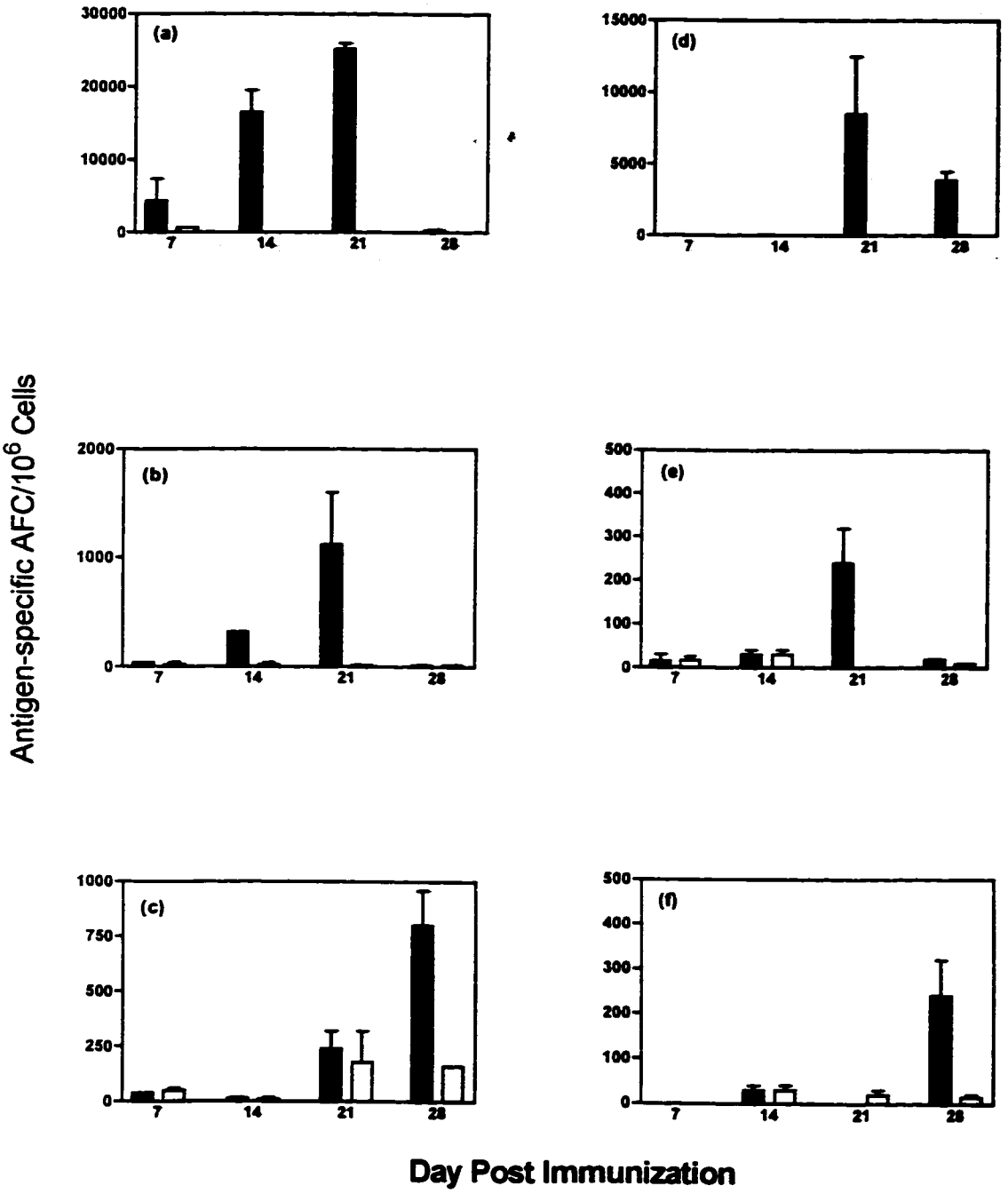
Table 2. Proliferative responses in Peyer's patches (PP) and spleen (SPL) following intragastric (i.g.) immunization with various forms of human serum albumin (HSA)

Inoculant	Cell Source	HSA (µg/mL) *in vitro*	^{3}H-thymidine incorporation (cpm)		
			Day 4	Day 10	Day 16
TS-PDMS-grafted MPs	PP	1000	646±81	1976±448	2090±446
		500	195±11	1003±69	1760±347
		250	184±9	397±118	514±41
		0	164±9	181±11	290±49
	SPL	1000	186±20	873±127	4282±1203
		500	191±11	603±104	1927±406
		250	227±29	795±17	829±44
		0	192±16	215±13	195±17
Soluble HSA	PP	1000	187±13	596±45	500±16
		500	240±51	331±29	357±43
		250	214±15	317±35	176±4
		0	186±15	247±20	186±29
	SPL	1000	206±10	307±52	225±32
		500	199±27	207±9	231±17
		250	279±47	285±21	186±9
		0	208±65	315±45	213±9

Groups of 10 mice were immunized i.g. on days 0, 7 and 14 with 50 µg of HSA incorporated into TS-PDMS-grafted microparticles (MPs) or HSA in 0.2 M $NaHCO_3$. On day 4, 10 or 16 animals were sacrificed and lymphocytes isolated from PP or SPL were examined for ^{3}H-Tdr incorporation following *in vitro* incubation for 96 h with or without HSA (250-1000 µg/mL) with ^{3}H-Tdr added to cultures in the final 24 h. Results are expressed as the mean cpm of quadruplicate cultures ± SEM.

Figure 6.

Appearance of human serum albumin (HSA)-specific spot-forming cells (SFCs) following intragastric (i.g.) immunization with various forms of HSA. Groups of 10 mice were immunized i.g. on days 0, 7 and 14 with 50 μg of HSA entrapped in TS-PDMS-grafted microparticles (MPs, solid bars), HSA in 0.2 M $NaHCO_3$ (hatched bars) or buffer alone. On days 7, 14, 21 and 28 animals were sacrificed and lymphocytes isolated from Peyer's patches (PP, a & d), mesenteric lymph nodes (MLN, b & e) and spleens (SPL, c & f) were evaluated for the presence of HSA-specific IgM (a-c) and IgG (d-f) SFCs using an ELISPOT. Results represent the mean number of spots per 1×10^6 pooled cells in duplicate wells containing at least two-fold more spots than wells incubated with pooled cells from buffer-treated animals.


(a)
(b)
(c)
(d)
(e)
(f)
Antigen-specific AFC/10^6 Cells
Day Post Immunization

4.4.4 Lymphocyte Cytokine Production Following Intragastric Immunization with Microentrapped HSA

It was previously shown that the isotype HSA-specific sera antibody responses induced after i.g. immunization with HSA-containing TS-PDMS-grafted MPs was predominantly IgG1 (Chapter 3, Heritage *et al.*, 1996). Since isotype directly reflects the cytokine microenvironment acting on B cells, spontaneous (non-restimulated) cytokine secretion by mucosally- and systemically-derived lymphocytes following i.g. immunization with soluble or microentrapped HSA was examined (Table 3). When compared to mice immunized i.g. with soluble HSA, those given MPs i.g. contained appreciable numbers of IL-4-secreting lymphocytes in the PP 2 wk after primary i.g. immunization; later, IL-4 secretion declined in PP and rose in SPL cells. Conversely, spontaneously secreting IFN-γ cells were rarely observed in PP or SPL following i.g. immunization with either soluble or microentrapped HSA. However, both IFN-γ and IL-4 secreting cells were detected following stimulation with conconavalin A (Con A) for 3 days *in vitro* (data not shown), confirming both that the ELISPOTS were working and the multipotent cytokine potential of MALT-derived lymphocytes. Thus, i.g. immunization with HSA-containing TS-PDMS-grafted MPs incited higher numbers of gut-associated lymphocytes to secrete cytokines known to support IgG1 synthesis.

Concomitant with these observations, HSA-specific IgG1 sera responses in animals given HSA i.g. in MPs were significantly greater than those detected in the

sera of soluble HSA-treated mice ($p<0.0002$) (Table 4). Indeed, anti-HSA IgG1 responses in this latter group were below the limit of ELISA detection. Sera anti-HSA IgG1 titres were greatly enhanced in animals given HSA in MPs following i.p. antigen boosting, while there was no analogous increase in IgG1 responses in the sera of animals primed i.g. with either soluble HSA or buffer alone. Additionally, oral immunization with soluble HSA or HSA-containing TS-PDMS-grafted MPs failed to stimulate appreciable HSA-specific sera IgG2a responses following i.g. immunization. Compared to buffer-treated mice, there was also no significant increase in sera IgG2a responses in mice given soluble or microentrapped HSA following a systemic HSA boost. These data support the notion that i.g. immunization with HSA-containing TS-PDMS-grafted MPs, but not soluble HSA, selectively primed GALT-derived lymphocytes involved in a systemic humoral recall response following i.p. antigen challenge.

Table 3. Detection of spontaneously-secreting IFN-γ and IL-4 spot forming cells (SFCs) in Peyer's patches (PP) and spleens (SPL) following intragastric (i.g.) immunization with soluble or microentrapped human serum albumin (HSA).

Inoculant	Cell Source	Day	Cytokine-specific SFCs/10^6 cells	
			IFN-γ	IL-4
TS-PDMS-grafted MPs	PP	7	ND	ND
		14	5	360
		21	5	280
	SPL	7	ND	ND
		14	ND	30
		21	ND	150
Soluble HSA	PP	7	ND	ND
		14	ND	10
		21	ND	5
	SPL	7	ND	ND
		14	10	ND
		21	ND	ND

Groups of 10 BALB/c mice were immunized i.g. on days 0, 7 and 14 with 50 μg of HSA incorporated into TS-PDMS-grafted microparticles (MPs), HSA in 0.2 M $NaHCO_3$ or buffer alone. On days 7, 14 and 21 animals were sacrificed and lymphocytes isolated from PP and SPL were evaluated for IFN-γ or IL-4 secretion using an ELISPOT. The results shown represent one of two experiments and demonstrate the mean number of spot per 1 x 10^5 pooled cells in duplicate wells containing at least two-fold more spots than wells incubated with pooled cells from buffer-treated animals.

ND; not detectable.

Table 4. Sera anti-human serum albumin (HSA) reciprocal end-point titre antibody responses elicited by intragastric (i.g.) immunization with various forms of HSA, followed by intraperitoneal (i.p.) challenge with HSA.

Inoculant	Prechallenge		Post Challenge	
	IgG1	IgG2a	IgG1	IgG2a
TS-PDMS-grafted MPs	581±194	33±19.5	26,100±7,961	499±340
Soluble HSA	ND	8.6±4.6	8,550±3,765	578±337
$NaHCO_3$	ND	ND	3,240±1,069	195±75

Groups of 10 mice were immunized i.g. on days 0, 7 and 14 with 50 μg of HSA incorporated into TS-PDMS-grafted microparticles (MPs), HSA in 0.2 M $NaHCO_3$ or buffer alone. On day 19, animals were immunized i.p. with 100 μg of HSA in solubilized in phosphate-buffered saline (PBS). Sera obtained on day 15 (prechallenge) or day 33 (post challenge) were evaluated for the presence of HSA-specific IgG1 and IgG2a using an ELISA. Results are expressed as the mean of reciprocal end-point titres representing the greatest serum dilutions giving optical density (OD) values exceeding two times buffer-treated values ± SEM.

4.5 DISCUSSION

Mucosal and systemic humoral immune responses can occur independently in response to mucosal and parenteral antigens, respectively (Clancy *et al.*, 1983; Mestecky *et al.*, 1978; Ogra & Karzin, 1971). Nevertheless, antigen administered by either of these routes can modify responsiveness to subsequent immunization by another route, an example being the modulation of mucosal antitoxin responses to enterically administered CT following parenteral cholera toxoid priming. These observations are relevant to immunizing against mucosal infections, as parenteral vaccination will prime (Svennerholm *et al.*, 1984) for or boost (Pierce & Gowans, 1975; Svennerholm *et al.*, 1984) mucosal immune responses *in vivo*. Despite concern that mucosal immunization might require prior parenteral antigen exposure to elicit strong circulating humoral immunity, it was demonstrated that i.g. immunization alone stimulates sera antibody responses in the absence of parenteral sensitization (Chapter 3, Heritage *et al.*, 1996). In this work, a low antigen dose (10-50 µg/mouse) in TS-PDMS-grafted MPs, elicited robust sera IgG and IgA responses which, following an oral MP boost, were substantially enhanced (Chapter 3, Heritage *et al.*, 1996). These results suggested that the initial MP immunization protocol provoked humoral recall capability.

In the present study, it was demonstrated that mucosal MP administration enhanced specific antibody responses *in vivo* elicited by parenteral boosting. Mice given microentrapped, but not soluble, HSA i.g. demonstrated enhanced HSA-

specific sera IgG responses following systemic antigen challenge. Indeed, even i.g. immunization with 50-fold greater doses of soluble HSA failed to stimulate equivalent sera antibody responses following i.p. antigen challenge, thus demonstrating the priming efficacy of i.g. administered TS-PDMS-grafted MPs for systemic antigen challenge.

This study strongly suggests that the i.g. administration of HSA-containing MPs stimulated mucosal immunity via PP. Following i.g. immunization with a low dose of HSA-containing MPs, but not soluble HSA, antigen-specific proliferation of PP cells was noted and reached a maximum after a third i.g. immunization. In contrast, antigen-specific lymphocyte proliferation was not observed in gut LP lymphocytes following i.g. immunization with MPs (data not shown). These results are consistent with those found by others (Eldridge *et al.*, 1990; Ermak *et al.*, 1995; Jani *et al.*, 1992; LeFevre *et al.*, 1985; Pappo & Ermak, 1989) demonstrating particulate antigen uptake into PP. HSA-specific IgM-secreting SFCs were initially detected in PP cell isolates following a single i.g. MP immunization and increased following 3 i.g. immunizations. These results support the observation that 3 oral MP immunizations are required to elicit maximal sera antibody titres and that oral immunization with soluble HSA fails to generate sera antibody responses (data not shown).

It was demonstrated that i.g. immunization with HSA-containing TS-PDMS-grafted MPs generated systemic humoral immunity *in vivo* which was augmented

by a parenteral antigen boost. These results suggested PP-stimulated lymphocytes might have migrated to systemic lymphoid compartments following i.g. MP administration. This hypothesis was supported by demonstrating lymphocyte activation in PP following i.g. MP administration and the subsequent appearance of lymphocyte proliferation in MLN and splenic tissue. A shift in specific IgM SFCs from PP and MLN cell isolates to SPL tissue was observed following i.g. MP immunization. The paucity of specific IgG SFCs in PP, MLN or SPL cells shortly after i.g. MP administration suggests gradual B cell maturation in PP, resulting in isotype switching and migration of IgG-committed plasmacytes from the GALT to systemic sites. PP, and possibly MLN, could serve as depots for oral administered HSA-containing MPs (Eldridge *et al.*, 1990; Jenkins *et al.*, 1994). Although others have demonstrated that orally administered MPs might directly stimulate systemic lymphoid tissues following transport through the mesenteric lymphatics (Jenkins *et al.*, 1994), the time delay between PP and SPL cell activation in the present studies suggests that MPs or released HSA is not directly stimulating systemically-situated lymphocytes. Rather, orally-administered MPs might accumulate in the GALT, resulting in sustained stimulation and eventual emigration of IgG-committed HSA-specific lymphocytes. However, it also possible that the observed systemic priming resulted from trafficking of MP-containing APCs from the GALT to systemic sites, where specific lymphocytes could then be activated. This notion is supported by data demonstrating both the phagocytosis of particulates by a variety of APCs,

including B lymphocytes (Vidard *et al.*, 1996), macrophages (Joel *et al.*, 1978; Tabata & Ikada, 1990; Vidard *et al.*, 1996), DCs (Inaba *et al.*, 1993; Reis e Sousa *et al.*1993), and the migration of GALT-derived APCs to peripheral sites (Liu & MacPherson, 1991, 1993; Mayrhofer *et al.*. 1986; Tabata & Ikada, 1990).

Whereas oral administration of HSA-containing MPs primed mice for an enhanced HSA-specific sera IgG (in particular IgG1) response following a parenteral HSA boost, specific circulating IgA levels were not enhanced (data not shown). This is in contrast to the previous study demonstrating enhanced sera IgG and IgA responses following an oral antigen challenge (Chapter 3, Heritage *et al.*, 1996). These results suggest that MP-stimulated IgA-committed PP lymphocytes are not restimulated following i.p. HSA challenge, a finding consistent with the view that mucosally-stimulated plasmacyte precursors are known to selectively localize to mucosal, but not to systemic sites (McDermott & Bienenstock, 1979). Indeed, after i.g. MP immunization, abundant numbers of HSA-specific SFCs were detected in PP and MLN cell isolates; however, no specific IgA SFCs were detected in SPL preparations up to 28 days following i.g. immunization (data not shown).

The present data supports the notion that oral immunization with microentrapped HSA stimulates a subset of T_H cells, hallmarked by the appearance of IL-4-secreting lymphocytes (Table 2). T_H cells are classified into two subsets depending on their cytokine profile. T_H1 cell clones exclusively produce IL-2, IFN-γ, and lymphotoxin and help to generate IgG2a responses, whereas T_H2 cells

synthesize IL-4, IL-5, IL-6 and IL-10 and provide help in mounting IgA, IgE and IgG1 responses (Mosmann *et al.*, 1986; Mosmann & Coffman, 1989). Although Xu-Amano *et al.* (1993) demonstrated that mature PP T cells are multipotent and can become either T_H1 or T_H2 cells following polyclonal T cell activation *in vitro*, they and others have demonstrated also that antigen delivery to the GALT preferentially stimulates T_H2-type cells (Jain *et al.*, 1996; Xu-Amano *et al.*, 1992,1993). The present results confirm and extended previous work (Xu-Amano *et al.*, 1993), demonstrating a shift of T_H2 -type cytokine secretion from GALT (PP) to systemic (SPL) lymphoid compartments following i.g. immunization with HSA-containing TS-PDMS-grafted MPs (Table 3). This cytokine microenvironment was likely responsible for driving the predominately IgG1 sera response observed after i.g. MP administration, which was boosted following the parenteral antigen boost (Table 3), perhaps via migration of PP-derived T cells. Although the mechanism responsible for the observed MP-induced T_H2 cell activity is unknown, recent studies suggest that both naive T cell precursors (Rincón *et al.*, 1997) and other non-T IL-4-producing cells (Moqbel *et al.*, 1995; Paul *et al.*, 1993) could be responsible for initiating differentiation of T_H2 cells from multipotent precursors.

TS-PDMS-grafted MPs are a useful addition to mucosal and systemic vaccine delivery systems. Mucosal priming with these novel polymer-grafted starch MPs might improve current systemic vaccine protocols by stimulating elements of the common mucosal immune system in addition to potentially reducing the need

for repeated systemic vaccination boosts.

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CHAPTER 5

COMPARISON OF MURINE NASAL-ASSOCIATED LYMPHOID TISSUE AND PEYER'S PATCHES

PREFACE

Electron microscopic and flow cytometric studies were conducted by Drs A.L. Arsenault and D. Snider, respectively, both from the department of Pathology, McMaster University.

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5.1 ABSTRACT

The nasal mucosal is the first site of contact with inhaled antigens. However, the nature of local immune responses and the role of the NALT in those responses have rarely been studied. To characterize the cells involved in mucosally-derived immune responses, NALT and PP cells from normal mice, and mice immunized i.g. or i.n. with CT, were isolated and analysed. Compared with PP cells, unstimulated NALT cells contained a higher proportion of T cells. The CD4:CD8 ratio in NALT cell preparations was less than that observed in PP and more closely resembled that seen in SPL. Additionally, the total B cell frequency in NALT cell isolates was 20% lower than that observed in PP cell preparations. Although NALT and PP cell isolates contained both mature B cells and cells undergoing activation to express surface IgA, unlike PP, NALT showed no significant frequency of IgA-switched cells. Following i.n. immunization with CT, toxin-specific IgA SFCs were detected in NALT cell preparations. The numbers of these cells correlated with CT-specific IgA in nasal washes (NWs), but not in GWs or sera, thus suggesting local nasal production of antigen-specific mucosal antibodies. There was no evidence of anti-CT SFCs in NALT or CT-specific antibody in NWs following i.g. CT administration. These results support the notion that nasal mucosal antibody production is best achieved via direct stimulation of IgA-committed, NALT-derived B cells.

5.2 INTRODUCTION

Mammals and aves evolved distinct respiratory and GI lymphoid compartments which participate in the defence of associated and distant mucosae. Respiratory lymphoid structures include the BALT, and lung parenchymal and alveolar cells in the respiratory tract (Bienenstock & Clancy, 1994). The GALT is comprised of the PP, LP and possibly SLN in the intestine (Kraehenbuhl & Neutra, 1992). In humans (Brandtzaeg, 1984) and certain other species (Hameleers *et al.*, 1989; Loo & Chin, 1974; Mair *et al.*, 1987), the oropharyngeal lymphoid tissues (Waldeyer's ring), including the adenoid and the bilateral tubule, palatine and lingual tonsils (Goeringer & Vidić, 1987) might also participate in respiratory and gastrointestinal tract defence (Hameleers *et al.*, 1990, 1991; Nedrud *et al.*, 1987; Tamura *et al.*, 1989; Wu & Russell, 1993). Although similarities in the structure and function of the BALT and GALT have been explored in humans and animals (Croitoru & Bienenstock, 1994), similar studies of Waldeyer's Ring have been restricted largely to humans as structural and/or functional equivalents have not been defined in most animals. Since the nasal mucosa is the first site of contact with inhaled antigens, it is possible that a nasal analogue of the BALT, GALT or Waldeyer's ring exists in mammals which contains lymphocytes capable of participating in the defence of respiratory and other mucosal sites. For example, in rats (Spit *et al.*, 1989), hamsters (Kuper *et al.*, 1992) and mice (Belai *et al.*, 1977; Ichimiya *et al.*, 1991; Asanuma *et al.*, 1995; Reuman *et al.*, 1989), paired lymphoid structures, termed the NALT are found at the entrance of the nasopharyngeal duct.

Structural and functional evaluation of NALT has been challenging, largely due to difficulties in accessing its anatomic location, although the presence of M cells in the lymphoepithelium overlying NALT in this site (Spit *et al.*, 1989), suggests functional similarities to BALT and GALT. However, whether NALT is an analogue of human BALT, GALT or Waldeyer's ring remains unclear.

In this work, a rapid and precise method for isolation of NALT to compare the composition and immune responsiveness of NALT and PP. The results show that NALT contains cell populations expected in a immune inductive site. However, whether the NALT is a BALT or GALT equivalent remains uncertain; although NALT had the capacity to respond to nasally-administered antigen, it contained distinct frequencies and ratios of immune cells when compared to PP.

5.3 MATERIALS AND METHODS

5.3.1 Animals

Female BALB/c mice (Harlen Sprague Dawley Inc., Indianapolis, IN) entered experiments at 6-8 wk of age and were allowed food and water *ad libitum*.

5.3.2 Collection and Preparation of Sera and Cells

Individual blood samples were obtained via the retro-orbital plexus. For sera evaluation, insoluble material was removed by centrifugation and sera were stored at -70°C until used. Pooled peripheral blood lymphocytes (PBLs) were obtained by

centrifuging fresh blood, containing 0.1M ethylenediaminetetraacetic acid, over Ficoll-Paque (Pharmacia, Uppsala, Sweden) (Fotino *et al.*, 1971).

Pooled NALT cell suspensions were prepared using a dissection procedure. Mice were sacrificed by cervical dislocation, decapitated and the lower jaw and tongues removed. After rinsing with ice-cold HBSS to remove blood, the heads were immobilized with pins on a wax dissection slab to reveal the upper palate. Using a dissection microscope (magnification 160 diameters; Model M3B, Wild Leitz Canada, Willowdale, ON) and a fibre optic WMB illuminator (Minebea Co., Ltd, Thailand), palates were excised as illustrated in Figure 1 using a No. 15 scalpel blade (Lance Blades, Sheffield, England). Following the incisions, palates were gripped behind the incisor teeth with fine forceps and gently pulled toward the molar teeth while using the scalpel to gently free tissue between the palates, jawbones and nasal septums. Palates were placed immediately into a 24 mM Petri dish containing 1.0 mL of ice-cold HBSS and the NALT (visible under low angle fibre optic illumination) was teased gently into the medium to release cells. NALT cell suspensions from individual animals were pooled and cells were washed twice with HBSS by centrifugation (200xg for 10 min at 4°C) in HBSS and resuspended in RPMI-1640 media supplemented with 5% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine and 1% HEPES, pH 7.4 (operationally termed RPMI-5).

Pooled SPL and PP were isolated from decapitated carcases and placed into

ice cold HBSS. SPL cell suspensions were obtained by crushing organs between the frosted ends of two microscope slides. Cell suspensions of PP were obtained by stirring the tissues for 15 min in HBSS containing 1.5 mg/mL of Dispase II (Boehringer Mannheim, Mannhein, Germany) at 37°C. The supernatant overlying PP was collected and the remaining tissue was incubated for an additional 15 min in fresh collagenase solution. The supernatant was collected and the remaining tissues were gently teased apart using forceps. The dissociated cell suspension and supernatant were filtered through nylon mesh (BSH Thompson, Scarborough, ON) to remove adipose tissue and large cellular aggregates. Single cell SPL and PP suspensions were washed twice with HBSS by centrifugation. Erythrocytes and dead cells were removed using Ficoll-Paque and lymphocytes were resuspended in RPMI-5. Viability of cell preparations routinely exceeded 90% as judged by ethidium bromide/fluorescein diacetate staining (Edidin, 1970; Mohr *et al.*, 1980).

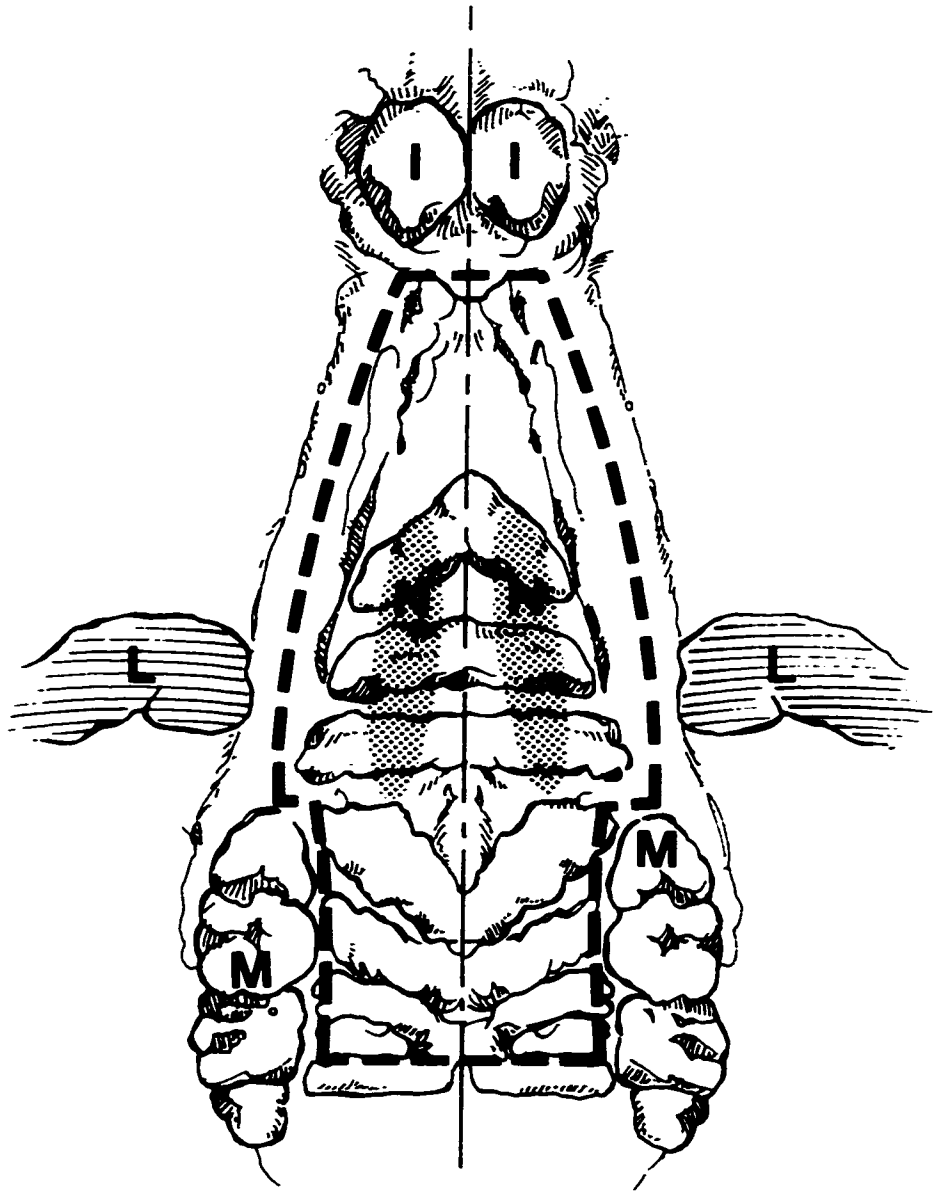
5.3.3 Electron Microscopy

Tissue specimens from 6 mice were fixed in 2.0% gluteraldehyde in 0.1M sodium cacodylate for 1 h, rinsed in 0.2M sodium cacodylate buffer and postfixed for 1 h in buffered 1.0% OsO_4. Tissues were then dehydrated in a graded series of ethanol. For SEM, specimens were critical point dried, sputter-coated with gold and viewed in a Philips 501B scanning electron microscope. For transmission electron microscopy (TEM), dehydrated specimens from 6 additional mice were solvent

exchanged in propylene oxide, embedded in Spurr's resin. Sections were stained with uranyl acetate and viewed in a JEOL 1200 EX transmission electron microscope. Representative images were photographed and printed.

Figure 7.

Anatomic location of nasal-associated lymphoid tissue (NALT) in *Mus musculus*. Illustrated view of ventral surface of upper palate. **I**, incisor tooth; **L**, ligament; **M**, molar tooth; **N**, NALT located on the dorsal surface of upper palate within the stippled area. Broken line describes the incision to remove upper palate.


L
L
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5.3.4 Flow Cytometry

Pooled cells from untreated animals were phenotypically characterized using a FACScan flow cytometer and Lysys-II software (Becton Dickinson, San José, CA). A minimum of 5000 cells were analysed for each phenotypic characteristic. Three colour staining for T cell subsets was performed using fluorescienated (FITC) anti-CD4 mAb (clone GK1.5 (Marrack *et al.*, 1983), prepared in our laboratory), phycoerythrin-conjugated (PE-) anti-CD8β mAb (clone 53-5.8, Pharmingen, San Diego, CA) and biotinylated anti-CD8α mAb (clone 53.6-72, Pharmingen) followed by streptavidin-peridinin chlorophyll protein (Becton Dickinson, Sparks, MD). B cells were detected with a FITC-anti-B220 mAb (clone 6B2 (Coffman & Weissman, 1981), prepared in our laboratory), FITC-goat anti-mouse IgA (Southern Biotechnology Associates, Birmingham, AL) and PE- goat anti-mouse IgM (Southern Biotechnology Associates). Macrophages were identified by two colour staining with PE-anti-mouse CD11b (clone MAC-1, Pharmingen) and FITC-anti-mouse IA/E (clone M5) (Bhattacharya *et al.*, 1981).

B and T cells were analysed within a lymphocyte gate defined by forward and side light scatter. An enlarged mononuclear gate was used to define the macrophage population. Equivalent gates were used in analyses of cells derived from different tissues for comparison. Dead cells were excluded from analyses on the basis of light scatter in reference to propridium iodide staining. Background staining was controlled by labelled isotype controls or biotinylated rat IgG

(Pharmingen) and never exceeded 1.0% of cells. The results represented the percentage of positively stained cells in the total cell population exceeding the background staining signal.

5.3.5 Immunizations

Groups of 6 mice each were immunized i.g. or i.n. on days 0 and 10. CT was chosen as it is a potent mucosal immunogen (Tamura *et al.*, 1989; Wu & Russell, 1993). Animals received 10 µg of CT i.g. in 500 µl of 0.2 M $NaHCO_3$ (to neutralize gastric acidity) or vehicle alone using PE50 tubing (Becton Dickinson) or i.n. in 10 µl of PBS, pH 7.2, distributed between the two nares.

5.3.6 Collection of Mucosal Washings

Mice were exanguinated by cardiac puncture and CT-specific immunoglobulin IgA and IgG in NWs or GWs was measured. Tracheas were exposed by dissection, ligated with a 3.0 silk suture (Ethicon, Somerville, NJ) and PE-50 polyethylene tubing (Becton Dickinson) was inserted, via the oropharynx, into the nasopharyngeai cavity. Contents of the nasal passages from individual mice were washed out of the nares with 0.5 mL of ice-cold enzyme inhibitor solution (Chapter 3, Heritage *et al.*, 1996). Individual GWs were obtained as previously described (Chapter 3, Heritage *et al.*, 1996). NWs and GWs were stored at -70°C until used.

5.3.7 Enumeration of Cholera Toxin-specific Spot-forming Cells

An ELISPOT was used to detect CT-specific SFCs in pooled NALT and PP preparations. Duplicate serial dilutions of single cell suspensions (beginning at 1 x 10^6 cells/100 µl/well) were examined using NC microtitre plates (Millititer HA; Millipore Corp., Bedford, MA) previously incubated for 2 h at 37°C with 100 µl/well of CT (10 µg/mL in PBS), washed with PBS and treated then with 250 µl/well of 0.1% gelatin in PBS. After a 8 h incubation at 37°C, wells were washed three times each with PBS and PBS containing 0.05% Tween-20 (PBS/Tween). The wells were incubated overnight at 4°C with 100 µl of biotinylated-goat anti-mouse IgA, IgG or IgM (heavy chain-specific; Southern Biotechnology Associates) diluted in PBS/Tween-20 containing 0.1% gelatin (dilution buffer). The wells were washed three times each with PBS, PBS/Tween and incubated for 1 h at room temperature with AP-conjugated streptavidin (Southern Biotechnology Associates) in dilution buffer. After washing the wells four times with PBS, spots were visualized by incubating with 100 mM $NaHCO_3$, 1.0 mM $MgCl_2$, pH 9.8) containing 0.15% w/v BCIP and 0.3% w/v NBT. Colour development in the wells was halted by thoroughly rinsing the wells with tap water. After drying at room temperature, individual SFCs were enumerated with the aid of a dissecting microscope. The results represent the mean numbers of spots per 1 x 10^6 cells in duplicate wells containing at least two-fold more spots than wells incubated with cells from PBS-treated animals.

5.3.8 Measurement of Cholera Toxin-specific Antibody Responses

An ELISA was used to measure CT-specific antibodies in individual NWs, GWs and sera as previously described (Chapter 3, Heritage *et al.*, 1996). Following the addition of halving or one third dilutions of sera or mucosal washings to CT-coated microtitre plates, anti-CT antibodies were quantified using AP-conjugated goat anti-mouse IgG1, IgG2a or IgA (heavy chain-specific; Southern Biotechnology Associates) and 1.0 M diethanolamine buffer, pH 9.8, containing 50 mM $MgCl_2$ and 1.0 mg/mL *p*-nitrophenylphosphate. The results were expressed as reciprocal end-point titres representing the greatest sera dilutions giving OD values exceeding two times normal mouse sera mean values.

5.3.9 Statistical Analyses

Statistical analyses of data were done using GraphPad Prizm® Version 2.00 (GraphPad Software, SanDiego, CA). One-way ANOVA, Tukey test pair-wise multiple comparisons and unpaired Student's *t*-tests were used to detect and compare mean differences between treatment groups. A level of significance of 95% was chosen for all tests.

5.4 RESULTS

5.4.1 Isolation of NALT

Figure 8 shows that following microdissection, paired NALT aggregates were

clearly visible on the palate, near the entrance of the nasopharyngeal duct. NALT cells were readily isolated, yielding approximately 1 x 10^6 viable cells per palate. Since most of the overlying epithelium remained attached to the nasopharynx, few ciliated cells were noted by either SEM (Fig. 8), in cell suspensions as assessed by light microscopy or by TEM of cell pellets (data not shown). Further, TEM analyses of NALT cells did not reveal the presence of any apoptotic lymphocyte nuclei (data not shown). Contamination of NALT cell preparations by erythrocytes was judged to be negligible. These results indicated that an adequate number of NALT cells could be attained for study.

Figure 8.

Scanning electron micrographs (SEMs) of nasal-associated lymphoid tissue (NALT) in *Mus musculus*. **A**, view of the dorsal surface of upper palate revealing NALT in correspondence to stippled areas in figure 7. **B**, magnified left NALT structure in **A** rotated and viewed at 60° angle. **C**, magnification of **B** near centre of NALT structure.


A
500 µm
B
C
10 µm
250 µm

5.4.2 Flow Cytometry

5.4.2.1 T Cell Composition of NALT

The similarity of NALT to other mucosal and systemic lymphoid tissues was assessed (Table 5). NALT isolates contained greater proportions of T cells than PP (50% *vs* 30%, respectively), of which a greater percentage were CD4+ as compared to CD8+ T cells ($p<0.0005$). The CD4:CD8 cell ratio in the NALT (3.7:1) was closer to that noted in the SPL (2.7:1), yet was less than that observed in PP and PBL isolates (10:1 and 30:1, respectively). Similar numbers of CD8αα+ T cells, a unique subset common to intestinal epithelia (Guy-Grand *et al.*, 1991), were detected in PP and PBL. Interestingly, no significant numbers of CD8αα+ T cells were found in NALT cell isolates, in contrast to PP ($p<0.001$) and similar to that seen in SPL cell isolates. Thus if viewed on the basis of overall T cell frequency and phenotype, NALT T cell populations most closely resembled SPL isolates rather than those found in PP preparations.

5.4.2.2 B Cell Composition of NALT

Although NALT cell preparations contained greater numbers of T cells than PP, table 5 shows that the total B cell frequency of NALT was 15% lower than that found in PP ($p<0.05$). However, NALT and PP cell populations contained similar numbers of mature, IgM+IgA- B cells, which were significantly greater than that observed in SPL cell isolates ($p<0.01$ and $p<0.001$ compared to NALT and PP,

respectively). Cells co-expressing surface IgA and IgM were detected at low but similar frequencies in NALT, PP and SPL cell preparations. These cells likely represented activated B cell types containing both IgM and IgA mRNA which have yet to undergo gene rearrangement preceding the exclusive expression of surface IgA. Low frequencies of IgM-igA+ B cells (committed entirely to IgA production) were detected in PP and PBL, but neither NALT nor SPL isolates had detectable levels of these cells. These results indicated that although NALT contained both normal mature B cells and cells undergoing expression of surface IgA, unlike PP, NALT did not contain IgA switched cells. Thus, the normal murine NALT might not be as active as PP if viewed in terms of the differentiation and expansion of IgA-committed B cell populations.

5.4.2.3 Macrophage Composition of NALT

Macrophage frequencies in NALT and other tissues were identified via cell surface staining with CD11b (Mac-1)- and Ia (MHC class-II)-specific antibodies. As shown in Table 5, small but detectable frequencies of macrophages were observed in NALT and SPL cell isolates which were significantly higher than those in PP or PBL cell isolates ($p<0.05$). The close resemblance of NALT macrophage and T cell content to that of SPL might have a functional bearing on development of cell-mediated immune responses in NALT.

Table 5. Comparison of surface phenotypes on mononuclear cell subsets in nasal-associated lymphoid tissue (NALT) and other lymphoid tissues.

Cell Subset	Cell Source (% cells)			
	NALT	PP	PBL	SPL
T Cells				
$CD4^+$	32.0 ± 6.7	22.3 ± 1.1	75.3 ± 0.8	32.3 ± 10.3
$CD8\alpha\beta^+$	8.6 ± 1.7	1.9 ± 0.2	2.5 ± 0.3	12.1 ± 4.9
$CD8\alpha\alpha^+$	N.D.	3.6 ± 1.1	2.2 ± 0.6	N.D.
B cells				
$B220^+$	55.3 ± 8.7	69.8 ± 2.2	11.9 ± 0.7	45.0 ± 8.5
IgM^+IgA^-	48.8 ± 4.0	55.7 ± 1.8	7.2 ± 0.7	30.8 ± 11.2
IgM^+IgA^+	4.2 ± 0.8	5.7 ± 0.4	1.8 ± 0.2	3.0 ± 1.2
IgM^-IgA^+	N.D.	3.4 ± 0.6	2.0 ± 0.3	N.D.
Macrophages				
$CD11b^+Ia^+$	4.5 ± 0.8	2.0 ± 0.4	1.8 ± 0.2	7.2 ± 2.0

Figures represent the arithmetic mean of percent positive cells ± S.D., for 4 independent experiments. For Peyer's patches (PP), peripheral blood lymphocytes (PBL) and spleens (SPL), each isolate was pooled from two mice. For NALT, each isolate was pooled from ten mice.

N.D., not detectable. Values are not different from fluorescent background controls that were typically 0.5-1.0%.

5.4.3 Detection of Antigen-specific Spot-forming Cells in NALT and Peyer's Patches Following Intranasal or Intragastric Immunization

Since Table 5 indicated that NALT and PP contained disparate frequencies of various cell populations, a direct comparison of the functional characteristics of these mucosae-associated tissues was conducted. Groups of mice were immunized i.n. or i.g. with CT, and NALT- and PP-derived cell populations were subsequently examined for the presence of CT-specific SFCs. Table 6 shows that following two i.n. or i.g. immunizations with CT, IgA producing CT-specific SFCs were detected in the local lymphoid compartments. I.n. immunization with CT resulted in appreciable numbers of CT-specific, IgA-producing SFCs in the NALT. However, i.g. immunization using the same dose of CT resulted in almost 3-fold more CT-specific IgA-producing SFCs in isolated PP. Over this short period of time, there was no evidence that anti-CT SFCs appeared at distant mucosal sites, as i.g. or i.n. administration of CT did not result in anti-CT SFCs in NALT or PP tissue, respectively. Additionally, whereas i.g. immunization with CT elicited solely an IgA plasmacyte response in PP, antigen-specific plasma cells producing IgG and IgM, in addition to IgA, were detected in the NALT following i.n. immunization with the same dose of CT (Table 6). These results demonstrated that stimulation of antigen-specific nasally-derived B cells is best accomplished by i.n. immunization.

Table 6. Cholera toxin (CT)-specific spot-forming cells (SFCs) in nasal-associated lymphoid tissue (NALT) and Peyer's patches (PP) following intranasal (i.n.) or intragastric (i.g.) CT administration.

Immunization	Source	CT-specific SFC/10^6 Cells		
		IgA	IgG	IgM
i.n.	NALT	868	10	30
	PP	ND	ND	ND
i.g.	NALT	ND	ND	ND
	PP	2398	ND	ND

Groups of 6 mice were each immunized i.n. or i.g. on days 0 and 10 with 10 µg of CT of in phosphate-buffered saline (PBS) or 0.2 M $NaHCO_3$ respectively. Animals were sacrificed on day 14 and lymphocytes isolated from NALT and PP were evaluated for the presence of CT-specific IgA, IgG and IgM SFCs using an ELISPOT assay. Table represents mean numbers of CT-specific SFCs per 1 x 10^6 cells in duplicate wells containing at least two-fold more spots than control wells incubated with cells from PBS-treated mice in a representative experiment.

ND; not detectable

5.4.4 Antigen-specific Antibody Responses Following Intranasal or Intragastric Immunization

Sera and mucosal secretions from groups of animals immunized i.n. or i.g. with CT were examined for the presence of CT-specific antibodies. Table 7 shows that 4 days after secondary i.n. or i.g. immunization with CT, anti-CT IgG1 sera titres were comparable between both groups of animals. Sera anti-CT IgG2a responses were, however, 6-fold greater in animals receiving CT i.n. Additionally, while CT-specific IgA was not detected in the sera of i.n. or i.g. immunized animals, CT-specific IgA was detected in mucosal washes of animals immunized i.n. or i.g. (Fig. 9). Specifically, i.g. CT administration elicited a robust CT-specific mucosal IgA response in intestinal, but not nasal secretions ($p<0.05$) while i.n. immunization stimulated nasal but not intestinal specific IgA production ($p=0.09$). Thus, similar to the detection of CT-specific SFCs in NALT and PP (Table 6), administering CT at one mucosal site stimulated local mucosal IgA production but failed to provoke detectable levels of CT-specific IgA in mucosal secretions at distant sites.

The lack of detectable levels of CT-specific sera IgA following i.n. or i.g. CT administration, together with the presence of antigen-specific mucosal IgA and CT-specific IgA SFCs detected in either mucosal lymphoid compartment (table 6) suggested local production of CT-specific mucosal antibodies.

Table 7. Antigen-specific serum antibody responses following intranasal (i.n.) or intragastric (i.g.) immunization with cholera toxin (CT).

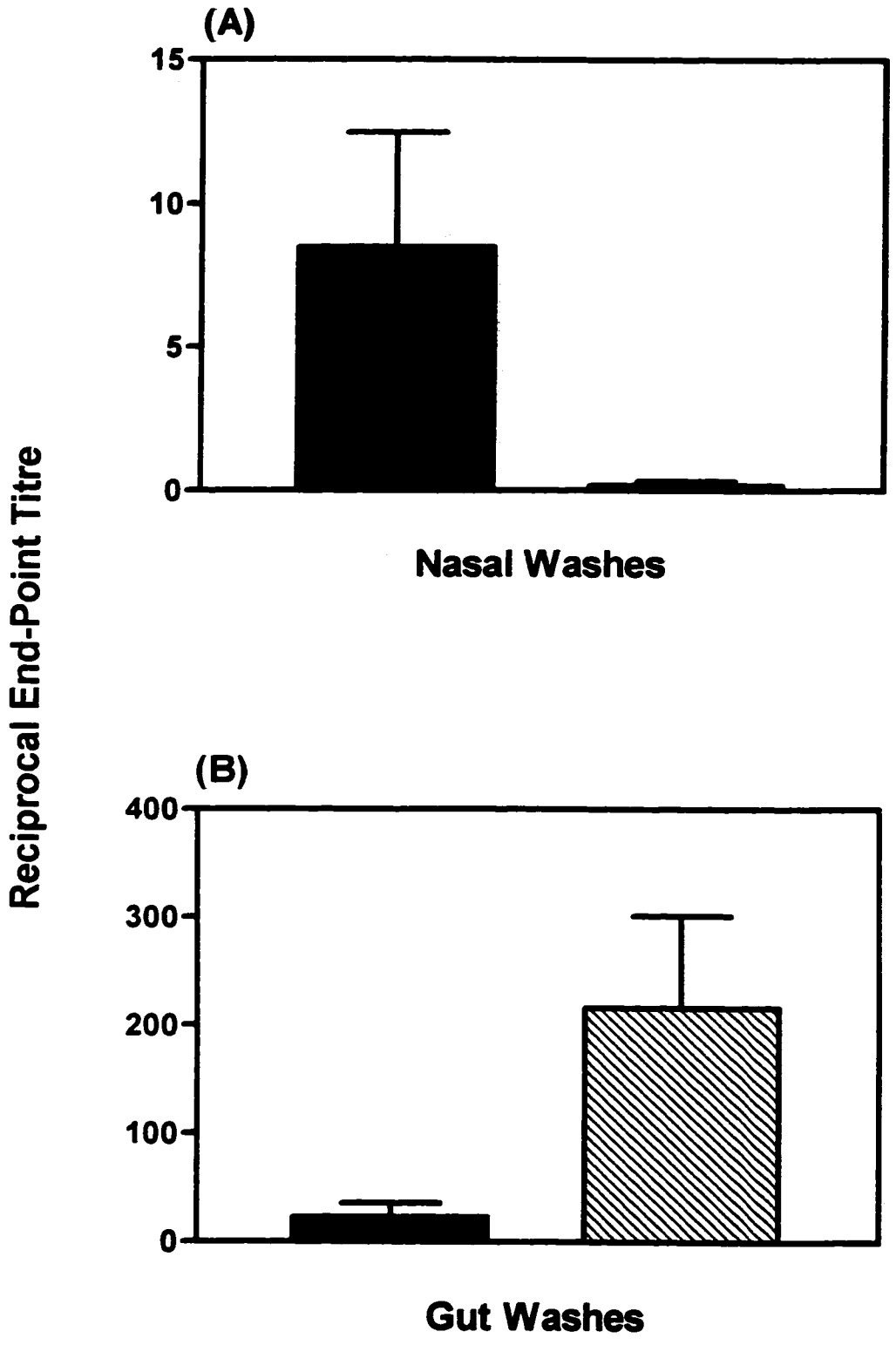
Immunization	CT-specific Reciprocal Antibody Titre		
	IgG1	IgG2a	IgA
i.n.	21,600 ± 6750	1216 ± 616	ND
i.g.	20,250 ± 5123	200 ± 81	ND

Mice were immunized i.n. or i.g. on days 0 and 10 with 10 µg of CT in phosphate-buffered saline (PBS) or 0.2 M $NaHCO_3$ respectively. Sera obtained on day 14 were evaluated for the presence of CT-specific IgG1, IgG2a and IgA using an enzyme immunoassay. Results are expressed as mean reciprocal end-point titres (± SEM) representing the greatest sera dilutions giving optical density values exceeding two times normal mouse control sera mean values

ND; no detectable CT-specific serum antibody response. The limit of detection was a 1:50 dilution.

Figure 9.

Cholera toxin (CT)-specific IgA antibodies in mucosal secretions following intranasal (i.n.) or intragastric (i.g.) immunization with CT. Groups of 6 mice were immunized i.n. (solid bars) or i.g. (hatched bars) on days 0 and 10 with 10 μg of CT in phosphate-buffered saline (PBS) or 0.2 M $NaHCO_3$, respectively. On day 14 animals were sacrificed and nasal (a) and intestinal (b) washes were evaluated for the presence of CT-specific IgA using an enzyme immunoassay. Results are expressed as mean reciprocal end-point titres (± SEM) representing the greatest sera dilutions giving optical density values exceeding two times normal mouse control sera mean values.


(A)
15
10
5
0
Nasal Washes
Reciprocal End-Point Titre
(B)
400
300
200
100
0
Gut Washes

5.5 DISCUSSION

The anatomic location of the NALT (Fig. 7) has precluded its precise removal and analysis. Inexact methods have been employed to isolate NALT cells (Asanuma *et al.*, 1995; Reuman *et al.*, 1989) allowing for potential contamination from the adjacent nasal mucosa, LP and vasculature. This difficulty was overcome by developing an accurate dissection method to removal NALT tissue. NALT cell preparations were judged to be free of unwanted cell contamination as a result of careful dissection. This was corroborated by disparate frequencies of various cell populations observed in NALT and PBL cell preparations (Table 5).

When compared to systemic (SPL, PBL) and mucosal (PP) cell preparations, it was shown that NALT cell isolates from unimmunized mice contained disparate frequencies and phenotypes of immune cell populations. Although NALT and PP cell preparations contained similar numbers of IgM+IgA- and IgM+IgA+ B cells, when compared to PP cell isolates, NALT-derived B cells comprised a smaller proportion of total lymphocytes ($p<0.05$). Additionally, unlike PP preparations, NALT isolates contained no IgA-switched (IgM-IgA+) B cells ($p<0.05$). In unpublished studies, IgA-committed B cells in NALT cells isolated from various ages of naive mice (6-20 wk of age) was also undetectable. This suggested that, even in the presence of environmental antigens, murine NALT might not be as active as PP, possibly reflecting exposure to a lower antigenic load.

NALT and PP cell preparations also contained distinct T cell and

macrophage populations. Compared to PP, NALT cell isolates contained higher frequencies of T cells and macrophages and displayed a lower CD4:CD8 T cell ratio (10:1 *vs* 3.7:1, respectively). Indeed, the frequencies and ratios of CD4+ and CD8αβ+ cells in NALT preparations most closely resembled spleen cell isolates. Also similar to SPL cell preparations, NALT cell isolates contained no significant numbers of CD8αα+ T cells. This is in contrast to PP which contained small, but significantly higher numbers ($p<0.001$) of CD8αα+ T cells, which highlights another dissimilarity between these mucosal-associated lymphoid structures. The relatively high frequency of CD8αβ+ T cells also observed in unstimulated NALT cell isolates, compared to PP ($p<0.05$), might reflect a greater contribution of cytotoxic T lymphocytes or CD8+ regulatory cells to NALT tissue. Cells with a macrophage phenotype (CD11b+ Ia+) in NALT and SPL cell isolates were significantly higher in frequency that those in PP ($p<0.05$ and $p<0.001$, respectively) possibly reflecting a greater contribution of cell-mediated immune responses in NALT tissue. Indeed, following i.n. influenza virus immunization, Tamura *et al.* (1996) observed cellular immune responses in crude NALT preparations that correlated with i.n. viral clearance. Thus, if viewed solely on the basis of immune cell content and ratio, NALT bears a greater resemblance to SPL than PP.

Although normal NALT cell isolates contained few IgA-secreting B cells (data not shown), following i.n. immunization with CT, CT-specific IgA SFCs were readily detected in NALT tissues. This suggested that B cell isotype switching and

differentiation occurs in the NALT following direct antigenic stimulation. Also, PP cells displayed increased numbers of CT-specific IgA SFCs after i.g. CT administration, albeit at higher levels than observed in NALT cell isolates. Following i.g. or i.n. CT immunization, there was no evidence that CT-specific SFCs dispersed to the NALT or PP, respectively. These findings question whether stimulated GALT-derived lymphocytes migrate to the nasal mucosa and suggest that stimulation of NALT is best achieved by nasal antigen administration.

Despite detecting CT-specific IgA SFCs in NALT isolates following i.n. immunization, antigen-specific IgA was not observed in the sera of these animals. However, toxin-specific IgA was detected in nasal, but not gut, washes following i.n. CT administration ($p=0.09$). These results suggest that i.n., but not i.g., immunization stimulates local antigen-specific mucosal IgA production via the selective stimulation of IgA-committed NALT-derived B cells. Similarly, i.g. CT administration elicited a robust CT-specific mucosal IgA response in intestinal but not in nasal secretions ($p<0.05$), suggesting regionalised activation of the mucosal immune system. Indeed, Husband and Gowans (1978) observed the selective migration of IgA plasma cell precursors to the intestinal LP, but not PP, following the adoptive transfer of MLN cells. This homing highlights the potential compartmentalization of the different tissues comprising the common mucosal immune system (Croitoru & Bienenstock, 1994) and suggests that local immunization at one mucosal site might best stimulate the production of local

mucosal immunity.

Although i.n. immunized animals failed to generate detectable CT-specific sera IgA responses, i.n. CT administration stimulated robust antigen-specific sera IgG1 and IgG2a responses. In contrast, i.g. immunized mice elicited chiefly an IgG1 antigen-specific sera response. These results suggest that different types of T_H cell activity may be dependent upon the route of inoculation. T_H cells are classified into two subsets depending on their cytokine profile. T_H1 cell clones exclusively produce IL-2, IFN-γ and lymphotoxin and help to generate IgG2a responses, whereas T_H2 cells synthesize IL-4, IL-5, IL-6 and IL-10 and provide help in mounting IgA, IgE and IgG1 responses (Mosmann *et al.*, 1986; Mosmann & Coffman, 1989). The present work suggests that both T_H1 and T_H2 cells in the NALT were likely responsible for driving the sera antibody responses observed after i.n. CT administration. Increased frequencies of CD4+ and/or CD8αβ+ T cells in NALT isolates, compared to PP, might be a source of T_H1 cytokines capable of promoting antigen-specific IgG2a responses as Tamura *et al.* (1996) detected IFN-γ production from crude preparations of nasally-derived CD4+ T cells.

NALT has been studied primarily in rat and mouse models (Kuper *et al.*, 1992). Immunohistochemical characterization of rat NALT has shown that B and T cells are distributed in distinct areas with a high CD4:CD8 T cell ratio and a predominance of B over T cells (Koornstra *et al.*, 1992). Although murine NALT has not yet been well described immunohistologically, these initial studies suggest that,

in mice, NALT is distinct from that found in rats and, if examined solely on immune cell content and subset ratios, more closely resembles SPL and not PP. Nevertheless, the capability of NALT to elicit specific IgA responses locally suggests that this structure might represent a unique mucosal lymphoid tissue which is capable of expressing both mucosal and systemic immune responses.

Despite growing interest in using the i.n. route for mucosal vaccine delivery, little attention has addressed the local site(s) and mechanism(s) which might be responsible for the induction of such responses. In humans, the participation of oropharyngeal lymphoid tissues (Waldeyer's ring) in i.n. immune responsiveness is unclear. However, Ogra (1971) reported that combined tonsillectomy and adenoidectomy in children resulted in diminished polio virus-specific antibody levels in nasopharyngeal secretions. In agreement with these studies, preliminary studies in our laboratory show that, in young BALB/c mice (less than 9 wk of age), the phenotypes and frequencies of NALT cells is comparable to those seen in adult animals (11- 20 wk of age) demonstrating the maturity and possible immune responsiveness of NALT even in young mice (data not shown). Despite a paucity of studies, it is clear that rodent NALT also has clear potential as an immunocompetant inductive site and might represent a functional equivalent of Waldeyer's ring in humans. Investigations of the types of precursor cell populations which can be stimulated in the murine NALT structure are now needed. Studies are currently underway to investigate the interaction of cells within this tissue with a

model protein antigen.

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CHAPTER 6

INTRANASAL IMMUNIZATION OF MICE WITH POLYMER-GRAFTED STARCH MICROPARTICLES ACTIVATES LYMPHOCYTES IN THE NASAL-ASSOCIATED LYMPHOID TISSUE AND DRAINING LYMPH NODES

PREFACE

The synthesis and characterization of TS-PDMS and other silicones was conducted by Dr. M. Brook, Department of Chemistry, McMaster University.

A formal communication of this work was published as: Heritage P.L., Brook M.A., Underdown B.J. & McDermott M.R. (1998) Intranasal immunization with polymer-grafted starch microparticles activates the nasal-associated lymphoid tissue and draining lymph nodes. *Immunology* **93**, 249-256.

6.1 ABSTRACT

Waldeyer's ring is located at the juncture of the respiratory and alimentary tracts where it is bombarded by inhaled and ingested antigens. However, knowledge of its exact function or consequences of its removal is incomplete. Recently, the murine NALT has been reported to have functional similarities to Waldeyer's ring and, thus, might be a suitable model to examine the function of oronasopharyngeal lymphoid tissues. To explore the capability of NALT to incite local mucosal and systemic immunity, mice were immunized i.n. with TS-PDMS-grafted MPs, an inoculant previously shown to induce robust systemic and mucosal humoral immunity following i.g. administration. It was demonstrated that i.n. immunization with low doses of microentrapped, but not soluble, HSA evoked robust circulating IgG responses ($p<0.05$). Additionally, NALT cells isolated from MP-treated mice proliferated *in vitro* when restimulated with HSA ($p<0.05$), suggesting that i.n. immunization with HSA-containing MPs incited specific immunity via NALT cell activation. Coinciding with these observations, after i.n. MP administration HSA-specific SFCs were observed in NALT, and later pCLN and SPL, suggesting that the observed MP-induced specific systemic antibody responses emanated from the NALT. It was showed also that i.n. immunization with HSA-containing TS-PDMS-grafted MPs stimulated IL-4-secreting lymphocytes in the NALT. This cytokine microenvironment was probably responsible for driving the IgG1 sera response observed after i.n. MP administration, via the migration of NALT-derived

IgG1-committed B cells. Interestingly, unlike i.g. MP administration, i.n. immunization with HSA-contain MPs did not evoke detectable specific IgA any lymphoid tissue examined or in nasal secretions, likely reflecting differences between NALT and other MALT.

6.2 INTRODUCTION

The upper respiratory tract is the first site of contact with inhaled or aspirated antigens and is receiving considerable attention as a route for mucosal vaccine delivery (Kiyono, 1997). Here, lymphoid structures include the BALT (Bienenstock & Clancy, 1994) and, in humans (Brandtzaeg, 1984) and several other species (Hameleers *et al.*, 1989; Loo & Chin, 1974; Mair *et al.*, 1987), the oronasopharyngeal lymphoid tissues (Waldeyer's ring) including the pharyngeal, palatine and lingual tonsils (Goeringer & Vidić, 1987). Since these lymphoid tissues together are believed to participate in upper respiratory tract immunity (Pabst & Tschernig, 1997), they are likely important targets for i.n. vaccines. However, few studies have focussed on the local site(s) and mechanism(s) which might be responsible for the induction of local mucosal and systemic immune responses following i.n. immunization. Although Waldeyer's ring probably encounters antigens initially, the importance of this tissue to local immune responses is unclear. However, Ogra (1971) reported that childhood tonsillectomy resulted in diminished polio virus-specific antibody levels in nasopharyngeal secretions, thus indicating the

involvement of Waldeyer's ring in respiratory immunity.

A possible equivalent of Waldeyer's ring has been described in the nasopharynx of the rat (Spit *et al.*, 1989), hamster (Kuper *et al.*, 1992) and mouse (Asanuma *et al.*, 1995; Belai *et al.*, 1977; Heritage *et al.*, 1997; Ichimiya *et al.*, 1991; Reuman *et al.*, 1989; Wu *et al.*, 1996) appearing as paired lymphoid accumulations, termed the NALT, near the entrance of the nasopharyngeal duct (Chapter 5, Heritage *et al.*, 1997). In rats, the NALT has been shown to drain preferentially to the pCLN (Proctor *et al.*, 1973) which elicits secretory and systemic immunity (Kuper *et al.*, 1992). Structural and functional evaluation of NALT has been challenging, largely due to difficulties in accessing its anatomic location. However, rapid and precise NALT isolation methods have been developed recently (Chapter 5, Heritage *et al.*, 1997; Wu *et al.*, 1996) and these studies revealed that although NALT cells responded to a potent mucosal antigen (CT), the frequencies and ratios of NALT cells were distinct from those noted in PP, a well characterized MALT. However, despite its unique phenotype among mucosal and systemic lymphoid tissues, NALT cells expressed both IgA and IgG responses following i.n. antigen administration (Chapter 5, Heritage *et al.*, 1997; Wu *et al.*, 1996).

The nature of antigens administered to the nasal mucosa and NALT influences the resulting specific immune responses. Although inhaled particulates impacting into the mucous layer of the nasal mucosa (Proctor *et al.*, 1973) might be cleared rapidly by ciliary action, particulates are also selectively delivered into NALT

via M cell transcytosis (Kuper *et al.*, 1992). Particulate antigens given i.n. could initiate the migration of NALT-derived lymphocytes to mucosal and systemic sites via the pCLN (Koornstra *et al.*, 1991). In contrast, soluble antigens penetrate the entire nasal epithelium (Kuper *et al.*, 1992), reaching the sCLN which, in turn, drain to the pCLN (Koornstra *et al.*, 1991; Tilney, 1971). Unlike antigens directly stimulating the pCLN, those stimulating the sCLN, given i.n., have not been shown to stimulate secretory immunity (Kuper *et al.*, 1992). Thus, particulate antigens are most efficacious in provoking mucosal and systemic immunity following i.n. administration.

Biodegradable MPs enhance and prolong mucosal and systemic antibody responses following oral immunization (Challacombe *et al.*, 1992; Eldridge *et al.*, 1990; Heritage *et al.*, 1996; Marx *et al.*, 1993; Moldoveanu *et al.*, 1993), presumably by temporarily protecting microentrapped antigens from degradation (Moldoveanu *et al.*, 1993) and facilitating M-cell uptake and transport into PP (Eldridge *et al.*, 1990; Ermak *et al.*, 1995; Frey *et al.*, 1996; Jani *et al.*, 1992; LeFevre *et al.*, 1985; Pappo & Ermak, 1989). Although i.n. administered antigens are probably degraded minimally by respiratory tract secretions, microentrapped antigens might be preferentially transported by M-cells overlying the NALT, establishing an antigen depot to induce systemic and secretory immunity via the pCLN. It was previously demonstrated that HSA-containing, biocompatible starch MPs grafted with the silicone polymer TS-PDMS stimulated both secretory and circulating antigen-

specific humoral immunity following i.g. immunization (Chapter 3, Heritage *et al.*, 1996), via PP lymphocyte activation (Chapter 4, Heritage *et al.*, 1998). In the present study, it was investigated whether HSA-containing TS-PDMS-grafted MPs, given i.n., could also induce specific mucosal and systemic humoral immunity. The results showed that i.n. immunization with low doses of microentrapped, but not soluble, HSA activated lymphocytes preferentially in the NALT and pCLN. However, unlike i.g. MP administration (Chapter 3, Heritage *et al.*, 1996), animals given HSA-containing MPs i.n. elicited strong systemic, but not mucosal, antibody responses, suggesting a unique role for NALT in the murine immune system.

6.3 MATERIALS AND METHODS

6.3.1 Microparticles

HSA (Fraction V; Sigma, St. Louis, MO) was incorporated into TS-PDMS-grafted MPs using an emulsion-based process (Chapter 3, Heritage *et al.*, 1996). These MPs routinely contained 5-6% w/w protein.

6.3.2 Immunizations

Groups of 10 female BALB/c mice, age 6-8 wk (Harlen Sprague Dawley Inc., Indianapolis, IN) were immunized i.n. on days 0, 7 and 14 with 10 µg of soluble or microentrapped HSA in 10 µl of PBS, pH 7.2, or vehicle alone distributed between the 2 nares.

6.3.3 Collection and Preparation of Sera and Cells

Individual blood samples were obtained via the retro-orbital plexus. For sera evaluations, insoluble material was removed by centrifugation and sera were stored at -70°C until used.

Pooled pCLN, sCLN and SPL were placed into ice-cold HBSS. SPL, pCLN and sCLN cell suspensions were prepared by crushing the tissues between the frosted ends of 2 microscope slides. Carcases were decapitated and pooled NALTs were isolated as previously described (Chapter 5, Heritage *et al.*, 1997). Single cell NALT, pCLN, sCLN and SPL suspensions were washed twice with HBSS by centrifugation. Erythrocytes and dead cells were removed using Ficoll-Paque (Fotino *et al.*, 1971) and lymphocytes were resuspended in RPMI-1640 media supplemented with 5% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine and 1% HEPES. Cell viabilities routinely exceeded 90% as judged by ethidium bromide/fluorescein diacetate staining (Edidin, 1970; Mohr *et al.*, 1980).

6.3.4 Measurement of Sera HSA-specific Antibody Responses

An ELISA was used to measure HSA-specific antibodies in sera samples (Chapter 3, Heritage *et al.*, 1996). Briefly, duplicate serial dilutions of sera were incubated on HSA-coated microtitre plate wells. HSA-specific antibodies were

quantitated by OD measurements (405 nm) using heavy chain-specific, AP-conjugated goat anti-mouse IgG, IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL). Sera prepared from buffer-treated animals were used to establish baseline OD values. The results were expressed as mean reciprocal end-point titres (± SEM) representing the greatest sera dilutions giving OD values exceeding 2 times buffer alone mean values.

6.3.5 Lymphocyte Proliferative Assay

Duplicate NALT cell cultures (5 x 10^5 cells/well) were prepared in 96-well round-bottom, sterile plates in the presence or absence of HSA (250-1000 µg/mL) for 96 h at 37°C with 5% CO_2 in air. One µCi of ^{3}[H]Tdr (740.0 GBq/mmol; Dupont/NEN, Mississauga, ON) was added to each well for the final 24 h of culture. Cells were harvested with a PhD Harvester (Cambridge, MA) and [^{3}H]Tdr incorporation was measured by standard liquid scintillation counting methods. The results were expressed as mean cpm (± SEM) from duplicate cultures.

6.3.6 Enumeration of IL-4 and IFN-γ-secreting Cells

An ELISPOT was used to detect cytokines released by pooled NALT, pCLN, sCLN and SPL cells *in vitro*. To detect IL-4-secreting cells, duplicate serial dilutions of single cell suspensions (beginning at 1 x 10^5 cells/100 µl/well) were examined using NC microtitre plates (Millititer HA; Millipore Corp., Bedford, MA) previously

incubated overnight at 4°C with rat anti-mouse IL-4 (clone 11B11; Pharmingen, SanDiego, CA) at 2 µg/mL in 0.1 M $NaHCO_3$, pH 8.2 (coating buffer), washed with PBS and then treated with 250 µl/well of 0.1% gelatin in PBS. After an 8 h incubation at 37°C, the wells were washed 3 times each with PBS and PBS containing 0.05% Tween-20 (PBS/Tween). The wells were incubated for 2 h with 100 µl of biotinylated rat anti-mouse IL-4 (clone BVD6-24G2; Pharmingen) diluted to 2 µg/mL in PBS/Tween containing 0.1% gelatin (dilution buffer). The wells were washed 3 times each with PBS and PBS/Tween and incubated for 30 min at 37°C with AP-conjugated streptavidin (Southern Biotechnology Associates) in dilution buffer. After washing the wells 4 times each with PBS and PBS/Tween, SFCs were visualized (Chapter 5, Heritage *et al.*, 1997). The results represent the mean number of SFCs per 1 x 10^6 cells (± SEM) from duplicate wells containing at least 2-fold more SFCs than wells containing cells from buffer-treated mice. To detect IFN-γ-specific SFCs, rat anti-mouse IFN-γ (clone R46A2; Pharmingen) (10 µg/mL) and biotinylated rat anti-mouse IFN-γ (clone XMG1.2; Pharmingen) (4 µg/mL) were similarly used.

6.3.7 Enumeration of HSA-specific Spot-forming Cells

A modification of a previously described ELISPOT (Chapter 5, Heritage *et al.*, 1997) was used to detect HSA-specific SFCs in pooled NALT, pCLN and SPL cell preparations. Briefly, duplicate serial dilutions of single cell suspensions (beginning

at 1 x 10^5 cells/100 µl/well) were examined using NC plates incubated with 100 µl/well of HSA (100 µg/mL in PBS). After incubating the wells with AP-conjugated goat anti-mouse IgG1 (heavy chain-specific; Southern Biotechnology Associates), SFCs were visualized (Chapter 5, Heritage *et al.*, 1997). The results represent the mean number of HSA-specific SFCs per 1 x 10^6 cells (± SEM) in duplicate wells containing at least 2-fold more SFCs than wells incubated with cells from buffer-treated animals.

6.3.8 Statistical Analyses

Figures and tables represent data from one of at least two independent experiments. Statistic analyses of data were done using GraphPad Prizm® Version 2.00 (GraphPad Software, SanDeigo, CA). One-way ANOVA, Tukey test pair-wise multiple comparisons and unpaired Student's *t*-tests were used to detect and compare mean differences between treatment groups at a significance level of 95%.

6.4 RESULTS

6.4.1 Intranasal Immunization with Microentrapped HSA Stimulates Sera anti-HSA Antibody Responses

It was previously shown that i.g. immunization with low doses (10-50 µg/mouse) of HSA-containing TS-PDMS-grafted MPs induced antigen-specific sera IgG and IgA responses which were statistically higher than those elicited following

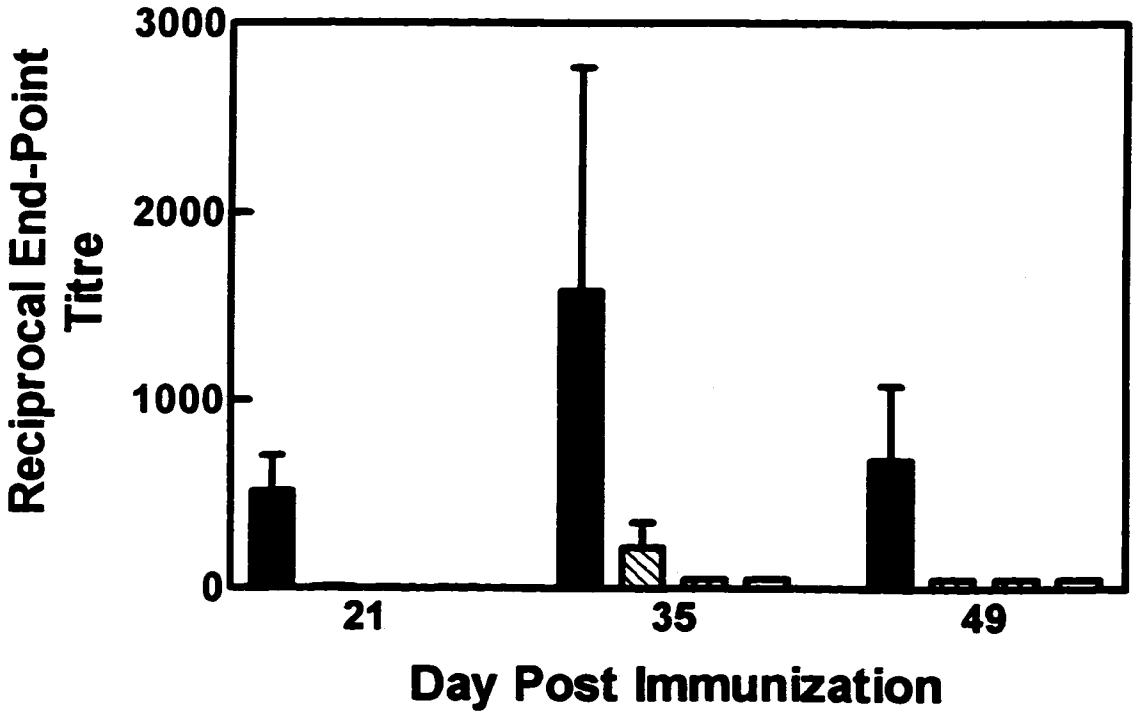
i.g. immunization with soluble HSA (Chapter 3, Heritage *et al.*, 1996). To examine the immunogenicity of microentrapped HSA administered directly to the nasal mucosae, anti-HSA sera antibody responses were measured following i.n. immunization with HSA-containing TS-PDMS-grafted MPs, soluble HSA or buffer alone. Figure 10 shows that compared to immunization with HSA alone, anti-HSA serum IgG antibody titres induced by comparable doses (10 µg/mouse) of HSA delivered i.n. via TS-PDMS-grafted MPs were significantly greater at all time points examined ($p<0.05$), with maximum responses observed on day 35. Indeed, i.n. immunization with microentrapped HSA stimulated greater levels of specific sera IgG than was previously observed after i.g. MP administration (Chapter 3, Heritage *et al.*, 1996). Unlike i.g. MP immunization (Chapter 3, Heritage *et al.*, 1996), however, mice given HSA-containing MPs i.n. did not generate detectable levels of anti-HSA sera IgA (data not shown). Additionally, i.n. MP administration did not result in detectable levels of specific IgA in nasal secretions (data not shown). Animals immunized i.n. with soluble HSA failed to produce detectable anti-HSA sera IgG and IgA or IgA in nasal secretions. Together, these representative results demonstrated that unlike soluble antigen, i.n. administration of HSA-containing TS-PDMS-grafted MPs evoked robust HSA-specific humoral IgG responses. The failure of i.n. MP administration to stimulate sera or mucosal anti-HSA IgA responses, however, supports earlier findings (Chapter 5, Heritage *et al.*, 1997) that i.n. immunization expresses unique immune responses compared to those evoked

after comparable i.g. immunization protocols.

Figure 10

Sera anti-human serum albumin (HSA) antibody responses following intranasal (i.n.) immunization with various forms of HSA. Groups of 10 mice were immunized i.n. on days 0, 7 and 14 with 10 μg of HSA incorporated in TS-PDMS-grafted microparticles (MP, solid bars), HSA in phosphate-buffered saline (PBS, hatched bars) or buffer alone (open bars). Sera obtained on days 21, 35 and 49 were evaluated for the presence of HSA-specific IgG using an ELISA. Results are expressed as mean reciprocal end-point titres (± SEM) representing the greatest sera dilutions giving optical density values exceeding twice the buffer-alone mean values.

*, significantly enhanced in MP immunized animals compared to those receiving soluble HSA or buffer; $p<0.05$.


Reciprocal End-Point Titre
3000
2000
1000
0
21
35
49
Day Post Immunization

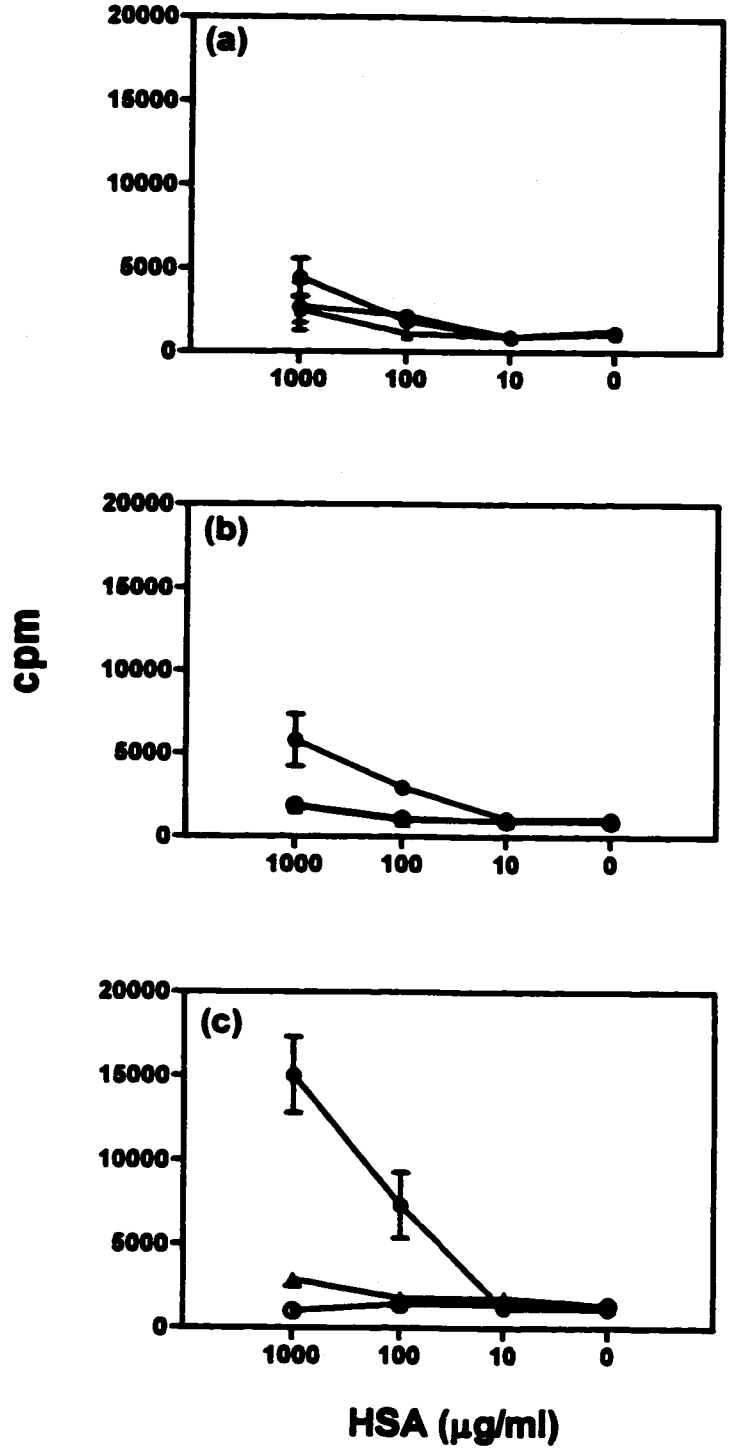
6.4.2 HSA-specific Lymphocyte Proliferation in NALT Following Intranasal Microparticle Administration

Since i.n. administered particulates localize in the NALT(Kuper *et al.*, 1992), whether the enhanced systemic humoral immune responses observed following i.n. MP immunization (Fig. 10) might have resulted from activation of HSA-specific NALT cells was examined. As seen in Figure 11a, 7 days after a single i.n. immunization with microentrapped, but not soluble HSA, modest HSA-specific NALT cell proliferation was detectable following *in vitro* restimulation with a high dose (1000 µg/mL) of HSA. The NALT cell proliferative response was enhanced 7 days after a second i.n. MP immunization (Fig. 11b), at which time MP-induced proliferation was significantly higher than that induced following i.n. immunization with soluble HSA ($p<0.05$). HSA-induced NALT cell proliferation rose to a maximum one week following a third i.n. MP administration (Fig. 11c) as judged by the efficacy of lower *in vitro* restimulatory doses of HSA correlating with higher levels of ^{3}[H]Tdr incorporation ($p<0.05$). Compared to animals given buffer alone, significant levels of HSA-specific proliferation was not detected in NALT cells from animals immunized i.n. with soluble HSA. Additionally, no antigen-specific proliferation was detected in PP cell preparations following i.n. immunization with HSA-containing grafted MPs or soluble HSA (data not shown), arguing against the possibility that circulating immune responses observed after i.n. MP administration resulted from HSA reaching and stimulating the GALT.

Figure 11

Human serum albumin (HSA)-specific proliferative responses in nasal associated lymphoid tissue (NALT) following intranasal (i.n.) immunization with various forms of HSA. Groups of 10 mice were immunized i.n. on days 0, 7 and 14 with 10 μg of HSA incorporated into TS-PDMS-grafted microparticles (MPs, closed circles), HSA in phosphate-buffered saline (PBS, closed triangles) or PBS alone (open circles). On days 7 (a), 14 (b) or 21 (c) animals were sacrificed and lymphocytes isolated from NALT were examined for [^{3}H]Tdr incorporation following *in vitro* incubation for 96 h with or without HSA (250-1000 μg/mL), with [^{3}H]Tdr added to cultures in the final 24 h. Results are expressed as the mean cpm (± SEM) of duplicate cultures.

*, significantly enhanced in MP immunized animals compared to those receiving soluble HSA or buffer; $p<0.05$.


(a)
(b)
(c)
20000
15000
10000
5000
0
1000
100
10
0
cpm
HSA (µg/ml)

6.4.3 Enhanced Spontaneous Cytokine Production by NALT and Cervical Lymph Node Cells Following Intranasal Microparticle Administration

Lymphocytes emigrate from the NALT and adjacent nasal mucosa via the CLN which drain the neck and head (Kuper *et al.*, 1992). Therefore, since particulate antigens stimulate posterior but not superficial CLN activity (Kuper *et al.*, 1992), spontaneous cytokine secretion by NALT, pCLN, sCLN and SPL cells following i.n. immunization with soluble or microentrapped HSA was compared.

Table 8 shows that 7 and 14 days following i.n. immunization with microentrapped or soluble HSA, there were 14-fold greater numbers NALT cells spontaneously secreting IL-4 from MP-immunized mice compared to animals immunized i.n. with soluble HSA ($p<0.05$). Increased numbers of IL -4 secreting cells were also detected in pCLN (7-fold), sCLN (5-fold) and SPL (5-fold) isolates from mice receiving microentrapped HSA compared to those given soluble HSA. Although IL-4 SFCs were still detectable in NALT, pCLN and sCLN cell isolates 3 wk post i.n. immunization, SPL cell cultures from mice given HSA-containing MPs i.n. contained the greatest numbers of IL-4 SFCs. Indeed, the number of spontaneously secreting IL-4 SFCs from SPL cell preparations from MP-immunized mice was significantly greater than that measured in SPL cells from mice immunized i.n. with soluble HSA ($p<0.05$).

In contrast to the appreciable numbers of IL-4 SFCs, cells spontaneously secreting IFN-γ were not detectable in any cell cultures 7 days following primary i.n.

immunization with microentrapped or soluble HSA and pCLN isolates failed to produce IFN-γ at any time interval examined in either immunization group. Small numbers of IFN-γ-secreting cells were observed in NALT, sCLN and SPL cell isolates of animals receiving MPs i.n., but never at levels significantly greater than that noted in animals immunized with soluble HSA. These results indicated that a single i.n. immunization with microentrapped, but not soluble, HSA resulted in enhanced numbers of spontaneously secreting IL-4 SFCs in NALT and, following multiple i.n. MP immunizations, there was a shift in IL-4 secretion from the mucosal (NALT) toward the systemic (SPL) compartment of the immune system.

6.4.4 Dissemination of HSA-specific Spot-forming Cells From NALT Following Intranasal Immunization with Microentrapped HSA

It was previously shown that HSA-specific sera antibody responses induced after i.g. immunization with HSA-containing MPs were dominated by IgG1 (Chapter 3, Heritage *et al.*, 1996), likely because i.g. MP administration incited high numbers GALT-situated lymphocytes secreting IL-4 (Chapter 4, Heritage *et al.*, 1998), a cytokine known to support IgG1 synthesis. Since i.n. MP administration also stimulated enhanced IL-4 secretion (Table 8), whether this cytokine microenvironment influenced the isotype of HSA-specific responses in mucosal (NALT, pCLN, sCLN) and systemic (SPL) lymphoid tissues was explored.

Figure 12 shows that 21 days post i.n. MP administration, IgG1-producing

SFCs were detected in the local lymphoid compartments. I.n. immunization with HSA-containing MPs, but not soluble HSA, resulted in appreciable numbers of IgG1-producing SFCs in the NALT (Fig. 12a) 3 wk post immunization. Over this time period there were small, but detectable numbers of specific SFCs in pCLN (Fig. 12b) and SPL (Fig. 12c), but not in sCLN cell isolates (data not shown). By 4 wk post immunization, HSA-specific IgG1 SFCs were no longer detectable in NALT cell isolate (Fig. 12a), although specific IgG1 SFCs in pCLN and SPL cell preparations (Fig 12b and 12c, respectively), were considerably elevated in MP-immunized mice. Indeed, by day 28 there were significantly higher numbers of IgG1-secreting, HSA-specific SFCs in SPL cell isolates from MP-treated mice compared to those receiving soluble HSA i.n. ($P<0.05$). HSA-specific IgA, IgG2a, IgG2b and IgG3 SFCs were not detectable in mucosal or systemic lymphoid tissues at any time interval examined (data not shown). These results suggest that i.n. administration of microentrapped HSA activated antigen-specific B cells in NALT, which subsequently migrated to the SPL via the pCLN.

In a manner consistent with these results, HSA-specific IgG1 sera responses in animals immunized i.n. with HSA-containing MPs were significantly greater than those detected in the sera of animals receiving soluble HSA ($p<0.05$) (Table 9). Indeed, anti-HSA IgG1 responses in this latter group were below the limit of ELISA detection. Additionally, i.n. immunization with either soluble or microentrapped HSA failed to incite anti-HSA IgG2a sera responses. These representative data support

the notion that i.n. immunization with microentrapped antigen induces specific systemic humoral immunity via the activation and dissemination of NALT-derived lymphocytes.

Table 8. Spontaneous secretion of IL-4 and IFN-γ by lymphocytes following intranasal (i.n.) immunization with soluble human serum albumin (HSA) or HSA entrapped in TS-PDMS-grafted microparticles (MPs).

Inoculant	Cell Source	Cytokine-specific SFCs/10^6 cells					
		Day 7		Day 14		Day 21	
		IL-4	IFN-γ	IL-4	IFN-γ	IL-4	IFN-γ
Grafted MPs	NALT	357±17*	ND	221±17*	51±17	57±38	28±9
	pCLN	170±68	ND	119±17	ND	20±20	ND
	sCLN	136±68	ND	136±68	68±0	28±9	10±10
	SPL	136±102	ND	119±17	34±34	285±57*	38±19
Soluble HSA	NALT	25±25	ND	98±98	12±12	14±14	7±7
	pCLN	ND	ND	ND	ND	ND	ND
	sCLN	ND	ND	73±25	ND	21±7	ND
	SPL	ND	ND	ND	ND	63±35	5±5

Groups of 10 mice were immunized i.n. on days 0, 7 and 14 with 10 μg of HSA incorporated into TS-PDMS-grafted MPs, HSA in phosphate-buffered saline (PBS) or buffer alone. On days 7, 14 or 21 animals were sacrificed and lymphocytes isolated from nasal associated lymphoid tissue (NALT), peripheral cervical lymph nodes (pCLN), superficial cervical lymph nodes (sCLN) and spleens (SPL) were evaluated for spontaneous IL-4 and IFN-γ secretion using an ELISPOT. The results shown represent the mean number of spot-forming cells (SFCs) per 1 x 10^6 cells (± SEM) in duplicate wells containing at least 2-fold more spot than wells incubated with cells from buffer-treated animals.

ND, no detectable IL-4 or IFN-γ SFCs.

* significantly enhanced in MP immunized animals compared to those receiving soluble HSA; $p<0.05$.

Figure 12

Appearance of human serum albumin (HSA)-specific spot-forming cells (SFCs) in nasal associated lymphoid tissues (NALT), posterior cervical lymph nodes (pCLN) and spleens (SPL) following intranasal (i.n.) immunization with various forms of HSA. Groups of 10 mice were immunized i.n. on days 0, 7 and 14 with 10 µg of HSA entrapped in TS-PDMS-grafted microparticles (MPs, solid bars), HSA in phosphate-buffered saline (PBS, hatched bars) or buffer alone. On days 14, 21 or 28 animals were sacrificed and lymphocytes isolated from NALT (a), pCLN (b) and SPL (c) were evaluated for the presence of HSA-specific IgG1 SFCs using an ELISPOT. Results represent the mean number of spots per 1 x 10^6 cells (± SEM) in duplicate wells containing at least 2-fold more spots than wells incubated with cells from buffer-treated animals. No HSA-specific SFCs were detected in cultures of superficial cervical lymph nodes (sCLN) at any time interval (data not shown).

*, significantly enhanced in MP immunized animals compared to those receiving soluble HSA; $p<0.05$.

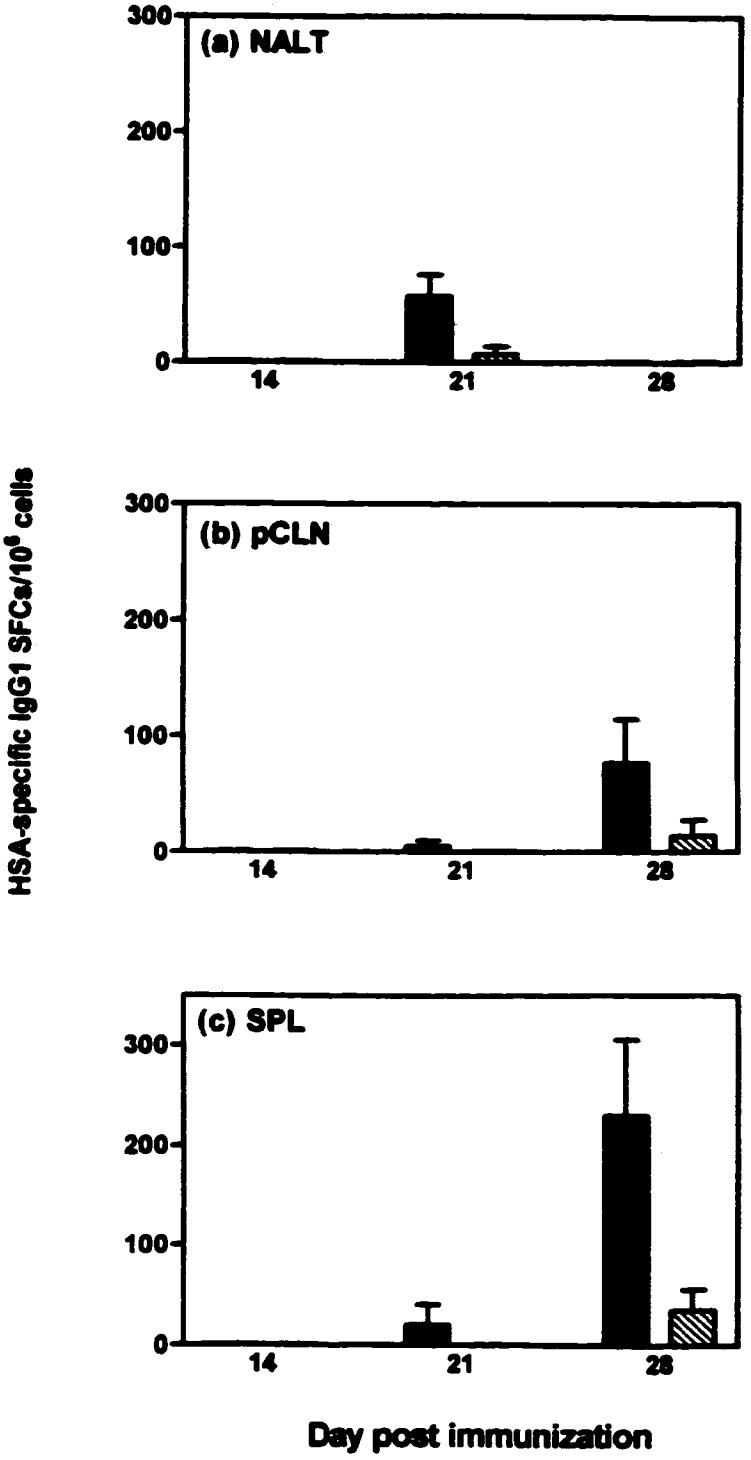

(a) NALT
(b) pCLN
(c) SPL
300
200
100
0
14
21
28
HSA-specific IgG1 SFCs/10^6 cells
Day post immunization

Table 9. Sera anti-human serum albumin (HSA) reciprocal end-point titre antibody responses elicited by intranasal (i.n.) immunization with HSA incorporated into TS-PDMS-grafted microparticles (MPs) or soluble HSA.

	HSA-specific reciprocal end-point titre (mean ± SEM)					
	Day 21		Day 35		Day 49	
Inoculant	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a
Grafted MPs	199±129	ND	780±398*	ND	2090±1183*	ND
Soluble HSA	ND	ND	ND	ND	ND	ND

Groups of 10 mice were immunized i.n. on days 0, 7 and 14 with 10 µg of HSA entrapped in TS-PDMS-grafted MPs, HSA in phosphate-buffered saline (PBS) or PBS alone (data not shown). Sera obtained on days 21, 35 and 49 were evaluated for the presence of HSA-specific IgG1 and IgG2a using an ELISA. Results are expressed as mean reciprocal end-point titres (± SEM) representing the greatest sera dilutions giving optical density values exceeding 2 times buffer alone mean values.

ND, no detectable HSA-specific serum antibody response. The limit of detection was a 1:50 dilution.

*, significantly enhanced in MP immunized animals compared to those receiving soluble HSA; $p<0.05$.

6.5 DISCUSSION

Waldeyer's ring, including the pharyngeal, palatine and lingual tonsils, is located at the juncture of the respiratory and alimentary tracts where it is bombarded by inhaled and ingested antigens. Although Waldeyer's ring shares similarities with other MALT (Scadding, 1990), knowledge of its exact function or consequences of its removal is incomplete. Recently, the NALT, a possible Waldeyer's ring equivalent found in the nasopharynx of several species (Asanuma *et al.*, 1995; Belai *et al.*, 1977; Heritage *et al.*, 1997; Ichimiya *et al.*, 1991; Kuper *et al.*, 1992; Reuman *et al.*, 1989; Spit *et al.*, 1989; Wu *et al.*, 1996), is reported to have functional similarities to Waldeyer's ring (Heritage *et al.*, 1997; Spit *et al.*, 1989) and, thus, might be a suitable model to examine the function of oronasopharyngeal lymphoid tissues.

Although description of rat NALT is considerable (Hameleers *et al.*, 1989; Koornstra *et al.*, 1991; Spit *et al.*, 1989), studies addressing the structure and immune responsiveness of mouse NALT have only been undertaken recently (Chapter 5, Heritage *et al.*, 1997; Wu *et al.*, 1996,1997). These studies suggest that mouse NALT is distinct from that found in rat (Chapter 5, Heritage *et al.*, 1997) and, compared to other mouse mucosal and systemic lymphoid tissues it contains dissimilar frequencies and ratios of cells (Chapter 5, Heritage *et al.*, 1997; Wu *et al.*, 1996). However, despite these differences, i.n. CT administration activated mouse NALT (Chapter 5, Heritage *et al.*, 1997), evoking both mucosal and systemic

responses which suggested that mouse NALT is an immunocompetant inductive site.

To further explore the capability of NALT to incite local mucosal and systemic immunity, mice were immunized i.n. with TS-PDMS-grafted MPs, an inoculant previously shown to induce robust systemic and mucosal humoral immunity following i.g. administration (Chapter 3, Heritage *et al.*, 1996; Chapter 4, Heritage *et al.*, 1998). It was suspected that since the NALT lymphoepithelium contains M cells (Kuper *et al.*, 1992), i.n. immunization with TS-PDMS-grafted MPs might evoke specific humoral immunity via the selective transcytosis of TS-PDMS-grafted MPs into NALT. In concordance with this premis, these results demonstrated that i.n. immunization with low doses of microentrapped, but not soluble, HSA evoked robust circulating IgG responses (Fig. 10). This finding indicated that TS-PDMS-grafted MPs could enhance the immunogenicity of a nasally administered soluble antigen.

These findings demonstrate that i.n. administered HSA-containing MPs induces specific immunity via NALT cell activation. Following a single i.n. immunization with microentrapped, but not soluble, HSA only NALT cells proliferated *in vitro* when restimulated with HSA (Fig. 11). This response reached a maximum after 3 i.n. MP immunizations. Coinciding with these observations, after i.n. MP administration, numerous HSA-specific SFCs were observed in NALT, and later pCLN and SPL, suggesting that the observed MP-induced specific systemic antibody responses emanated from the NALT. Additionally, HSA-specific SFCs

were not detectable in the sCLN at any time interval following i.n. MP immunization, confirming previous findings (Proctor *et al.*, 1973) showing the selective drainage of NALT cells exclusively in the pCLN after i.n. administration of a particulate antigen. In contrast, HSA-specific proliferation was not detect in PP cells following i.n. HSA administration, suggesting that specific circulating antibodies were not induced because inhaled HSA-containing MP were swallowed, activating the GALT.

Our data supports the notion that, like oral immunization (Chapter 4, Heritage *et al.*, 1998), i.n. immunization with HSA-containing TS-PDMS-grafted MPs stimulates a subset of T_H cells in NALT, hallmarked by the appearance of IL-4-secreting lymphocytes (Table 8). T_H cells are classified into two subsets depending on their cytokine profile. T_H1 cell clones exclusively produce IL-2, IFN-γ and lymphotoxin and help to generate IgG2a responses, whereas T_H2 cells synthesize IL-4, IL-5, IL-6 and IL-10 and provide help in mounting IgG1 responses (Mosmann & Coffman, 1989). Although previous studies showed that i.n. immunization with CT, or its B subunit, stimulates both T_H1 and T_H2 cells in the NALT (Chapter 5, Heritage *et al.*, 1997; Wu *et al.*, 1996) and draining lymph nodes (Wu *et al.*, 1997), the present work demonstrates that i.n. MP administration preferentially stimulates T_H2-type cells. This cytokine microenvironment was probably responsible for driving the IgG1 sera response observed after i.n. MP administration (Table 9), likely via the migration of NALT-derived IgG1-committed B cells (Fig. 12).

The representative results in this study demonstrated MP-induced B cell

activity predominantly in posterior, but not superficial CLN, following i.n. MP administration. However, specific IgA was not detectable in any lymphoid tissue examined or in nasal secretions following i.n. immunization with microentrapped HSA (data not shown). This suggests that either pCLN in mice are not intermediate in evoking sIgA responses in the nasopharynx or that TS-PDMS-grafted MPs are incapable of stimulating this arm of the murine immune system. However, since it was previously demonstrated that i.g. immunization with comparable doses of microentrapped HSA evoked specific intestinal IgA responses, the inability to detect HSA-specific IgA in nasal secretions following i.n. immunization with HSA-containing TS-PDMS-grafted MPs likely reflects differences between murine PP and NALT and not a unique inability of TS-PDMS-grafted MPs to evoke secretory immunity in the nose. Unlike the findings in this study, it was demonstrated previously that i.n. CT administration evoked both local mucosal and systemic specific antibody responses, likely due to B cell activation in the NALT (Chapter 5, Heritage *et al.*, 1997). This dichotomy implies that immunizing i.n. with highly immunogenic and adjuvant substances, such as CT, might not be suitable when attempting to delineate typical immune responses and cellular interactions in the NALT. CT is not representative of the type of antigens usually accessing the nasal mucosa and, therefore, using more relevant mucosal vaccine antigens, such as TS-PDMS-grafted MPs might be more suitable when scrutinizing the role of NALT in nasally-induced immune responses.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1 PREAMBLE

Despite recent interest in developing novel MP-based antigen delivery systems for mucosal vaccination, existing MP formulations possess a number of liabilities which potentially preclude their widespread use. In particular, the most widely examined MPs, composed of PLG, necessitates the use of harsh organic solvents and high temperatures in fabrication, thus suggesting that only antigens which are highly immunogenic and stable throughout the MP formation process could be used for PLG MP vaccine delivery (Alonso *et al.*, 1994). Furthermore, although PLG MPs have promise as a mucosal vaccine delivery system, it is not clear whether this MP formulation can reproducibly elicit mucosal humoral (Cahill *et al.*, 1995; Ray *et al.*,1993) or systemic cellular immunity (Partidos *et al.*, 1996) following mucosal immunization. Additionally, milligram amounts of costly PLG-entrapped antigens must also be repeatedly administered to mice to elicit efficacious mucosal immune responses (Challacombe *et al.*, 1992; O'Hagan *et al.*, 1993). Indeed, in a recent human clinical trial, more than 4 mg of orally-administered microentrapped antigen was required to elicit only modest immune responses (Tacket *et al.*, 1994). Nevertheless, there is good evidence to suggest that if immunogenically stable vaccine material can be incorporated into biocompatible/biodegradable MPs and delivered to various mucosal surfaces, this may be an effective way to elicit protective systemic and mucosal immunity.

In the studies described in this work, a novel MP delivery technology was

developed using starch, a well-studied, biologically acceptable and non-toxic material when given orally or parenterally (Whistler *et al.*, 1984; Laccourreye *et al.*, 1993). It was hypothesized that the hydroxyl groups of starch, which can facilitate compatibility with a wide range of protein antigens, would make it attractive as a vaccine carrier. However, despite these advantages, starch is susceptible to degradation in the gut. Thus, to deliver biologically-active components, antigen-containing starch MPs were grafted with a hydrophobic silicone which, it was thought, would protect microentrapped antigen from the deleterious environment found in the GI tract, facilitate MP uptake by M cells overlying MALT and/or act as an adjuvant or immunopotentiator.

TS-PDMS was chosen as a MP grafting agent for a number of reasons. First, TS-PDMS is similar to Simethicone®, a pharmaceutically acceptable excipient used as an anti-foaming agent in antacids (The United States Pharmacopoeia, 1994) which is stable and non-reactive in aqueous environments. However, TS-PDMS possesses reactive triethoxysilyl end groups that are capable of spontaneously coupling to the abundant hydroxyl groups on starch. By grafting antigen-containing MPs with TS-PDMS, it was postulated also that mucosally-administered soluble proteins and/or peptides would be protect from degradation, be sampled more effectively by MALT-situated M cells and stimulate robust mucosal and systemic immunity.

7.2 ASSESSMENT OF THE HYPOTHESIS

To overcome difficulties associated with existing MP formulations, a novel hydrophobic biocompatible/biodegradable MP vaccine delivery vehicle was created, capable of entrapping a wide variety of antigens under mild conditions. It was presumed (see Section 2.2) that TS-PDMS-grafted starch MPs would protect mucosally-administered, encapsulated antigens from dilution or the deleterious effects of the acidic and enzymatically hostile environments found in the respiratory and GI tracts, respectively. Moreover, antigen-containing MPs less than 10 μm in diameter should be selectively transported into mucosal inductive sites, resulting in the generation of robust antigen-specific disseminating mucosal and circulating humoral immune responses. Thus, the hypothesis stated in Section 2.2 was supported based on the findings detailed within this work.

7.3 EXAMINATION OF THE OBJECTIVES OF THE EXPERIMENTAL WORK

Upon assessment of the studies reported in this work it is clear that the objectives, detailed in Section 2.3, were met.

7.3.1 Creation of A Novel Microparticle Vaccine Delivery System

The novel silicone polymer-grafted starch MP delivery system described in this work fulfilled the criteria outlined in Section 2.3.1. Firstly, silicone oil and starch, the major components of the MP carrier system were chosen because they are

substances which are biologically and chemically well-characterized, non-toxic and non-immunogenic in biological systems. Secondly, the successful entrapment of a soluble protein antigen (HSA) into TS-PDMS-grafted MPs was demonstrated at a core loading (5-6% w/w protein) consistent with that found by others using different MP fabrication methods (Chapter 3). Additionally, MP fabrication was accomplished under mild conditions, allowing for the successful entrapment of a variety of protein and peptide antigens without any demonstrable loss in immunogenicity (Chapter 3). Grafting of starch MPs with the hydrophobic silicone polymer TS-PDMS was done to provide a barrier to gut fluids and to facilitate interaction with mucosal lymphoid tissue. Indeed, Brook *et al.* (1997) demonstrated that under acidic conditions, protein release from MPs was retarded when MPs were grafted with TS-PDMS. Furthermore, the observation TS-PDMS-grafted MPs had a diameter of 4.3 ± 3.0 μm (Chapter 3) would, theoretically, further facilitate their interaction with mucosae-associated M cells. Lastly, although protein release from TS-PDMS-grafted MPs compared to ungrafted MPs was hindered (Chapter 3, Fig. 2), microentrapped protein was still released from TS-PDMS-grafted MPs demonstrating that the MP composition allowed for entrapped proteins to readily leach from the MP matrix. Overall, these observations suggested that TS-PDMS-grafted MPs fulfilled all of the requirements of an efficacious MP-based mucosal vaccine delivery system.

7.3.2 Demonstration of Immunity Following Intragastric Microparticle Administration

To be considered an attractive alternative to current mucosal MP vaccine delivery vehicles, TS-PDMS-grafted MPs should incite both local mucosal and systemic humoral immunity following i.g. administration of low doses of microentrapped antigen (see Section 2.3.2). The studies in this report clearly demonstrated that oral immunization with very low doses of TS-PDMS-grafted MPs stimulated both systemic and mucosal humoral immune responses (Chapter 3). Furthermore, serum antibody titres were augmented after an oral (Chapter 3) or systemic (Chapter 4) booster immunization, suggesting that the initial immunization protocol stimulated systemic immunologic memory, an advantage when developing successful mucosal immunization strategies. In addition to stimulating specific serum antibody responses, i.g. immunization with TS-PDMS-grafted or ungrafted MPs resulted in specific sIgA responses in the gut (Chapter 3); this is in contrast to soluble HSA which was incapable of inciting specific intestinal immunity following i.g. administration. Indeed, the intestinal IgA antibody responses observed in these studies were comparable with or exceeded those responses demonstrated by several other laboratories. Thus, compared to soluble antigen, TS-PDMS-grafted MPs had immunopotentiating activity when delivered orally.

7.3.3 Examination of the Pathway of Microparticle-induced Immunity

Following Intragastric Immunization

As detailed in Section 2.3.3, to manipulate MP-induced immunity at mucosal surfaces, the mechanism(s) responsible for inciting antigen-specific local and systemic immunity, following oral MP administration, must be first ascertained. The studies described in this work strongly suggested that i.g. administration of TS-PDMS-grafted MPs stimulated mucosal immunity via involvement with PP. Following i.g. immunization with a low dose of HSA-containing MPs, but not soluble HSA, antigen-specific proliferation of PP cells was observed (Chapter 4). Lymphocyte proliferation was subsequently observed in MLN and splenic tissue. In contrast, antigen-specific lymphocyte proliferation was not observed in gut LP lymphocytes following i.g. immunization with MPs, suggesting that MP-induced immunity was incited by MP uptake and processing solely by PP. A shift in specific IgM SFCs from PP and MLN cell isolates to SPL tissue following i.g. MP immunization was also observed (Chapter 4). The paucity of specific IgG SFCs in PP, MLN or SPL cells shortly after i.g. MP administration suggested gradual B cell maturation in PP, resulting in isotype switching and migration of IgG-committed plasmacytes from the GALT to systemic sites. Thus, PP and possibly MLN, could serve as depots for oral administered HSA-containing MPs (Eldridge *et al.*, 1990; Jenkins *et al.*, 1994).

While robust antigen-specific systemic humoral immune responses were observed after a third i.g. immunization with relatively low amounts of

microentrapped HSA, maximal sera HSA-specific IgA and IgG responses occurred following oral boosting. Moreover, it was demonstrated also that a parenteral antigen boost could augment TS-PDMS-grafted MP-generated systemic IgG responses. Surprisingly, systemic antigen challenge failed to boost HSA-specific sera IgA titres following i.g. immunization with microentrapped or soluble antigen. These results suggested PP-stimulated IgA lymphocytes migrate to mucosal lymphoid compartments following i.g. MP administration, while antigen-specific PP-stimulated IgG plasmacytes had the propensity to migrate to both mucosal and systemic lymphoid compartments, a finding consistent with the view that mucosally-stimulated plasmacyte precursors are known to selectively localize to mucosal, but not to systemic sites (McDermott & Bienenstock, 1979).

7.3.4 Demonstration of Immunity Following Intranasal Microparticle Administration

Oral delivery of vaccines has been widely studied mainly due to the ease of vaccinating via this route and it is well accepted that PP can disseminate immune sensitized cells to mucosal and systemic effector sites. However, i.n. immunization also has a long history, having been used as a route of variolation in ancient times. More recently, this route of immunization has been intermittently used since at least the 1970s (Polly *et al.*, 1975) and has recently attracted increased attention because of its apparent greater efficiency in inducing mucosal and systemic

immune responses (Abraham, 1992; Langermann *et al.*, 1994; Tamara *et al.*, 1989; Russell & Wu, 1991; Wu & Russell, 1993; Quiding-Järbrink *et al.*, 1995) than the more conventional regimens of enteric immunization. As particulate antigens might be preferentially transported into mucosal inductive sites, following i.n. immunization, it was hypothesized that TS-PDMS-grafted MPs, given i.n., could induce enhanced mucosal and systemic humoral immune responses compared to soluble antigen alone. It was shown that i.n. immunization with low doses of microentrapped, but not soluble, HSA evoked robust circulating specific IgG responses (Chapter 6), indicating that TS-PDMS-grafted MPs could enhance the immunogenicity of an i.n. administered soluble antigen. Indeed, i.n. immunization with microentrapped HSA stimulated greater levels of specific sera IgG than was observed after i.g. MP administration with equal amounts of microentrapped antigen. However, unlike i.g. TS-PDMS-grafted MP immunization, antigen specific IgA was not detected in local mucosal secretions or sera following i.n. immunization with microentrapped antigen, suggesting that i.n. immunization expresses unique immune responses compared to those evoked after comparable i.g. immunization protocols.

7.3.5 Examination of the Pathway of Microparticle-induced Immunity Following Intranasal Immunization

Although i.n. administered antigens are probably degraded minimally by

respiratory secretions compared to soluble antigens, microentrapped antigens might be preferentially transported by M cells overlying the NALT, establishing an antigen depot to induce systemic and secretory immunity via the pCLN. However, only following the development of a precise NALT isolation technique (Chapter 5), could the pathway of i.n. administered TS-PDMS-grafted MPs be examined. It was demonstrated that i.n. immunization with low doses of microentrapped, but not soluble HSA, evoked robust circulating IgG responses via NALT cell activation (Chapter 6). Numerous antigen-specific SFCs were observed in the NALT and later the pCLN and SPL, confirming the selective drainage of NALT cells exclusively to the pCLN following i.n. administration of a particulate antigen. Although B cell activity was observed in the pCLN following i.n. MP administration, no specific IgA was detected in any lymphoid tissue examined or in nasal secretions following i.n. immunization with TS-PDMS-grafted MPs. This suggests that either pCLN in mice are not intermediate in evoking sIgA responses in the nasopharynx, in contrast to that seen in the rat (Kuper *et al.*, 1992), or that TS-PDMS-grafted MPs are incapable of stimulating this arm of the murine immune system. However, since it was previously demonstrated that i.g. immunization with comparable doses of TS-PDMS-grafted MPs evoked intestinal IgA responses, the inability to detect antigen-specific IgA in nasal secretions probably reflected differences between murine NALT and PP and not a unique inability of TS-PDMS-grafted MPs to evoke secretory immunity in the nose.

7.4 POSSIBLE MECHANISM(S) OF MICROPARTICLE-INDUCED IMMUNITY

Studies have demonstrated that M cells overlying MALT (PP, BALT and NALT) are involved in the uptake and transport of particles and, in particular, MP-uptake by PP-associated FAE has been repeatedly demonstrated. Via the oral route, MPs appear to be preferentially delivered to APCs in PP (Eldridge *et al.*, 1989), with MPs being almost exclusively delivered to the mesenteric lymph (and MLN) within these migrating APCs which can facilitate the subsequent dissemination of MPs to the systemic compartment (Eldridge *et al.*, 1989; Jenkins *et al.*, 1994; LeFevre & Joel, 1984; Wells *et al.*, 1988). Thus, if TS-PDMS-grafted MPs are to be used successfully as antigen delivery systems, selective uptake by M cells overlying MALT and their processing by underlying APCs in PP is very important.

APCs situated in the invaginated pocket at the basolateral membrane of M cells likely play an important role in antigen processing and presentation of mucosally-administered TS-PDMS-grafted MPs. Potential APCs include MHC class II-expressing B lymphocytes, macrophages and DCs which localize at M cell basolateral surfaces in response to homing signals. Several investigators have demonstrated that all of the APC phenotypes located in the M cell pocket are capable of processing and presenting a wide variety of particulates, suggesting that mucosally-administered TS-PDMS-grafted MPs might also incite mucosal immunity

via these cells. For example, Tabata & Ikada (1990) observed that macrophages can engulf a wide range of MPs. In general, regardless of the biodegradable nature of the MPs themselves, hydrophobic MPs between 1-2 μm in diameter were preferentially engulfed into phagocytic vacuoles. Phagocytic uptake of the protozoa *Giardia muris* by macrophages in the intestine has been demonstrated also by Owen *et al.* (1981). Macrophages beneath the basal LP in PP extended pseudopods into the epithelium to trap invading *G. muris* and enclosed them in phagolysosomes. Macrophage uptake of MPs was also observed by Wells *et al.* (1988) following injection of fluorescent particles (1.09 and 0.89 μm) of two different colours into separate isolated jejunal loops in dogs. Seven days after particle implantation, the MLNs draining the isolated intestinal segments were excised and examined. Nearly all MLN-situated phagocytes contained particles of only one colour, indicating that these phagocytes ingested the beads at the only site where the colours were separate (i.e. in the intestinal tract) and subsequently transported them to the draining lymph nodes. In their extensive studies on the chronic uptake of particulates into PP, LeFevre & Joel (1984) found the majority of particles were sequestered into macrophages after uptake, and these macrophages were thought to be responsible for the transport of MPs from the dome to distant regions of the PP and into the MLN. Eldridge *et al.* (1990) have also shown the uptake of MPs into PP and their subsequent transport into MLN in phagocytic cells. The fate of MPs after uptake into PP was shown to be very size dependant and peak numbers

of MPs (less than 5 μm) were detectable in the SPL 14 days post-administration. Seifert & Sass (1988) have also repeatedly demonstrated the uptake of various particulates by M cells and their subsequent transport in the mesenteric lymph of rats in macrophages. In our studies, the time delay between PP and SPL cell activation suggests also that TS-PDMS-grafted MPs or released HSA is not directly stimulating systemically-situated lymphocytes. Rather, orally-administered TS-PDMS-grafted MPs might accumulate in the GALT, resulting in sustained stimulation and eventual emigration of MP-containing macrophages and/or IgG-committed HSA-specific B lymphocytes.

Whether FAE-associated B lymphocytes in various MALT can efficiently process and present MP-associated antigens is still speculative. Kovacsovics-Bandowski *et al.* (1993) demonstrated that while macrophages could readily internalized particulate antigens (in the form of 0.5-1.5 μm OVA-linked iron beads) and present MP-associated antigens to T cells, T and B cell lines, however, could not present the particulate antigen. These studies confirmed the work of Galelli *et al.* (1993) who demonstrated also that macrophages, but not B lymphocytes, could present particulate antigens to T cells. In contrast, Vidard *et al.* (1996) found that B lymphoblastoid cell lines and LPS-activated B cells could present 4 μm polystyrene bead-associated OVA up to 10^5-fold more efficiently compared to soluble antigen. Indeed, particulate antigens could also be presented efficiently by unstimulated B cells when bound to surface Ig (Vidard et al., 1996). Presentation

of OVA-linked beads by B cells required antigen processing and 4 um MPs were clearly visible within both B cell hybridoma and lymphoblastoid cells. However, in general, macrophages presented particulate antigen 10 to1000-fold more efficiently and could also present a much wider size range of particulate antigens. The discrepancies found by these different studies might be explained in several ways. First, relative to macrophages, B cells are more restricted in the size of MP that they can present and do not generate antigenic epitopes as efficiently. Also, different B cell populations vary in their capacity to present MP antigens. Altogether, these studies suggest that PP-situated B lymphocytes may be capable of processing and presenting mucosally administered TS-PDMS-grafted MP-associated antigens albeit not as effectively as macrophages.

DCs, an APC also present in the subepithelial areas of PP, are well-documented as being able to present processed antigens in T cell dependant areas of lymph nodes and, at least in rodents, DCs are the only APC able to activate resting T cells (Metlay *et al.*, 1989) Immature DCs are usually the most active in antigen capture, including some uptake of particulates (Inaba *et al.*, 1993; Reis e Sousa *et al.*, 1993) For example, Mayrhofer *et al.*(1986) showed that during a *Salmonella* infection, specific antigens were present in cells with the morphology of DCs in lymph draining the intestine. Liu & MacPherson also demonstrated that DCs in the intestine acquire soluble antigens that are either injected directly into the intestinal lumen or administered orally and that, within a few hours, these DCs

migrate into peripheral lymph carrying antigen in a form that can be presented to sensitized T cells in an MHC class II-restricted, CD4-dependant manner (Liu & MacPherson, 1991, 1993). Thus, all of the APC phenotypes found in the invaginated pocket on the basolateral surface of M cells have been demonstrated to process and present particulate antigens and could provide a means for TS-PDMS MP-induced immunity at mucosal surfaces.

7.5 ANCILLARY FINDINGS

It is now well-established that T_H cells are of central importance in regulating mucosal immunity and, indeed, constitute the largest cellular component of the mucosal immune system. T_H cells are classified into two subsets depending on their cytokine profile. T_H1 cell clones exclusively produce IL-2, IFN-γ, and lymphotoxin and help to generate IgG2a responses, whereas T_H2 cells synthesize IL-4, IL-5, IL-6 and IL-10 and provide help in mounting IgA, IgE and IgG1 responses (Mosmann *et al.*, 1986; Mosmann & Coffman, 1989). Although Xu-Amano *et al.* (1993) demonstrated that mature PP T cells are multipotent and can become either T_H1 or T_H2 cells following polyclonal T cell activation *in vitro*, they and others (Jain *et al.*, 1996b; Xu-Amano *et al.*, 1992,1993) have demonstrated also that antigen delivery to the GALT preferentially stimulates T_H2-type cells. The results in the studies described in this report confirm and extended previous work (Xu-Amano *et al.*, 1993), demonstrating a shift of Th2 -type cytokine secretion from GALT (PP) to

systemic (SPL) lymphoid compartments following i.g. immunization with HSA-containing TS-PDMS-grafted MPs (Chapter 4). This cytokine microenvironment was likely responsible for driving the predominately IgG1 sera response observed after i.g. MP administration, which was boosted following an oral (Chapter 3) or parenteral antigen boost (Chapter 4). Although the mechanism responsible for the observed MP-induced T_H2 cell activity is unknown, recent studies suggest that both naive T cell precursors (Rincón *et al.*, 1997) and other non-T IL-4-producing cells (Moqbel *et al.*, 1995; Paul *et al.*, 1993) could be responsible for initiating differentiation of T_H2 cells from multipotent precursors.

The present data support the notion that, like oral immunization (Chapter 4), i.n. immunization with HSA-containing TS-PDMS-grafted MPs stimulates a subset of T_H cells in NALT, hallmarked by the appearance of IL-4-secreting lymphocytes (Chapter 6). Although previous studies showed that i.n. immunization with CT, or its B subunit, stimulates both T_H1 and T_H2 cells in the NALT (Chapter 5; Wu *et al.*, 1996) and draining lymph nodes (Wu *et al.*, 1997), the present work demonstrates that i.n. MP administration preferentially stimulates T_H2-type cells. This cytokine microenvironment was probably responsible for driving the IgG1 sera response observed after i.n. MP administration (Chapter 6), likely via the migration of NALT-derived IgG1-committed B cells.

7.6 MODEL OF INTERACTION BETWEEN MICROPARTICLE-ASSOCIATED

SILICONE AND PROTEIN

Based on the relative quantities of reagents used in MP fabrication, it was suspected that in silicone-grafted, antigen-containing starch MPs, the dominant interaction would be between the starch and silicone. However, it was not possible to make silicone-grafted MPs, using either PDMS or TS-PDMS, in the absence of protein (data not shown), thus demonstrating that protein (for example, HSA) was clearly playing a pivotal role in the silicone-grafting of MPs. This suggested that, of the components present in the initial emulsion, the protein was best able to stabilize the oil-water interface as proteins contain both polar and hydrophobic moieties (Brook *et al.*, 1997). Upon exposure of the aqueous droplet to acetone/silicone during MP formation, the HSA exposed at the oil-water interface might interact with the silicone polymer. Therefore, the ability to make stable MPs only in the presence of protein might be attributable to the rapid establishment of a silicone-protein interaction; the starch might not react/interact with the silicone sufficiently quickly to stabilize the particle droplet prior to aggregation. At the same time, the protein might also strongly interact with the starch, presumably through hydrophilic interactions (e.g., hydrogen bonding). Therefore, as there is no evidence that the protein leaches from the starch into the silicone phase, the protein is likely acting to adhere the silicone and starch to one another.

The model of silicone-grafted MPs proposes that TS-PDMS, compared to PDMS, forms a physically-adherent film to protein molecules which serves to better

protect MPs from biological degradation in the gut and/or facilitates MP/protein interaction with the mucosal immune system; MPs grafted with PDMS cannot form a bond between the biopolymer and silicone (Brook *et al.*, 1997). Despite this prediction, when comparing MPs fabricated with either PDMS or TS-PDMS, no obvious difference was observed between the rate of protein release from either MP type (Brook *et al.*, 1997). However, compared to ungrafted MPs, both PDMS and TS-PDMS provided a hydrophobic barrier on the surface of MPs as evidenced by the slower protein release (Chapter 3). Additionally, changes in pH also appeared to not have an effect on protein release when either PDMS or TS-PDMS was used as a grafted agent (Brook *et al.*, 1997). These results indicated that although TS-PDMS was capable of enhancing immunoresposivness to mucosally-administered microentrapped antigens, it must have done so via a unique physicochemical relationship between the protein antigen and silicone in a starch matrix which was not discernable using standard physicochemical techniques.

Although the present studies demonstrated similar *in vitro* behaviour by both PDMS- and TS-PDMS-grafted MPs, as evidenced by slower protein release (Chapter 3; Brook *et al.*, 1997), following i.g. immunization MPs grafted with PDMS were capable of stimulating only feeble systemic antigen-specific humoral immune responses (Chapter 3) and were incapable of inciting specific mucosal immunity (data not shown). In contrast, TS-PDMS-grafted MPs elicited both detectable antigen specific sIgA and circulating humoral immunity following i.g. administration

(Chapter 3). These findings suggest that, although PDMS and TS-PDMS behaved remarkably similar in physicochemical analyses (suggesting similar protein/silicone interactions in both cases), their differing biological activity indicates that if there is a protein-PDMS association, it is insufficiently strong to survive the relatively brutal biological conditions found in the GI tract; both free and silicone-associated proteins are likely degraded. Thus, PDMS might act to provide an efficient, but only temporary, hydrophobic barrier to the protein/starch MPs. Like PDMS, TS-PDMS can also form a relatively weak association between itself and protein. However, a covalent bond between the two polymers can also arise (via transesterification or, less likely, a transamination process). Alternatively or subsequently, the protein-silicone bonds can hydrolyze to give physical adhering, cross-linking silicone film. Thus, it is proposed that the TS-PDMS forms a physically-adhering film or covalent bond to the protein molecules and that the grafting of a functionalized silicone polymer to MPs creates a structure that is capable of rapidly releasing protein for processing and presentation to the mucosal immune system. Starch is likely not involved in this process as it is composed of linear and branched carbohydrate molecules and is not readily soluble in aqueous buffer below 50-60°C (Whistler *et al.*, 1984). However, the starch matrix might provide a physical structure on which protein/silicone interactions can occur and/or might serve to trap protein prior to its interaction with silicone. Whatever its function, the observation that ungrafted MP could enhance specific systemic and mucosal humoral immunity following i.g.

administration, compared to soluble antigen, suggests that the observable adjuvant effect of this novel MP formulation likely also involves a depot effect afforded by the starch MP formulation alone.

7.7 MURINE NALT AS A MUCOSAL INDUCTIVE SITE

Although Waldeyer's ring likely plays an important role in local immunity in the upper respiratory tract, the precise role of this tissue in local and systemic immunity is not known. Studies of Waldeyer's ring have been largely restricted to humans and, until recently, functional equivalents had not been described in suitable experimental animal models. However, a putative Waldeyer's ring equivalent, termed NALT, has been identified in rats (Hameleers *et al.*, 1989; Koornstra *et al.*, 1992; Van der Ven *et al.*, 1993) and effective immunization of rodents by the i.n. route is considered to be the result of the stimulation of NALT. However, although NALT is considered to be the equivalent of Waldeyer's ring in humans, the exact process of generating immune responses when NALT is exposed to antigen is not clear because cells from this tissue could not be isolated homogeneously. Thus, a rapid and precise method for isolate NALT from mice to study its immune function and cell populations was developed and reported in this work (Chapter 5).

Lymphoid cell populations in the murine NALT were found to be different from those observed in PP. NALT contained a higher proportion of T cells and

lower proportion of B cells relative to PP (Chapter 5; Wu *et al.*, 1996). Indeed, the CD4:CD8 ratio in NALT cell preparations was less than that observed in PP and more closely resembled that seen in SPL. Of the CD4+ T cells, NALT included more CD45RB^{hi} and fewer CD45RB^{lo} cells compared to PP, indicating that NALT T_H cells contained more naive and fewer memory cells than PP T_H cells (Wu *et al.*, 1996). This may be the result of a smaller antigen load in the nasal cavities than in the gut. Additionally, unlike PP, no CD8αα+ T cells were observed in NALT and NALT contained increased frequencies of CD4+ and/or CD8αβ+ T cells (Chapter 5). Additionally the total B cell frequency in NALT cell isolates was 20% lower than that observed in PP (Chapter 5). Also, although NALT and PP cell isolates contained both mature B cells and cells undergoing activation to express mIgA, unlike PP, NALT showed no significant frequency of IgA-switched cells. Thus, NALT might not be as active as PP if viewed in terms of the differentiation and expansion of IgA-committed B cell populations but, nonetheless, contains all of the cell phenotypes required of a mucosal inductive site.

Despite observations that "normal" murine NALT may not be as active as PP (Chapter 5; Wu *et al.*, 1997), it has been clearly demonstrated that murine NALT cells can respond to antigenic stimulation (Chapters 5 and 6; Wu *et al.*, 1996, 1997). After i.n. CT administration, antigen-specific SFCs of all isotypes appeared in the NALT (Chapter 5; Wu *et al.*, 1996), but IgA-secreting cells were particularly elevated. These results indicated that isotype switching, differentiation and

maturation of B lymphocytes might have occurred in the NALT on exposure to antigen. Additionally, the numbers of specific IgA SFCs correlated with CT-specific IgA in nasal, but not gut washes or sera (Chapter 5), thus suggesting local nasal production of antigen-specific mucosal antibodies. However, there was no evidence of anti-CT AFCs in NALT or CT-specific antibodies in nasal washes following i.g. CT administration. These results support the notion that nasal mucosal antibody production is best achieved via direct stimulation of IgA-committed, NALT-derived B cells.

Although antigen-specific SFCs in NALT were demonstrated to be predominantly of IgA isotype following i.n. CT administration (Chapter 5; Wu *et al.*, 1997), the systemic immune response was much enriched with the IgG class after i.n. immunization. The exact mechanism responsible for the observed elevation of specific serum antibody response is not clear, but recent studies suggested that switched IgG B cells might receive additional help after migrating out of the NALT. Indeed, studies on draining lymph nodes of NALT showed that the CLN, in particular pCLN, are locations where the augmentation of antigen-specific IgG responses might occur (Chapter 6; Wu *et al.*, 1997). IFN-γ and/or IL-4 have been detected in sCLN and/or pCLN and from crude preparations of nasally-derived CD4+ T cells (Tamura *et al.*, 1996), and these cytokines are known to favour different subclasses of IgG responses. Indeed, the CLNs might be a source of T_H1 cytokines capable of promoting antigen-specific IgG2a responses as i.n. CT administration stimulated

both IgG2a and IgG1 CT-specific antibody responses in sera while, in contrast, orally administered CT stimulated solely a IgG1 response in serum.

7.8 COMPARISON OF NALT WITH BALT

Although both NALT and BALT might both be equally important in defence of the respiratory tract against pathogenic invasion, several differences exist between these two MALT, suggesting that they have divergent functions and importance in the defence of respiratory surfaces. First, NALT is found earlier in ontogeny than BALT (Hameleers *et al.*, 1989). NALT is compartmentalized into discrete T and B cell areas that can be distinguished clearly in rats from 10 days after birth (Hameleers *et al.*, 1989), while the same degree of BALT compartmentalization is not observed until 4 weeks after birth (Plesch *et al.*, 1983). Second, it has a more activated appearance than BALT in conventionally housed, untreated rats; this is apparent in better developed follicles and marked intraepithelial infiltration by leukocytes in NALT (Kuper *et al.*, 1990, 1992). The more activated appearance and earlier development of NALT, compared to BALT, is probably due to its strategic location with respect to incoming air and suggests that the upper respiratory tract is stimulated earlier than the lower respiratory tract. Additionally, since the frequency of BALT varies greatly between species, and is not found in the normal adult lung in humans (Pabst, 1992; Pabst and Gehrke, 1990), NALT (or equivalent oronasopharyngeal lymphoid structures in other species) might

play a pivotal role in local defence of the oronasopharynx.

7.9 COMPARISON OF NALT WITH PEYER'S PATCHES

Although both NALT and PP appear to be important to the defence of mucosal sites, they differ in several aspects. With regards to ontogeny, PP appear shortly before birth, earlier than NALT (Hameleers *et al.*, 1989). This may reflect a more central role for PP in the mucosal immune system or may simply result from the earlier exposure of the gut to foreign material. The ontogeny of these MALT components, however, suggest that NALT and PP are the main components in the defence of the respiratory tract and gut, respectively.

Although NALT and PP both appear to be important to the defence of mucosal sites, their functions may differ. PP may be the central tissue in the induction of secretory Ig synthesis, while NALT appears to be more involved in cellular and/or systemic humoral immunity. Specifically, several lines of evidence suggest that murine NALT might play a more important role than PP in inducing systemic immunity. First, If viewed solely on macrophage and T cell frequencies and phenotypes, murine NALT cell populations most closely resemble systemic lymphoid compartments (SPL) rather than those found in PP preparations (Chapter 5). Secondly, i.n. immunization with microentrapped HSA or CT stimulated greater levels of specific sera IgG than was observed previously after i.g. administration with similar dose of antigen (Chapters 5 and 6). Furthermore, unlike i.g. MP

immunization, mice given HSA-containing MPs i.n. did not generate detectable levels of sera IgA. Additionally, i.n. MP administration did not result in detectable levels of specific IgA in nasal secretions, suggesting that i.n. immunization with MPs expresses unique immune responses compared to those evoked after comparable i.g. immunization protocols and may be better able to disseminate systemic immune cells than PP.

Since i.n. immunization can induce stronger and earlier immune responses that i.g. immunization, in many circumstances, is the i.n. route of immunization preferable to the i.g. route for mucosally-applied vaccines? The choice of route to deliver mucosal vaccines may depend on where the establishment of immune memory capability to a particular pathogen is required. It is known that mucosal inductive sites can generate mucosal responses by disseminating immune activated cells to mucosal effector sites when properly stimulated by antigens, and that these respond upon restimulation and retain extended immunologic memory. However, it is not clear whether memory cells can reside in inductive sites other than the one originally stimulated by antigen, or to what extent they can respond to restimulation and generate recall responses if they migrate to mucosal-inductive sites other than the original. Several lines of evidence exist to suggest that immune cells do not migrate efficiently between inductive sites. First, few SFCs occur in palatine tonsils after following i.n. immunization in humans (Quiding-Järbrink *et al.*, 1995) and fewer T lymphocytes from NALT bind to HEVs of PP than to those of NALT in rats

(Koornstra *et al.*, 1992). Additionally, i.g. immunization was demonstrated to be less effective than i.n. immunization in protecting the trachea and lung against i.n. challenge with live *B. pertussis* or RSV (Kanesaki *et al.*, 1991; Shahin *et al.*, 1992). Although, direct evidence of interaction between mucosal-inductive sites is currently unavailable, mice are now adaptable to studying this phenomena where two major mucosal inductive sites, NALT and PP, are now equally accessible. Information collected from NALT and PP comparisons, in a variety of infectious models, should be helpful in determining the optimal route for a mucosal vaccine against a particular pathogen and will provide evidence and explanations for the compartmentalization of the mucosal immune system.

7.10 CONCLUDING REMARKS

MP delivery systems for oral vaccine administration are receiving considerable attention. To this end, this work describes the development of a novel polymer-grafted starch MP system which is capable of entrapping a wide variety of soluble antigens under mild conditions and which can elicit efficacious immune responses both orally and nasally, via MALT activation. The adjuvanticity of TS-PDMS-grafted MPs arises via a unique physiochemical relationship which occurs between protein antigen and silicone in a starch matrix. This leads to a predominantly T_H2-type immune response following mucosal MP administration of relatively small amounts of microentrapped antigen, resulting in enhanced systemic

and local mucosal humoral immune responses. However, despite the efficacy of mucosally-administered TS-PDMS-grafted MPs in inciting local and disseminating antigen-specific humoral immunity, it is unclear whether this novel MP vaccine delivery system can stimulate immunized animals also for specific CTL responses as has been demonstrated by several investigators who utilized PLG MPs (Kovacsovics-Bandowski *et al.*, 1993; O'Hagan *et al.*, 1993; Partidos *et al.*, 1997). Indeed, demonstrating that TS-PDMS-grafted MPs could incite both humoral and cellular immunity would strengthen their use as a novel mucosal vaccine delivery system.

Although the novel MP system described in this work may be advantageous for the delivery of small quantities of antigen nasally, whether TS-PDMS-grafted MPs are feasible for i.g. antigen delivery is questionable. Firstly, i.g. MP delivery consistently failed to elicit specific immunity in all inoculated animals examined. Also, following i.g. administration, the quantities of TS-PDMS-grafted MP-associated antigen required to stimulate robust immunity, although substantially less than that required with other MP formulations, is still large when compared to the animal's total body weight. Indeed, the dosage of TS-PDMS-grafted MPs given i.g. to mice would be the equivalent of a 70 kg human receiving over 150 g of MP-associated antigen orally if scaled in a linear manner. Whether, mucosal TS-PDMS-grafted MP delivery to larger species (e.g., humans and non-human primates) would require excessive MP doses remains to be determined. However, preliminary evidence in

monkeys and humans, using PLG MPs, suggests that larger species do not necessarily need to receive exorbitant amounts of MPs to elicit specific immune responses (Marx *et al.*, 1993; Tacket *et al.*, 1994; Tseng *et al.*, 1995). Studies using monkeys have demonstrated that intratracheal or i.g. administration of 100 µg of microencapsulated antigen, a dose frequently used in murine models, stimulated specific sera antibody responses (Marx *et al.*, 1993; Tseng *et al.*, 1995) and this response was protective in an infectious model (Tseng *et al.*, 1995). Moreover, Tacket *et al.* (1994) showed that i.g. immunization of human volunteers with 1 mg of microencapsulated *E. coli* pilus antigens (a dose far below that estimated by linearly scaling MP doses from murine studies) once weekly for 4 weeks stimulated local and systemic specific immunity. Indeed, volunteers with the highest antigen-specific jejunal fluid sIgA titres were among those volunteers who did not become ill following *E. coli* challenge. Thus, to determine whether TS-PDMS-grafted MPs have any practical use as a human mucosal vaccine delivery system, similar studies need to be conducted, evaluating doses of TS-PDMS-grafted MPs required to incite robust immune responses in larger animals.

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