Novel bioadhesive formulations for mucosal and parenteral delivery of vaccines

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To my family in loving memory of mother

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Novel bioadhesive formulations for mucosal and parenteral delivery of vaccines

by

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THESIS SUMMARY

In this work we have established the efficient mucosal delivery of vaccines using absorption enhancers and chitosan. In addition, the use of chitosan was shown to enhance the action of other known adjuvants, such as CTB or Quil-A. Collectively, the results presented herein indicate that chitosan has excellent potential as a mucosal adjuvant.

We have evaluated a number of absorption enhancers for their adjuvant activity *in vivo*. Polyornithine was shown to engender high serum immune responses to nasally delivered antigens, with higher molecular weight polyornithine facilitating the best results. We have demonstrated for the first time that vitamin E TPGS can act as mucosal adjuvant. Deoxycholic acid, cyclodextrins and acylcarnitines were also identified as effective mucosal adjuvants and showed enhanced immune responses to nasally delivered TT, DT and *Yersinia pestis* V and F1 antigens. Previously, none of these agents, common in their action as absorption enhancing agents, have been shown to have immunopotentiating activity for mucosal immunisation. We have successfully developed novel surface modified microspheres using chitosan as an emulsion stabiliser during the preparation of PLA microspheres. It was found that immune responses could be substantially increased, effectively exploiting the immunopotentiating characteristics of both chitosan and PLA microspheres in the same delivery vehicle. In the same study, comparison of intranasal and intramuscular routes of administration showed that with these formulations, the nasal route could be as effective as intramuscular delivery, highlighting the potential of mucosal administration for these particulate delivery systems.

Chitosan was co-administered with polymer microspheres. It was demonstrated that this strategy facilitated markedly enhanced immune responses in both magnitude and duration following intramuscular administration. We conclude that this combination shows potential for single dose administration of vaccines. In another study, we have shown that the addition of chitosan to alum adsorbed TT was able to enhance immune responses.

PLA micro/naonspheres were prepared and characterised with discreet particle size ranges. A smaller particle size was shown to facilitate higher serum IgG responses following nasal administration. A lower antigen loading was additionally identified as being preferential for the induction of immune responses in combination with the smaller particle size. This may be due to the fact that the number of particles will be increased when antigen loading is low, which may in turn facilitate a more widespread uptake of particles.

PLA lamellar particles were prepared and characterised. Adsorbed TT was evaluated for the potential to engender immune responses *in vivo*. These formulations were shown to generate effective immune responses following intramuscular administration.

Positively charged polyethylcyanoacrylate and PLA nanoparticles were designed and characterised and their potential as delivery vehicles for DNA vaccines was investigated. Successful preparation of particles with narrow size distribution and positive surface charge (imparted by the inclusion of chitosan) was achieved. In the evaluation of antibody responses to DNA encoded antigen in the presence of alum administered intranasally, discrimination between the groups was only seen following intramuscular boosting with the corresponding protein. Our study showed that DNA vaccines in the presence of either alum or Quil-A may advantageously influence priming of the immune system by a mucosal route. The potential for the combination of adjuvants, Quil-A and chitosan, to enhance antibody responses to plasmid encoded antigen co-administered with the corresponding protein antigen was shown and this is worthy of further investigation.

The findings here have identified novel adjuvants and approaches to vaccine delivery. In particular, chitosan or vitamin E TPGS are shown here to have considerable promise as non-toxic, safe mucosal adjuvants. In addition, biodegradable mucoadhesive delivery systems, surface modified with chitosan in a single step process, may have application for other uses such as drug and gene delivery.

Keywords: Vaccine delivery, chitosan, adjuvant, nasal delivery, absorption enhancers, bile salts, microspheres, cyanoacrylate, DNA vaccine, vitamin E TPGS, mucosal adjuvant.

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ABBREVATIONS

ABTS	(2,2 azino-bis) 3 –ethylbenzthia-zoline –6-sulphonic acid
ALUM	aluminium hydroxide
Ag	antigen
APCs	antigen presenting cells
BALT	bronchial-associated lymphoid tissue
BCA	bicinchoninic acid
β-gal	beta galactosidase
BSA	bovine serum albumin
CMC	carboxymethyl chitosan
CMV	cytomegalovirus
CMI	cell-mediated immunity
CT	cholera toxin
CTB	cholera toxin B subunit
CTL	cytotoxic-T-lymphocyte
DCM	dichloromethane
DT	diphtheria toxoid
DTH	delayed-type hypersensitivity
EDTA	ethylene-diamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
ELISPOT	enzyme linked immunosorbent assay
EPI	expanded programme of immunization
F1	fraction 1 antigen
FITC	fluorescein isothiocyanate
GALT	gut associated lymphoid tissue
HBsAg	hepatitis B surface antigen
HIV	human immunovirus
IFN	interferon
IFN-γ	interferon gamma
Ig	immunoglobulin
i.m.	intramuscular
i.n.	intranasal
ISCOM	immunostimulating complex
IL	interleukin
L121	pluronic L121block copolymer
LD_{50}	lethal dose required to kill50% of animals
Lf	limit of flocculation
LPS	liposaccharide
LT	lymphotoxin
MALT	mucosal associated lymphoid tissue
M cell	microfold cells
MDP	muramyl dipeptide
MHC	major histocompatability complex
Mw	molecular weight
MS	microsphere
NALT	nasal associated lymphoid tissue
NP	nuclear protein

OD	optical density
OVA	ovalbumin
O/W	oil-in-water
PBS	phosphate buffered saline
PBST	PBS containing 0.05% v/v Tween 20
PCS	photon correlation spectroscopy
pDNA	plasmid DNA
PEG	polyethylene glycol
PLA	polylactide
PLGA	polylactide-co-glycolide
PPs	peyer's patches
PVA	polyvinyl alcohol
PVP	polyvinyl pyrrolidone
Quil-A	saponin fraction
QS-21	saponin fraction
RES	reticuloendothelial system
V	recombinant V antigen
s.c.	subcutaneous
s.d.	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEB	staphylococcal enterotoxoid B
SEM	scanning electron microscopy
SIV	simian immunovirus
TB	tuberculosis
Th	T helper cells
TPGS	tocopheryl polyethylene glycol
TT	tetanus toxoid
VET	vitamin E TPGS
WHO	world health organisation
W/O	water in oil
W/O/W	water in oil in water
V/V	volume *per* volume
W/V	weight *per* volume
W/W	weight *per* weight

CHAPTER: 1

1. 0. INTRODUCTION

One of the most effective and most versatile means to combat infectious diseases is immunization, also called vaccination in honor of its first successful application. Vaccination has had the greatest impact of any medical intervention technique (Andre, 2001) with immunization programmes being potentially cost effective and able to arrest and possibly eradicate a number of infectious diseases e.g. small pox eradicated in 1980. Numerous vaccines have been and developed for a wide range of bacterial, viral and protozoan infections. However, due to a variety of factors vaccination has failed to realise its full potential. These reasons include a failure to elicit the desired immune response, toxicity, or vaccine instability. It is imperative that vaccine development continues in order to meet the challenge posed by both current and emergent diseases. This thesis presents data illustrating how vaccine efficacy can be enhanced by development of antigen delivery systems as adjuvants.

1.1. History of vaccination

In 1798 Edward Jenner published his method of preventing smallpox by inoculation with pus from cowpox (containing the virus *Vaccinia*). The science of vaccinology can be traced to the ancient Chinese who protected against smallpox by the process of variolation, in which small quantities of scabs from a lesion of an infected person were intranasally inoculated. Jenner's work was the first to be evaluated scientifically and was the first to establish the scientific precedent for using a related but less dangerous pathogen to engender immune responses that are cross protective against the more virulent pathogen. Although vaccination against smallpox was rapidly accepted throughout Europe, it took nearly 100 years before the appearance of the next vaccines: killed attenuated *Pasteurella multocida* for animals in 1880, anthrax for animals in 1881 and rabies vaccine in 1885, developed by Pasteur, bacterial cell vaccines for typhoid fever (Salmon and Smith in 1886 for animals, Pfeiffer and Kolle, and Almroth Wright

for humans in 1886), cholera by Kolle in 1896 and plague by Haffkine in 1887. This concept of weakening a pathogen (attenuation) to invoke the immune system to produce a response forms the basis of immunity elicited by the BCG (Bacilli Calmette Guerin) vaccine, first administered in 1921 and still in wide use today. The date of introduction of first generation vaccines for use in humans is shown in table 1.1 below.

Vaccine	**Year of introduction**
Small pox	1798
Rabies	1885
Plague	1897
Diphtheria	1923
Pertussis	1926
Tuberculosis	1927
Tetanus	1927
Yellow fever	1935
Injectable polio vaccine	1955
Oral polio vaccine	1962
Measles	1964
Mumps	1967
Rubella	1970
Hepatitis B	1981
Haemophilus influenzae type b	1985 §
Japanese encephalitis	1992 §
Varicella	1995 §
Lyme disease	1998 §
Rotavirus	1998 §

Table 1.1: The date of introduction of vaccines for use in humans for different diseases. § Vaccine licensed for use in United States (adapted from CDC MMWR 1999).

In the following century, the rate of new vaccine development has still been quite modest, bringing in improvements of existing vaccines and extending the number of vaccine-preventable diseases to some 25. Recent advancements in biotechnology and improved research methods, resulting in new understandings of the immunology of vaccination and the pathogenesis of diseases has expanded the application of vaccination strategies in the prevention and treatment of disease.

Vaccination against smallpox has another distinction: it was the first and so far is the only procedure that has been able to fully eliminate any disease, with the important consequences that this vaccine is no longer needed, and all smallpox-related costs, both humanitarian and financial, are saved globally. Polio, only about 50 years after the introduction of polio vaccination, is a good example of an attainable target for eradication and has received significant interest in this respect from the World Health Organization. Vaccine initiatives have resulted in virtually eliminating the virus from the Americas and in considerably reducing the worldwide prevalence of this potentially devastating pathogen.

In addition to the spectacular success of eradication of small pox, many success stories can be told of a remarkable reduction and local elimination of life-threatening diseases: tetanus, diphtheria, measles, rubella, *Haemophilus influenzae* type b. However, we have also seen that a disease believed to be well under control by a general vaccination program can resurge if the program is relaxed or circumstances otherwise change to favor the spread of infection or reduce the resistance of the population. Tetanus and diphtheria still constitute major public health problems in many areas of the world and tetanus kills 400,000 annually (Kumate 1997). The requirement for multiple injections of the currently licensed diphtheria and tetanus vaccines dictates that there is often comparatively poor coverage in countries where economic or logistical factors preclude this. For this reason, the WHO has indicated that the developments of improved immunization strategies for these diseases are a priority. In the case of diphtheria, this has been given added impetus following recent epidemics in Eastern Europe, which threaten to spread unchecked, exploiting the incomprehensive vaccine coverage that now exists in many industrialized populations.

1.2. Mucosal immunization

The development of systems for vaccination *via* mucosal routes is an essential prerequisite for the stimulation of protective immunity against the majority of enteric and mucosal pathogens of both man and animals. The majority of pathogens gain access to the body *via* the mucosal surfaces, orally, nasally or genitally. Many of these organisms, such as cholera, enterotoxigenic and enteropathogenic *E. coli*, Salmonella (particularly *S. typhi*) and many strains of *Shigellae* as well as rotavirus can cause life-threatening diarrhoea, particularly in developing countries or in areas of poor hygiene. Despite the obvious need for vaccines to confer protection at these sites, the majority of vaccines in use today is active against systemic pathogens or toxins, such as tetanus toxin, diphtheria toxin, pertussis and hepatitis B, and is given by intramuscular injection. The exception to this is the oral polio vaccine, which has been licensed for human use since 1960. While the parenteral route of vaccination is effective in the development of systemic immunity, the immunity thus generated does not generally extend to the mucosal surfaces (McGhee *et al.*, 1992). Hence in man, and many other animals, the major protective antibodies of the mucosal surfaces are derived from local synthesis and not from the systemic pool of antibodies (Mestecky *et al.*, 1997). It is widely believed that the production of these antibodies can only be generated through mucosal delivery of the antigen or vaccine. This route of vaccine delivery has added potential advantages in that it may be used for immunization against systemic infections, administration may be easier, it is potentially cheaper, and there is increased patient comfort and compliance (O'Hagan *et al.*, 1995).

Despite the need for mucosal vaccine technology and the perceived advantages of this, progress in the development of mucosal immunization has been limited. There are several reasons for the lack of success in developing a generic technology for oral vaccine delivery. Firstly, the intestine itself is designed to stop the uptake of foreign molecules such as intact vaccine antigens. Thus, the intestinal milieu is a highly proteolytic environment (essential for digestion), and the intestinal wall is almost completely impermeable to all but the smallest of molecules (which are normally the products of digestion). The existence of a 'common mucosal immune system' also allows one surface to be primed by antigen given *via* another mucosal route, so that oral immunization can be effective in protecting against infections of the respiratory and

urogenital tracts, as well as inducing passive immunity through breast-feeding. Finally, the success of the oral polio vaccine demonstrates the applicability of this route for stimulating protective immunity against systemic infections. Two distinct compartments of the immune system have been identified: i) the systemic, which comprises the bone marrow, spleen, and lymph nodes, and ii) the mucosal, which comprises lymphoid tissue associated with mucosal surfaces and external secretary glands (Mestecky *et al.*, 1997). Each compartment is associated with both humoral and cell-mediated responses. However, the qualitative nature of the immune responses induced in each compartment is different. Antibodies associated with the systemic compartment are mainly of the IgG isotype, which function to neutralize pathogens in the circulatory system. In contrast, antibodies in the mucosa are primarily secretary IgA, which function to prevent entry of the pathogen into the body *via* the mucosal surfaces (McGhee *et al.*, 1992). Systemic immunity cannot prevent entry of pathogenic organisms at mucosal surfaces. Thus, the development of an effective local immune response is essential for the prevention of most infectious diseases.

1.2.1. Mucosal immune system: anatomy

The mucosal immune system can be divided into inductive sites, where antigens are encountered, endocytosed and presented to B and T cells, and effector sites where antibody is secreted. The principal inductive sites are in the GI tract, the gut-associated lymphoid tissue (GALT), e.g., Peyer's patches (PP), appendix, mesenteric lymph nodes, small solitary lymphoid nodules and intraepithelial lymphocytes. In the respiratory tract, the bronchus-associated lymphoid tissue (BALT) and nasal-associated lymphoid tissue (NALT), e.g., palatine, lingual and nasopharyngeal tonsils, and in the genitourinary tract, the less well characterized rectal-associated lymphoid tissue of the large intestine. These highly specialized lymphoid tissues are collectively termed the mucosa-associated lymphoid tissue (MALT) (McGhee *et al.*, 1992). The most highly studied mucosal lymphoid tissue is the PP of the small intestine, and despite functional differences between different tissues, it serves as a good model for the functioning of the mucosa-associated lymphoid tissue. The PP contains a dome-shaped region highly enriched with lymphocytes, macrophages, and plasma cells. This is covered by an epithelial layer, which contains follicle-associated epithelial or microfold cells, and is

highly specialized for the uptake and transport of intact luminal antigens, such as proteins, bacteria, viruses, and small parasites, into the underlying lymphoid tissue. Beneath the dome of the PP are distinct follicles, which contain germinal centres where B-cell division occurs. Affinity maturation is known to occur at these localized sites. The majority of B cells producing secretary IgA are found here. Well-defined T cell areas are adjacent, which provide functional cytotoxic T-lymphocyte (CTL) and T helper (Th) cells to support IgA responses. Also present within the inductive sites are APCs, i.e., macrophages, dendritic cells, and B cells. Following antigenic stimulation in the inductive sites, antigen-specific B and T cells leave the PP *via* the efferent lymphatics, reach the systemic circulation *via* the thoracic duct, and hence disseminate to the effector sites. These are the lamina propria of the respiratory, GI and genitourinary tracts, and the excretory glands (lacrimal, salivary, and mammary). Under the influences of antigen, T cells and cytokines, the B cells clonally expand and become mature IgA plasma cells. The distribution pathway of cells from IgA inductive sites (e.g., BALT, GALT, NALT) to IgA effector sites (e.g., lamina propria regions of GI, genitourinary, and respiratory tracts) is termed the common mucosal immune system (see figure 1.1).

Figure 1.1: The common mucosal immune system (CMIS) with different compartments. GALT: Gut associated lymphoid tissue, NALT: nasal associated lymphoid tissue, BALT: bronchus associated lymphoid tissue, reproduced from McGhee *et al.*, 1992.

The hallmark of mucosal immunity is local production of secretory IgA antibodies which constitute more than 80% of all antibodies in MALT and which are induced, transported, and regulated by mechanisms distinct from those of the systemic response. Secretory IgA is thought to be of primary importance in host defense and acts not only to resist mucosal pathogens but also microorganisms causing systemic disease, many of which initially colonise mucosal surfaces. There appear to be three sites of IgA mediated mucosal defense. Firstly, in the lumen, where secretary IgA can neutralize viruses, bacterial toxins and enzymes and prevent viral attachment, microbial adherence and adsorption of antigen. Secondly, within epithelial cells, where dimeric IgA can bind to intracellular antigen and thirdly, within the lamina propria, where dimeric IgA can complex with antigen, and the immune complex thus formed can be transported to the

lumen. Furthermore, secretary IgA is resistant to proteolysis and has a high avidity for mucus and thus is well suited for the mucosal environment.

1.2.2. Current mucosal vaccines

Many mucosal vaccines in development are composed of live attenuated organisms. These are able to infect mucosal surfaces and are usually highly effective at inducing mucosal responses. Oral polio, *Salmonella typhi* Ty21a and *Vibrio cholerae* attenuated vaccines are currently licensed for use in humans. However, attenuated vaccines carry the risk of reversion to a virulent form, especially in immunocompromised individuals (Walker 1994, Vogel 1995). Furthermore, not all pathogens may be effectively attenuated. Synthetic or recombinant antigens are considered safer than traditional attenuated or inactivated whole pathogens (McGhee 1993). They are often poorly immunogenic and require adjuvants to enhance specific immunity. Cholera toxin (CT) and the closely related *Escherichia coli* heat-labile enterotoxin (LT) are the most commonly used mucosal adjuvants in animal models (Snider *et al.*, 1994). However, toxicity prevents their use in humans. Genetically detoxified mutants of CT or LT have been developed which appear to have little or no toxicity yet retain adjuvanticity in animal models (Pizza *et al.*, 2001), but the efficacy of these new adjuvants has yet to be shown in humans. Various vaccine delivery systems also in development are designed to keep the antigen intact until it reaches the inductive sites of the mucosal immune system including microspheres, liposomes, proteosomes, cochleates, and immunostimulating complexes (Chen 2000). Nevertheless, there is a great need to develop new approaches for mucosal immunization capable of inducing strong mucosal and systemic immune responses without associated toxicity. In addition, many of the current vaccines are not affordable in less developed countries, where they are most needed. The Children's Vaccine Initiative of the World Health Organization stated that an ideal vaccine should be affordable worldwide, heat stable, effective after a single dose, applicable to a wide range of diseases, administered by a mucosal route, and able to be given early in life (Ada 1995).

1.2.3. Routes of mucosal immunization

Many routes of mucosal immunization have been tested as potential alternatives to systemic (parenteral) delivery of vaccines. These include oral, intranasal (i.n), rectal/colonic, intravaginal/uterine and ocular routes of delivery. Exploring the potential of these routes of immunization together with the concept of the CMIS increase the options for vaccination.

1.2.3.1. Oral delivery of vaccines

Recently, it has been shown with poliovirus and rotavirus vaccines in healthy infants aged 5 to 25 weeks, that concurrent administration of two oral vaccines is feasible. While the oral route of immunization can effectively stimulate both mucosal and systemic immune responses, some non-specific barriers of effective antigen presentation exist in the gastrointestinal tract (O'Hagan *et al.*, 1995). These include the size and hydrophilicity of the antigen and the fact that such antigens have to withstand gastric acidity, intestinal enzymes, peristalsis, neutralizing adsorption onto non-digestible food components and breakdown by commensal micro flora and their metabolic by-products in the digestive tract (Nugent *et al.*, 1998). Such problems can be overcome by using suitable delivery systems. It is well known that one oral dose of certain protein antigens can also induce a state of unresponsiveness, termed oral tolerance and the absence of significant mucosal memory following oral immunization has often been noted (Kagnoff 1993). This process is highly dose dependent and at low doses, priming rather than tolerance may be induced. It has been established that a brief phase of sensitivity always precedes the development of oral tolerance. In contrast, the induction of oral tolerance can be useful to prevent and treat T cell mediated autoimmune diseases where the immune response is elicited against the body's own tissues. The ability of a vaccine to evoke oral tolerance could improve vaccine safety. For example, mucosal administration of a tolerogenic vaccine with the components of the vaccine that cause the immunopotentiating adverse effects would reduce or abolish these unwanted side effects of vaccination at the mucosal site (Ruedl *et al.*, 1996). Oral tolerance is also overcome by targeting to M-cells rather than normal eneterocytes.

1.2.3.2. Nasal delivery of vaccines

The mucosal surfaces of the respiratory tract are the initial sites of contact of pathogenic viruses and bacteria such as influenza A virus, adenovirus, *Haemophilis influenza* and *Streptococcus pneumonia*. Some of these diseases can result in major pandemics and show high morbidity and significant mortality, thus a protective mucosal response in the upper respiratory tract is critical to control or prevent further spread of the diseases caused by such pathogenic organisms (McGhee., 1993, Liang *et al.*, 2001). Numerous studies have shown that the i.n. route of immunization is more immunogenic than other mucosal routes for the induction of a local immune response in the respiratory tract and lung (Mestecky *et al.*, 1997). Intranasal immunization also facilitates immune response at distant mucosal sites (VanCott *et al.*, 1998) and has been shown to enhance the efficiency of parenteral vaccines (Treanor *et al.*, 1992). Intranasal delivery of antigens and/or vaccines can stimulate a mucosal and systemic immune response. Some non-specific barriers to effective antigen delivery exist. These include the size and/or conformation of the antigen, the transport across the epithelial membrane and mucous layer, and degradation by enzyme systems (O'Hagan and Illum 1990). Since minor changes in conformation can lead to changes in immunogenicity, antigen delivery systems must be chosen so as to prevent any potential changes in conformation that may occur on absorption across the respiratory mucosal barrier.

1.2.3.3. Rectal-colonic delivery of vaccines

The rectal route has not been as frequently exploited for vaccination as other mucosal routes. This route may avoid the acidic and proteolytic barriers of the upper gastrointestinal tract. The rectal mucosa is densely populated with lymphoid follicles and can generate immune responses to locally presented antigen in the rectal area and at distant mucosal sites of the respiratory, genital and gastrointestinal tracts (Walker 1994). Rectal administration of antigen has also been shown to stimulate systemic immune responses. For example, three doses of live vaccine *S. typhi* Ty21a administered to adult human volunteers by the rectal route engendered effective systemic immune responses (Forrest *et al.*, 1990).

1.2.3.4. Inravaginal/uterine delivery of vaccines

Intravaginal uterine delivery of vaccines has more recently been explored as a way of protecting the vagina against invading microorganisms. This is mainly because of the increased incidence of viral and bacterial sexually transmitted diseases (STD) and the efforts that are being made towards the development of a mucosal contraceptive vaccine. The immune system of the reproductive tract provides crucial protection against STD, yet it is the least understood part of the mucosal immune system with respect to the role of its immune cells, the role of CTLs, the contribution of serum-derived versus mucosally produced antigens (McGhee and Kiyono 1994). The lymphoid tissues of the female reproductive tract differ considerably from that of the gut, and possess unique immunological characteristics when compared with other mucosal tissues. For protection of the vaginal tract, it appears that both mucosal and systemic immunity have significant role to play (Hocini *et al.*, 1995). Recent studies clearly demonstrate this concept. Marxs and colleagues (Marx *et al.*, 1993) reported that macaques were protected against vaginal challenge with simian immunodeficiency virus (SIV) when immunised with formalin treated SIV in biodegradable spheres by intramuscular injection plus oral or intra tracheal administration. Mixed mucosal routes have also shown utility in vaginal/uterine and rectal immunization. This has been documented in macaque monkeys immunised with SIV gag p27 fused genetically to yeast like particles covalently linked to CT (Walker 1994).

1.2.3.5. Ocular delivery of vaccines

The ocular route of immunization has shown potential for the induction of a local secretory IgA antibody response against bacterial and viral antigens. This has been reviewed by Sullivan in detail, and the generation of immune resistance to and protection against challenge has been shown (Sullivan 1994). The magnitude of the induced response was shown to be enhanced by the use of adjuvants such as avridine (Peppard *et al.*, 1988; Nesburn *et al.*, 1994).

1.2.4. Comparative studies on mucosal routes of immunization

Comparative studies on routes of immunization have illustrated the phenomena of compartmentalization in the mucosal immune system (McGhee and Kiyono 1993). Separate mucosal inductive sites differ in their roles for the provision of the necessary B-cell precursors for secretory IgA responses, suggesting that the most effective route of immunization for the induction of a local and systemic antibody response must be sought for each specific foreign antigen. Comparative studies on the oral and i.n. routes of immunization have shown that i.n. immunization induces a stronger antibody response in serum and saliva than oral immunization, as has been demonstrated for influenza A virion and bacterial polysaccharide antigens (Wu and Russell 1997). These studies were performed using either an adjuvant or a delivery system to stimulate a mucosal immune response. It was also observed that oral immunization required a much higher dose of antigen and adjuvant than i.n. immunization. For example, the i.n. HA influenza vaccine was 100 times as effective as oral immunization for the induction of an antiviral IgA response in the respiratory washings and serum (Hirabayashi *et al.*, 1990).

1.2.5. Mucosal adjuvants

Most soluble antigens and antigens synthesized by recombinant DNA techniques stimulate a weak or poor immune response (Marinaro *et al.*, 1999). There is therefore a need to develop ways to augment the immune response generated by such antigens. Adjuvants, when administered concurrently with an antigen, can selectively and independently enhance different aspects of the antibody response, including the kinetics, duration, quantity, affinity, isotype and epitope specificity as well as the generation of neutralizing antibodies, cell-mediated responses and protection. Many substances have been studied as potential vaccine adjuvants including inorganic and organic surfactants, vesicles and water-soluble compounds. Compounds such as aluminium hydroxide, lipid A, phospholipids, alklyamines such as avridine, polymers, saponins, liposomes, ISCOMs, proteosomes, muramyl dipeptide, and cytokines have been shown to act as vaccine adjuvants by the parenteral route (Chen 2000., Gregoriadis 1994). Some of

these agents have also shown an adjuvant effect when administered *via* the mucosal route.

1.2.5.1. Bacterial enterotoxins

Cholera toxin (CT) and *E. coli* heat-labile toxin (LT) have been shown to act as potent mucosal immunogens and adjuvants (when delivered orally or intranasally). Both toxins are composed of two promoters, A and B. The B promoter of the toxin contains five identical, noncovalently associated polypeptide chains arranged in a ring-like configuration. The A promoter is composed of two polypeptide chains, A1 and A2, linked by a disulphide bond. Reduction of this bond is required for expression of enzymatic activity of CT (Snider 1995). The B chain complex mediates binding of the toxin to ganglioside GM1 expressed on the cell surface membrane of mammalian epithelial cells (Dertzbaugh and Elson 1993; Snider 1995). The adjuvant action of these enterotoxins has been demonstrated for a wide variety of common protein antigens following mucosal delivery. Oral immunization of CT admixed with protein antigen has been shown to stimulate both a systemic IgA and local IgA response to itself and the unrelated antigen. There is generally little or no immune response to oral feeding of antigen alone. The ability of CT to act as an intestinal immune adjuvant has been shown to depend on a number of factors. CT has to be administered concomitantly with and by the same route as the antigen (Freytag and Clements 1999). The dose of CT required for an adjuvant effect has been shown to vary depending on the antigen involved. A single dose of CT admixed with antigen induced a detectable amount of local IgA antibody, but repeat immunizations were required for a maximal local IgA response (Jackson *et al.*, 1993). The adjuvant effect of CT has been demonstrated repeatedly for CT admixed or conjugated with antigen. Conjugation of CT to antigen was shown to further enhance the immune response to the antigen. For example, conjugation of the food protein ovalbumin to CT or CTB converted the nature of the mucosal immune response from a suppressive to a stimulatory response (Stok *et al.*, 1994). A number of factors have to be considered in the use of CT as an adjuvant. CT generates a strong mucosal and systemic immune response to itself when co-administered with adjuvant. It has also been demonstrated that oral or intranasal immunization of some protein antigens, i.e. ovalbumin or hen egg lysozyme, admixed or conjugated with CT can induce the

production of IgE and a hypersensitive state in mice (Snider *et al.*, 1994). The induction of this hypersensitive state was shown to depend on the antigen tested and the strain of mouse used. The studies by Snider and colleagues (Snider *et al.*, 1994) also indicated that administration of antigen alone or antigen admixed with CTB failed to potentiate anaphylactic sensitivity. In this regard the use of CTB would seem a safer choice of mucosal adjuvant for human use although *in vitro* studies have indicated that CTB may have the same IgE potentiating function as CT but to lesser degree.

The heat-labile toxin of *Escherichia coli* has been shown to be quantitatively less toxic than CT (Walker 1994). LT has been used as an oral adjuvant admixed with a variety of protein and bacterial antigens. Results have shown that LT enhanced the local serum antibody response (Pizza *et al.*, 2001).

The respective B subunits of these toxins have also shown a mucosal adjuvant effect but only in the presence of trace amounts of CT. To date it has not been possible to separate the adjuvant role from the toxic effects of CT despite substantial *in vitro* and *in vivo* studies on the molecular basis of the adjuvant properties this agent. It can be concluded from these studies that enterotoxin adjuvanticity involves multiple aspects of the immune response at mucosal sites, including expansion of antigen-specific B and T cells, alteration of T cell cytokine production and changes in regulatory T cells. The adjuvant action of CT has also been shown to involve indirect effects on the immune system, i.e. CT has been reported to increase gut permeability to luminal antigens. A greater knowledge of the cellular events, i.e. the exact role of antigen presenting cells, T and B cell activation and cytokine production, following a primary mucosal immunization is necessary to further elucidate the adjuvant effect of the enterotoxins. Recent studies using gene fusion technology have shown promising results for the use of a chimeric CT molecule lacking the toxic effects of the A1 subunit as a potential mucosal adjuvant (Walsh 1993). Recent advancements in the use of a non toxic mutant of LT, LTK63 (serine-to-lysine substitution at position 63 in the A subunit) and LTR72, a mutant showing markedly reduced toxicity (alanine-to-arginine substitution at position 72 in the A subunit) have been significant as both of them are extremely active as mucosal adjuvants (Pizza *et al.*, 2001).

1.2.5.2. ISCOMS

Immunostimulating complexes (ISCOMs) were first described by Morein and coworkers (Morein *et al.*, 1984) they comprise of a mixture of the saponin Quil-A and cholesterol to form the cage-like structure of an ISCOM matrix. Proteins are incorporated in the ISCOM by hydrophobic interactions and in conjugation with the addition of phosphatidyl choline, a less rigid lipid than cholesterol. Quil-A serves as a 'built in' adjuvant making the ISCOM highly immunogenic (Sjolander and Cox 1998). The efficacy of ISCOMs in inducing antibody responses, cell-mediated immunity and protective immunity has been documented in several vaccine studies for parenteral and mucosal routes of immunization. Oral immunization of ISCOMs incorporating the protein antigen OVA have been shown to prime systemic IgG, delayed type hypersensitivity (DTH) and initiate CTL responses in addition to enhancement of local IgA responses in contrast to administration of antigen alone which is normally ineffective in these respects. These studies also showed that oral immunization with OVA ISCOMs did not prime for mucosal immunopathathology on re-exposure to the antigen (Mowat *et al.*, 1999). Intranasal immunization with ISCOMs incorporating influenza antigens have similarly shown the induction of potent antibody responses and CTLs (Brennan *et al.*, 1999). ISCOMs are able to prime both Th1 and Th2 $CD4^+$ T cell responses following subcutaneous or oral immunization (Sjolander and Cox 1998). This may be central to their potent adjuvant activities and confirms their potential for mucosal delivery. A unique feature of ISCOMs, in comparison to other adjuvants such as liposomes, is their ability to induce $CD8^+$ MHC class 1-restricted CTL responses, which has been demonstrated for the intranasal, oral and subcutaneous routes of immunization (Mohamedi *et al.*, 2001). Overall it can be concluded that mucosal delivery of ISCOMs has the potential to induce a full range of local and systemic immune responses. This contrasts with other existing mucosal adjuvants such as *Salmonella* or CT that have shown restricted Th1 and Th2 responses. More recently highly purified non-toxic saponins of Quil-A have also been identified that show potential for the induction of mucosal responses, allowing the use of saponins with optimal combination of mucosal adjuvant activity and less toxic (Kensil *et al.*, 1998).

1.2.5.3. Bacterial Lipopolysaccharide (LPS) and Lipid A

The LPS constituent of the outer membranes of gram-negative bacteria has multiple adjuvant effects on the immune system including the stimulation of macrophages and their production of cytokines, mitogenic activity for B cells, alteration of MHC class II expression on APCs, Interferon-γ production and the stimulation of cell-mediated immunity. An unusual feature of the adjuvant property of LPS is that it can be administered at a site different from the antigen (Ogawa *et al.*, 1986). Despite its many adjuvant effects it is too toxic and pyrogenic for human use. The adjuvant active component of LPS is lipid A. Many modified forms of lipid A have been prepared to find a less toxic but immunogenic derivative of this compound. Of these, monophosphoryl lipid A has shown many of the adjuvant effects of lipid A and LPS and was found to be safe for human administration (Dertzbaugh and Elson 1993).

There are few studies on the mucosal adjuvant effects of LPS and its derivatives. A mucosal adjuvant action was noted for MDP when it was incorporated in liposomes and delivered by the oral route while other studies have shown the induction of oral tolerance and mucosal hypo responsiveness to fed antigen (Childers *et al.*, 2000). These studies illustrate that the effects of LPS at mucosal surfaces are complex and require further study.

1.2.5.4. Bordetella pertussis

The principal adjuvant components of *Bordetella pertussis* are LPS and pertussis toxin (PT/pertussigen). PT, a protein exotoxin that is structurally distinct from CT and LT, binds to the surface of cells *via* sugar groups but it uses a different toxigenic mechanism than CT for the induction of an increased level of cAMP (Snider 1995). PT like LPS and lipid A can be given by a different route and at a different time to the foreign antigen and still exerts its adjuvant effects. The propensity of PT to produce adverse effects is a real concern and this limits its usefulness as an adjuvant even by mucosal routes.

1.2.5.5. Muramyl dipeptide (MDP)

Muramyl dipeptide (N-acetyl muramyl-L-ananyl-D-isoglutamine) is derived from the cell wall of Mycobacteria. It is the smallest structural component on the cell wall to have adjuvant activity and it is one of the active components of Freund's complete adjuvant. MDP and its derivatives have been shown to act as effective parenteral and mucosal adjuvants for many natural and synthetic antigens (Friedman and Warren 1984). Oral administration of MDP with particulate antigens of *Streptococcus mutans* showed an enhancement of the local IgA response that was protective in some cases against the mucosally associated disease, dental caries (Michalek *et al.*, 1983). A major drawback with the use of MDP is its untoward side effects, which prevent its use as an adjuvant in humans. Lipophilic derivatives of MDP have been synthesized and tested for mucosal adjuvanticity. Co-administration of some of these derivatives with the protein BSA has been shown to result in an enhancement of the mucosal response while others were only when incorporated with antigen into liposomes. A more recent study on the mucosal adjuvant effect of a derivative of MDP demonstrated a protective effect against lethal mucosal infection by Sendai virus and Rota virus (Fukushima *et al.*, 1996). The ultimate potential of such derivatives of MDP as mucosal adjuvants will depend upon their toxic-therapeutic ratio in humans.

1.2.5.6. Avridine

Avridine is a synthetic alkyl diamine (lipoidal amine) developed as an inducer of interferon production (Hughes *et al.*, 1992). Its adjuvant action was initially demonstrated by intradeuodenal administration of avridine with a reovirus antigen (Walker 1994). The uptake and retention of antigen were enhanced and an increase in the secretory immune response was observed.

1.2.5.7. Lysophosphatidyl glycerol

The absorption enhancer lysophosphatidyl glycerol (LPG) has been tested as a mucosal adjuvant. It was shown to be an effective adjuvant for intravaginally administered peptide antigen of the HIV-2 envelope glycoprotein gp 120 in rats (O'Hagan *et al.*,

1992). Both serum and vaginal wash IgA antibody responses were enhanced when LPG was used as a vaginal adjuvant.

1.3. Biodegradable particulate delivery systems

Biodegradable micro-and nanoparticles can act as efficient delivery systems in the context of vaccine administration (Eldridge *et al.*, 1989; Eldridge *et al.*, 1991; Eyles *et al.*, 2000). The use of such controlled release systems has the potential to reduce the frequency of vaccination required to establish long-term protection. Biodegradable micro-and nanoparticles can be classified as 'the monolithic' type with the antigen evenly dispersed throughout the polymeric matrix, or the 'reservoir type' with antigen in solution in cavities formed by the polymeric material surrounded by an outer polymer shell (Morris *et al.*, 1994).

Both natural and synthetic polymers have been used to encapsulate antigen as recently reviewed by Bowersock and Martin 1999. The ability of these particulate delivery systems to protect mucosally administered antigen and to be taken up by mucosal tissues has been studied with many vaccine candidates (Almeida *et al.*, 1993; Eyles *et al.*, 2000).

One of the most promising mucosal particulate delivery systems is formulated from poly (DL-lactide-co-glycolide) (DL-PLG). This is due to the proven biocompatibility and biodegradability of this copolymer and the history of safe use in humans (Eldridge *et al.*, 1993). Upon degradation *in vivo* the polymer undergoes non-enzymatic hydrolysis into the endogenous metabolites lactic and glycolic acids. The rate of degradation and thus antigen release is primarily influenced by the ratio of lactide to glycolide present and the size of the particle (Kofler *et al.*, 1997). Antigen is released from the particle both by diffusion and through matrix degradation. Studies in the gut have shown that tissue penetration is specific to PP and is restricted almost exclusively to particles <10 μm in diameter (Eldridge *et al.*, 1991). Time course studies on the fate of particles showed that the majority of those 0.5 μm diameter were transported through the efferent lymphatics within macrophages, while the majority of those 5 μm in diameter remained fixed in the PP. The exact means by which DL-PLG particles exert their immunopotentiating effects are uncertain but may include a depot effect, as with aluminium salt adjuvants, or the delivery of antigen directly to APCs or continuous

exposure of the foreign antigen in the host (Morris *et al.*, 1994). Studies in animals have shown the potential for mucosal delivery of antigens encapsulated in DL-PLG particles for the induction of a systemic immune responses and mucosal immune response at local and distal sites. The antigens incorporated included the toxoid staphylococcal enterotoxin B (SEB), OVA, *Bordetella pertussis* vaccine, plague subunit vaccines (V and F1 antigens) (Eldridge *et al.*, 1991; Jones *et al.*, 1995; Eyles *et al.*, 2000). In many cases a protective immune response was demonstrated (Jones *et al.*, 1996; Eyles *et al.*, 2000). Studies with influenza A virus and SIV in DL-PLG particles showed that a combination of parenteral and mucosal delivery was essential for the induction of a protective immune response (Marx *et al.*, 1993). Mucosal (oral or intratracheal) boosting of systemically primed animals has shown enhanced immune responses and provided protection against virus challenge (Israel *et al.*, 1999).

Tacket *et al.* explored the possibility of mucosal delivery of an *E. coli* antigen encapsulated in DL-PLG microspheres (Tacket *et al.*, 1994) with initial results showing that the delivery system was safe and immunogenic and protective in humans. However, one problem with the DL-PLG system is that in order to form a spherical particle the presence of organic solvents such as ethyl acetate is necessary. This process of microencapsulation works for peptide based drugs and antigens such as SEB but was found to be unsuitable for large or less stable immunogens such as enveloped viruses (Kiyono *et al.*, 1995). An alternative may be the use of a water-soluble biodegradable polymer. One such polymer, Polyphosphazene, is being researched for oral delivery of a HIV gp 120 antigen (Kiyono *et al.*, 1995). Additionally, sodium alginate has received attention as a readily available, inexpensive polymer that can be used to encapsulate a wide variety of antigens under mild conditions (Bowersock *et al.*, 1999). Other polymers that have shown an adjuvant effect when delivered by a mucosal route include polyacrylamide, poly (butyl-2-cyanoacrylate) and poly (aminoacids) (Haas *et al.*, 1996). Oral immunization of antigens adsorbed to polycrylamide of poly (alkyl cyanocrylate) particles in animals primed by the parenteral route showed a significant increase in mucosal secretory IgA relative to a soluble antigen control group (O'Hagan *et al.*, 1989; Cox and Coulter 1999). Failure to elicit a primary response was thought to be due to degradation of the antigens on the surface of the particles during passage through the gut. Studies with poly (L-lactic acid) particles demonstrated that particles

(average size 0.51-0.83 μm) delivered intranasally effectively stimulated a primary systemic response in guinea pigs (Almeida *et al.*, 1993). Other studies have demonstrated the potential for proteinoid microspheres, hydrogel microspheres and microparticles grafted in a silicone polymer for oral immunization (Haas *et al.*, 1996; Heritage *et al.*, 1998; Bowersock and Martin 1999).

Although mucosal delivery of particulate delivery systems has pronounced immunoenhancing effects, the M-cell uptake of orally administered particles is poor and most of the material is eliminated without uptake (Eldridge *et al.*, 1991). Thus, ensuring adequate uptake of particles for pulsatile release application is problematic. Another concern with the use of particles in vaccines is that their antigen release properties could theoretically present problems. Sustained release of small amounts of antigen over time could induce tolerance rather than stimulate immune responses. Furthermore, sustained or pulsatile release could potentially lead to adverse consequences if an allergic or an anaphylactic reaction occurred in association with microencapsulated vaccine. Once administered it would not be possible to remove the particle (Walker 1994). Many current approaches to vaccine delivery focus on either the production of a single-dose vaccine, or the exploitation of non-parenteral routes of administration (particularly nasal and oral routes) since both these approaches bring a number of potential advantages.

1.4. Development of single dose vaccines (SDV)

Single administration vaccines containing both priming and successive booster doses would greatly widen vaccine coverage, especially in developing countries where drop-out rates from the diphtheria, pertussis and tetanus (DPT) schedule can reach 70% in some countries (Aguado and Lambert 1992). Current combination vaccine preparations include DPT and MMR (measles, mumps and rubella). Vaccines currently available are thought to prevent about 33% of the mortality caused by infectious diseases in the 0 – 14-year age group but many existing vaccines require improvements. The wealth of new technology available may enable acellular pertussis vaccines to replace whole cell pertussis vaccine in DPT, hepatitis B and Haemophilis influenzae B to be added to DPT, and varicella to MMR. The impact that such a vaccine would have is depicted by the currently recommended WHO/EPI schedule for immunization of infants mainly in developing countries.

Single dose vaccines may also stimulate an enhanced neutralizing antibody response in comparison to multiple-dose formulations. Much of the work carried out to date for SDV has concentrated on parenteral delivery, however, for most vaccine scientists, the ultimate goal is a mucosally administered single dose vaccine for reasons described earlier. There is a wealth of published data that demonstrates the potential for mucosal SDV administrations. The most widely used approach to single dose vaccine development is the controlled- or sustained- release formulation. In particular, microspheres, liposomes, emulsions and ISCOMs, amongst other materials, have been investigated as vaccine delivery systems for both parenteral and non-parenteral routes of administration. The current status of development for single administration vaccines using controlled release technology has recently been summarized by Cleland 1999.

Vaccines based upon the biodegradable polymers of lactic and glycolic acids have been studied for several years. A controlled-release formulation could be developed to release antigen continuously or in predetermined pulses congruent with the optimum dosing schedule of the antigen. In particular, by utilizing mixtures of microspheres with differing lactide: glycolide ratios, as well as different microsphere sizes, formulations can be engineered to give discrete, pulsatile or sustained antigen (protein) release which forms the basis of single dose vaccine delivery systems (Ying *et al.*, 1995; Qiu and Zhu 2001).

1.5. Liposomes

The adjuvant activity of liposomes was initially highlighted by Allison and Gregoriadis 1974. In recent years the potential of liposomes as mucosal delivery systems and immunoadjuvants has been investigated. Liposomes are composed of a bimolecular sheet of phospholipid forming an enclosed vesicle surrounded by an aqueous solution. Liposomes may differ in their dimensions, composition (phospholipid and cholesterol content), charge and structure (multilamellar consisting of several lipid bilayers separated by thin aqueous phases or unilamellar liposomes have a single lipid bilayer) (Michalek *et al.*, 1992). In addition to immunogenicity, liposomes may have some important characteristics that are advantageous for the mucosal delivery of antigenic material (Alving 1992; Michalek *et al.*, 1992). Until recently researchers have generally accepted that a physical association between the liposome and antigen was necessary for

adjuvanticity to occur, involving antigen encapsulation into the aqueous phase of the vesicles, reconstitution of hydrophobic antigen in the liposomal membrane or adsorption onto their surface (Gregoriadis 1990; Gregoriadis 1994). Recent studies by de Hann and colleagues (de Haan *et al.,* 1995) have shown that physical association of the antigen with liposomes was not required for immune stimulation by the intranasal route. Co-administration of antigen with empty liposomes effectively stimulated significant systemic, and secretory antibody response for influenza, measles and poliovirus antigens. This has demonstrated that liposomes possess an additional adjuvant activity independent of physical association of the antigen with the liposomes. Many strategies have been employed in order to enhance the immunogenicity of liposomal antigen delivery systems. These have included the co-administration or coating with other agents such as chitosan, which was shown to have significant potential for intranasal delivery (Bramwell *et al.* 1999) and facilitate enhanced responses to mock challenge following initial oral dosing (Bramwell *et al.* 1999). Studies on the potential of liposomes as mucosal antigen delivery systems have been performed by the intranasal, oral, intragastric and rectal routes of administration. These studies demonstrated that, in general, liposomes containing encapsulated antigen induced a moderate increase in secretory IgA and in some cases this correlated with protection against disease. For antigens such as the HA and NA of influenza A virus and the antigens of *S. mutans* the immunoadjuvanticity of the liposomal formulation was sufficient to enhance the secretory immune response (Gregory *et al.,* 1986; Jackson *et al.,* 1990; de Haan *et al.,* 1995). By contrast, studies on oral and rectal administration of other antigens incorporated into liposomes have shown that co-administration of an immunoadjuvant, such as cholera toxin, muramyldipeptide, lipid A or avridine, was necessary for the induction of mucosal response (Pierce and Sacci 1984; Ogawa *et al.,* 1986; Michalek *et al.,* 1989; Zhou *et al.,* 1995).

1.6. Live recombinant bacterial vectors as antigen delivery systems

The idea of using live avirulent strains of bacteria as a mucosal antigen delivery system began with studies on the use of avirulent *Salmonella* mutants. These were used to present antigens by the oral route of administration (Curtiss *et al.,* 1989). The reasons for exploring the use of such delivery systems for mucosal immunization are three fold.

Firstly, live avirulent bacteria have the capacity to bind and to be taken up at mucosal sites of the body and thus can stimulate local and systemic immune responses. Secondly, bacterial strains can be attenuated to delete important virulent genes, creating bacterial delivery systems that are not able to revert to pathogenicity and thirdly, they can serve as carriers for recombinant protein genes or more recently, plasmid DNA encoding foreign antigens (Degreve *et al.*, 2000). Other live bacterial vectors to show potential as mucosal antigen delivery systems include live attenuated vectors of *Escherichia coli* (Kotloff *et al.*, 1992), *Bacillus Calmatte-Guerin* (BCG), an avirulent mycobacterium bovis strain (Romain *et al.*, 1993), *Shigellae* (Linde *et al.*, 1993) and *Yersinia enterocolitica* (Sory *et al.*, 1990). The use of non-pathogenic *Lactobacillus* as a delivery vector has also been explored (Pouwels *et al.*, 1996). Initial studies on oral administration of *Lactobacillus* recombinant vectors showed the induction of serum and cell mediated antibody responses, and memory to the foreign antigens expressed. This opens possibilities for the use of a safe non-pathogenic bacterial vector for mucosal delivery.

1.7. Live recombinant viral vectors as antigen delivery systems

Genes encoding immunologically active antigens have also been inserted into viruses. Viral vectors that have been studied for mucosal immunization include vaccinia, adenovirus and herpes simplex virus (Redman *et al.*, 1994; Walker 1994). Adenoviral vectors have shown particular promise for generating mucosal immune responses to many foreign antigens when administered by the intranasal and oral routes (Fooks 2000). From the studies to date, it can be concluded that mucosal vaccination with recombinant adenovirus vectors have effectively induced serum and T-cell mediated immune responses and in some cases these responses have correlated with protection. Adenovirus vectors have the advantages of stability, ease of propagation and administration. Thus making this delivery system potentially suitable for widespread vaccination. The potential of vaccinia virus vectors for mucosal delivery has been studied with antigens including the HA of influenza and respiratory syncytial virus achieving similar encouraging results (Jones *et al.*, 1986; Kanesaki *et al.*, 1991). While these studies showed the induction of protective immunity, pre-existing antibody to the viral vector can inhibit the titre and duration of antibody responses to secondary or

further immunizations. This may adversely affect the widespread use of such vectors if multiple applications are required.

1.8. Chitosan

Chitosan is a natural polysaccharide derived from crustacean shells. It is a biocompatible and slowly biodegradable polymer that has been widely used in controlled drug delivery (Rege *et al.*, 1999) and has received much interest as an effective non-toxic delivery vehicle for plasmid DNA (Roy *et al.*, 1999). Structurally, chitosan is a polymer with a glucose backbone where the alpha carbons of the monomers carry amino groups (see figure 1.2 below).

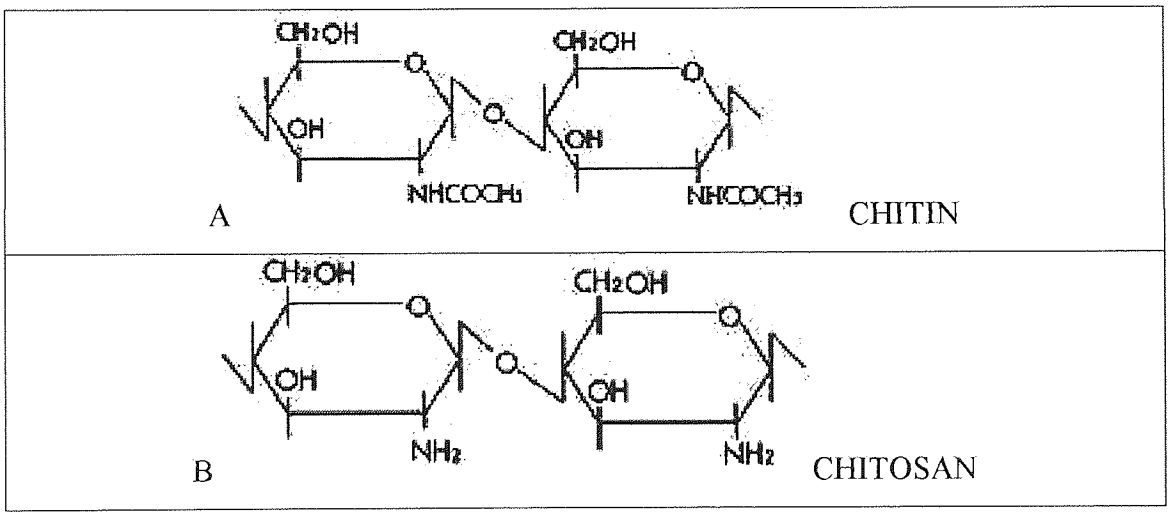


Figure 1.2: Chemical structure of chitin and chitosan.

The number of amino groups in the polymer depends on the degree of deacetylation and hence determines the charge density and related properties. Such a structure gives chitosan some unique physico-chemical properties. The amine groups are protonated at low pH (<6.5) and hence the polymer is highly cationic below physiological pH. This ensures that it can react electrostatically with negatively charged molecules or polymers (in particular plasmid DNA) under specific conditions. The availability of amine groups in the polymer is of additional advantage since it is possible to chemically attach cell targeting ligands to the polymer using simple amine cross linking (Roy *et al.*, 1999).

The apparent pKa of chitosan is 5.5. The rationale for using chitosan for the mucosal delivery of vaccines is not only the ability to complex plasmid DNA or vaccine antigens but also its mucoadhesive and transport enhancing properties in the nasal epithelium. Chitosan being a mucoadhesive polymer (Illum *et al.*, 1994), microparticles prepared in the presence of chitosan may have the potential to adhere to mucosal surfaces. *In vitro* studies show that chitosan can increase trans and paracellular transport of drugs across intestinal epithelial monolayers (Artursson *et al.*, 1994). This might also have beneficial effects on the transport of antigens and plasmid DNA across mucosal barriers (Jabbal-Gill *et al.*, 1998; Somavarapu 1998; Bacon *et al.*, 2000; van der Lubben *et al.*, 2001). Furthermore, chitosan is used, as an alternative therapy for dietary fat absorption, hence is already available, and widely used. Its safety and toxicity has been examined in animal models and in humans and chitosan has been shown to be both non-toxic and biodegradable (Ravi Kumar 2000).

1.9. DNA vaccines

Injection of naked plasmid DNA encoding an antigen results in synthesis of the protein by host cells and subsequent development of cellular and humoral immune responses against the antigen (Tang *et al.*, 1992; Ulmer *et al.*, 1993). Advances in the manipulation of the immune system utilising these DNA vaccines has provided a novel therapeutic approach which may have application in a variety of human diseases (Roy *et al.*, 1999; Lodmell *et al.*, 2000; Spack and Sorgi 2001). Recent studies have also shown that utilising intramuscular or intradermal DNA based immunization, modulation of aeroallergen induced IgE synthesis and airway hyper-responsiveness can be achieved (Hsu *et al.*, 1996). DNA vaccines are being studied extensively because of their potential advantages over conventional protein vaccines. Plasmid DNA is easier to purify in large quantities and does not involve complicated and low yield protein purification or viral manipulation. Moreover, it has been shown that DNA encoding for particular epitope of the antigen can generate $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells, advantageous for protection against certain pathogens and not effectively induced by other strategies. Furthermore, in genetic immunization, the antigen (protein) is synthesized inside the host cell and can undergo proper posttranslational modifications similar to that in viral infection. This enables the immune system to be presented with

the correct conformation of the antigen, which can also be modified to be retained intracellularly or secreted. Hence, this technology can potentially generate a more appropriate and effective immunological protection. The mechanism of DNA vaccination, in terms of the cells involved and the process of antigen presentation, is not fully understood. Several potential pathways have been proposed (Gregoriadis *et al.*, 1997; Robinson 1999) but this may be dependent upon the delivery system. Following immunization with DNA, somatic cells or probably some professional antigen presenting cells (APC) express the antigen. Cross priming may be involved, where antigen is transferred from transfected somatic cells to nearby APCs (Donnelly *et al.*, 2000) and the relevant epitopes are subsequently presented to T cells. A schematic representation of the generation of an immune response following intramuscular immunization is shown below in figure 1.3.

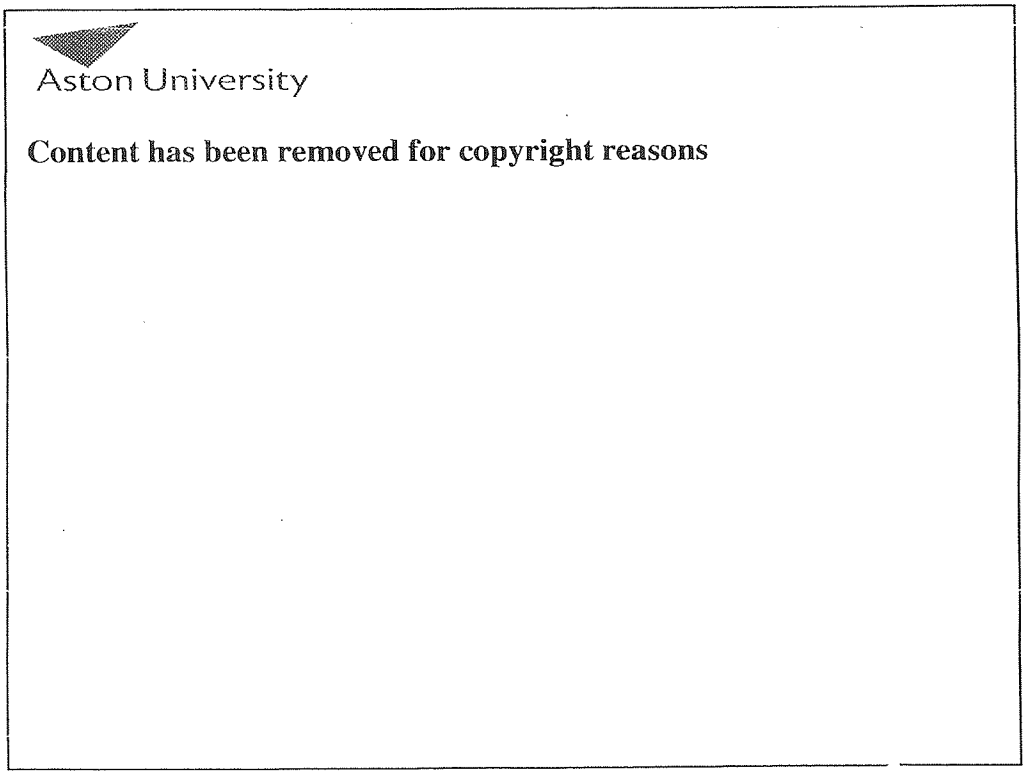


Figure 1.3: The generation of an immune response following intramuscular immunization with plasmid DNA. Taken from interactive biochemistry web site on 10/03/01 (WWW.HARCOURTCOLLEGE.COM).

Based on the route of DNA administration, the mechanism of antigen presentation and the co-stimulatory signals present, effective B cell driven antibody response and/or $CD8^+$ CTL responses are generated. It has also been shown that certain DNA sequences (e.g. CpG motifs) in the plasmid can act as adjuvants and modify and or potentiate the generated immune response (Klinman *et al.*, 1999; McCluskie *et al.*, 2001).

1.10. Aims and objectives

The overall aims of these studies were the design and characterization of safe mucosal adjuvants or delivery systems in order to facilitate effective mucosal delivery of vaccines and the improvement of immune responses to antigen delivered by other routes.

We hypothesized that positively charged and mucoadhesive PLA microspheres can be prepared using chitosan as a stabilizer during the preparation of micro/nanospheres. With this aim we proposed to study the effect of concentration of chitosan on the formation of microspheres and their physicochemical properties such as surface charge, particle size and antigen loading. We also evaluated the effect of chitosan in the preparation of PLA microspheres containing antigen following nasal delivery in mice.

It was hypothesized that the nasal uptake of particles will be influenced in the presence of chitosan and polyornithine and the particle uptake *in vivo* in the presence and absence of chitosan and polyornithine was evaluated. In conjunction with this, the effect of these agents on the *in vitro* transport of particles across Caco-2 cell monolayers was also investigated.

Microparticles and nanoparticles have been extensively studied as vaccine carriers for developing single shot vaccination and also for mucosal delivery of vaccines. To investigate the effect of particle size on immune response to nasally delivered microencapsulated vaccines, the effects of variations in the size of particles was examined.

We hypothesized that positively charged and mucoadhesive PLA microspheres can be prepared using chitosan as an emulsifier during the preparation of micro/nanospheres. With this aim we proposed to study the effect of concentration of chitosan on the formation of microspheres and their physicochemical properties such as surface charge,

particle size and antigen loading. We also evaluated the effect of chitosan in the preparation of PLA microspheres containing antigen following nasal delivery in mice.

Following the identification of chitosan as an effective mucosal adjuvant, we have undertaken a detailed study in order to investigate the factors, which influence the adjuvanticity of chitosan such as molecular weight and derivative. To this end we have tested chitosan chloride and chitosan glutamate of low and high viscosities, trimethyl chitosan of varying degrees of methylation (20, 40 and 60%). These chitosans were compared with another cationic agent, cationic starch. In addition, microspheres were prepared from chitosan base and chitosan chloride loaded with BSA as a model antigen. These formulations were evaluated following intranasal administration in the mouse model. The addition of saponin to alum-adsorbed antigens has been shown to potentiate immune responses. In order to evaluate the capability of chitosan to facilitate similar potentiating responses, alum adsorbed antigen was delivered in combination with chitosan and/or Quil-A.

The effect of formulations showing different *in vitro* antigen release profiles was evaluated *in vivo* following nasal and intramuscular administration, thus comparing route and effect of release kinetics on immune responses for single dose vaccines.

Absorption enhancers have been used widely as agents to enhance the mucosal delivery of proteins. We have proposed that these absorption enhancers may be useful for the mucosal (mainly nasal) delivery of vaccines. The rationale being that the main problem with the failure of especially subunit vaccines when delivered *via* mucosal surfaces is poor absorption of antigens. We have tested a series of absorption enhancers; bile salts, cyclodextrins, vitamin E TPGS, polyornithine, and acylcarnitine at various concentrations and molecular weights and evaluated the immune responses to nasally delivered diphtheria and tetanus toxoids, and V and F1 subunit antigens.

In order to achieve positively charged polyethylcyanoacrylate nanoparticles, the formation of polyethylcyanoacrylate nanoparticles from ethylcyanoacrylate monomer was evaluated in the presence of chitosan and this was tested as a potential carrier for DNA vaccines. The ability of alum to act as a mucosal adjuvant for DNA vaccines was also evaluated.

CHAPTER: 2

2.0. Methods and materials

2.1. Materials

2.1.1. Antigens

Ovalbumin fraction V (Mw 45 kDa), cholera toxin B subunit (Mw 11 kDa), were purchased from Sigma Chemical Company (Poole, UK). The fraction 1 (F1) and V subunit antigens of *Yersinia pestis* (Mw 17.5 kDa and 38 kDa respectively) were prepared by Dr. E.D.Williamson and her team at the Chemical and Biological Defence Establishment (Porton Down, UK). Tetanus toxoid and diphtheria toxoid were gifts from the serum institute of India, Pune, India. pRc/CMV-HBs (S) (or simply pCMV-S) expresses the hepatitis B surface antigen (small, or S, protein) under the control of the CMV immediate-early promoter and recombinant hepatitis B surface antigen subtype (HBsAg) were obtained from Aldevron, North Dakota USA. A schematic representation of this construct is shown below.

Figure 2.1: Map of HBsAg-expressing plasmid pRc/CMV-HBs (S), more commonly called pCMV-S. Expresses hepatitis B surface antigen (small, or S, protein) under the control of the CMV immediate-early promoter.The hepatitis B plasmid DNA construct used in these studies (image taken from www.aldevron.com on 10/03/01).

2.1.2. Polymers and other reagents

The polymers used in this thesis were Poly (L-lactide) 2 kDa and 100 kDa, supplied by Boehringer Ingelheim *via* Alpha Chemicals (Berkshire, UK) and also obtained from Polysciences (UK). Polyvinyl alcohol was supplied by Aldrich Chemical Co., Gillingham, Dorset, UK). All other chemicals and reagents were supplied by Sigma Chemical Company (Poole, UK) unless otherwise stated and were of Analar grade or equivalent. Double distilled water was used in all experiments. Microplates for the BCA protein assay and the enzyme-linked immunosorbant assay (ELISA) were supplied by Dynax (Dynax technologies, Billingshurst, W.Sussex, UK). Bio-rad (Heartfordshire UK) supplied all silver stain reagents, coomassie blue stain and molecular biology certified agarose. Pharmaceutical grade solvents (methanol, dichloromethane and isopropanol) were supplied by Fisher Chemicals (Loughborough UK). The HindIII DNA ladder was supplied by Helena Biosciences (Sunderland UK).

2.2. Methods

2.2.1. Preparation of microspheres

2.2.1.1. Preparation of microspheres by single emulsion method

Polylactide (PLA) microspheres were prepared by the solvent evaporation method. The preparation of single emulsion solvent evaporation has been well documented. This method has been modified from that used by Conti *et al.* (1992). The polymer was dissolved in the organic solvent dichloromethane (DCM). This organic solution was added, drop by drop, to 75ml of an aqueous solution containing various concentrations of chitosan (0.1 % w/v to 2.0 % w/v) or polyvinyl alcohol 1.5 % w/v (PVA) (PVA, Mw 13-23 kDa, 88% hydrolyzed; Aldrich Chemical Co., Gillingham, Dorset, UK) and homogenized for 8 minutes at 17,000 rpm using a Silverson homogenizer (Silverson machines, Chesham, Bucks, UK). This emulsion was continuously agitated overnight to allow the solvent to evaporate and the microspheres to harden. The hardened microspheres were collected by centrifugation at 31,000g using a JA-20 rotor on a Beckman J2-21 centrifuge (Beckman JA-20 centrifuge, Beckman Instruments Ltd., Bucks, UK). All batches were washed three times and resuspended in distilled water.

The microspheres were freeze-dried (Edwards Modulo freeze drier; BOC Ltd., Sussex, U.K) and stored in a desiccator at room temperature or at 4^0C as appropriate. In the production of protein-loaded microspheres the protein was suspended in the organic phase with the polymer. All solutions were cooled on ice for one-hour prior to use.

2.2.2. Preparation of microspheres by double emulsion solvent evaporation

Microspheres were prepared by a modified double emulsion solvent evaporation method described by Jeffery *et al.* (1993). The primary emulsion was prepared using an aqueous solution containing the protein (antigen) (1.0 –25 mg) and emulsifying agent (Chitosan or PVA) at different concentrations (0.1% w/v to 10% w/v) and the organic solvent dichloromethane (DCM) containing the polymer. The organic solution was previously cooled in an ice bath for an hour. The aqueous phase (1.5ml) was added; drop wise to the organic phase (5ml) and homogenized for 2 minutes at 17,000 rpm using a Silverson homogenizer (Silverson machines, Chesham, Bucks UK). This primary emulsion was then added drop wise to an aqueous solution of PVA or chitosan and further homogenized for 6 minutes as above to produce the final emulsion. The emulsion was continuously stirred at room temperature overnight to allow the solvent to evaporate and the microparticles to harden. The hardened microparticles were collected by centrifugation as described in section 2.2.2, washed three times in distilled water and freeze-dried.

2.3. Characterisation of microspheres

2.3.1. Particle size determination

The size of the microparticles was determined by using the Malvern Master Sizer/E. A sample (2-3mg) of microspheres was suspended in 0.2µm-filtered double distilled water and sonicated in a bath sonicator to disperse the samples before sizing. The instrument was fitted with a 45mm angle lens and a flow cell and the presentation used was for polystyrene in water (2PAD) all sizes are expressed as mean volume diameters, which are the diameter of spheres that would have the same volume as the particle.

2.3.2. Nanoparticle size determination

The size of nanoparticles was determined by photon correlation spectroscopy (PCS) (Malvern Instruments, Malvern, UK). The sample was dispersed in double distilled 0.2μm filtered water and all sizes quoted passed the minimum quality criterion advised by Malvern Instruments with regard to polydispersity, count rate, merit (signal to noise ratio) and other parameters. Values are represented as z average.

2.3.3. Morphology of microparticles

Scanning electron microscopy was used to analyze the size, morphology and surface properties of microspheres. A thin layer of microspheres was mounted onto aluminium stubs using adhesive carbon pads either as a dry powder of in a dilute suspension and dried onto the surface of the support. The samples are then coated with a thin layer (approx. 20nm) of gold under vacuum in an argon atmosphere using a sputter coater (Emscope SC500). The morphology is viewed using an electron microscope (Stereoscan 90- Cambridge Instruments). The photomicrographs of microspheres were stored electronically.

2.3.4. Surface charge of the particles

A major problem with all colloidal systems is their stability. Colloidal particles will generally be dispersed by Brownian motion. The surface properties of the particles play an important role in determining the colloidal behavior of microspheres and also on interaction with cell surfaces. The zeta potential of a particle is a measure of the surface charge on that particle and is also an indication of the likelihood of aggregation between the particles. If the zeta potential is highly negative or highly positive then the likelihood of the particles aggregating will be low because they will repel each other. A sample (approx.1mg) of microparticles was suspended in 10^{-3}M KCl to produce a dilute suspension. The electrophoretic motility of these particles in an electric field was measured using a zeta master (Malvern Instruments Ltd., Malvern, Worcs, UK). The average zeta potential (mV) was determined over five readings for each sample.

2.4. Determination of protein loading using the BCA assay

To determine the protein loaded into the microparticles the microspheres were digested to release the entrapped protein and a bichinchonic (BCA) assay was performed. A sample of microparticles (~5mg) was digested with 0.1M NaOH to release the protein. 10µl of each sample was added to a 96 well microtitre plate (4 wells for each sample) and 200µl of working BCA reagent was added to each one. The working reagent was made up as follows: Reagent solution A (aqueous solution of 1 % (w/v) bicinchoninic acid, 2 % (w/v) $Na_2CO_3.H_2O$, 0.16 % (w/v) disodium tartrate, 0.4 % (w/v) NaOH and 0.95 % $NaHCO_3$) and reagent solution B (4 % (w/v) $CuSO_4.5H_2O$ in double distilled water) were mixed together in a ratio of fifty volumes of solution A to one volume of solution B. The wells were incubated at 60°C for 30 minutes and the optical density was read at 570nm using a Dynax MRX microplate reader (Dynax Technologies, Billingshurst, West Sussex, UK). The concentration of protein in the sample was calculated based on a calibration curve prepared from protein standards treated in the same way as the samples. The concentration of the samples is calculated by putting the optical density obtained from the samples into the equation of the calibration curve as the y value. The concentration of protein in the sample allows us to calculate the actual loading obtained in the batch. The encapsulation efficiency is calculated by comparing the actual load to the theoretical load of protein used in the preparation of the microparticles as follows:

$$\text{Encapsulation efficiency} = \frac{\text{Actual load} \times 100}{\text{Theoretical load}}$$

2.5. Determination of the integrity of the encapsulated protein using SDS-Page

2.5.1. Preparation of the gels

The gel casting plates were secured on the casting rig. Water was then poured between the plates to check that they are secure and the gel will not run out when it is cast. The water was removed and 12% separating gels were prepared. The separating gels were

poured in between the plates and allowed to set. The stack gels (5%) were then prepared and poured on top of the separating gel. The combs, which mould the wells for the samples were added and the gel was allowed to set. If necessary, the gels were then placed in the fridge overnight to be run the next day.

2.5.2. Preparation of the samples and the running of the gel

The samples were prepared by adding 40µl of sample to 40µl of loading buffer (loading buffer composition: 10% w/v SDS 6ml, 50%v/v glycerol 4ml, double distilled water 8.72ml, 0.062M Tris (pH6.8) 1.24ml, 5%w/v bromophenol blue 40µl). These samples were vortexed and heated to 100°C for 5 minutes. The combs were removed from the plates leaving wells for the addition of samples. The gel-running rig is filled with normal strength running buffer and the samples (20µl) were loaded onto the gel. The gel was then run at a voltage of 60V for 190 minutes using a Bio-rad 300 power pack.

2.5.3. Staining of the gels (Coomassie Blue)

When the gel has finished running it must be stained to visualize the bands. If the concentration of protein present is high then the coomassie blue staining method may be used. The gels were destained for 3x 20 minutes in a destain solution (40% methanol, 10% glacial acetic acid, 50% water). The gels were then stained with coomassie blue stain (0.1% coomassie blue R250 stain, 50% methanol, 10% glacial acetic acid, 40% water) for 30 minutes. The destain procedure was then repeated and the gels were stored in distilled water and photographed using a white light transilluminator.

2.6. Release of protein from microparticles

Samples from microparticle batches (5-20mg) were suspended in release medium (PBS/SDS/NaN_3) for various time periods as indicated. Each sample and time point was prepared in triplicate. The release samples were kept at 37°C for the duration of the experiment. The samples were removed, centrifuged at 13,000 rpm using a micro centrifuge and a 100µl aliquot was taken and frozen until all aliquots were removed. At the end of the release period all aliquots were removed from the freezer and the amount

of protein present was determined using the BCA assay as described in section 2.4. The amount of protein released was represented as a percentage of the original protein load.

2.7. Agarose gel electrophoresis

Agarose gel electrophoresis is used to detect the size of DNA fragments present in a sample and hence the stability. The technique is based on the theory that when a voltage is applied negatively charged DNA will migrate towards the positive electrode. The larger the DNA fragment the slower it will migrate through the gel. The conformation of the DNA fragments can also be determined by this method since super helical circular, nicked circular and linear DNA of the same molecular weight will migrate at different rates. A 1% w/v agarose solution (approximately 50ml for a small gel) was prepared in 0.5 x TBE solution and was heated until it became clear. The solution was allowed to cool until it was hand hot and 5µl of a 10mg/ml ethidium bromide solution was added. Ethidium bromide intercalates between the stacked base pairs in the DNA, which allows it to be detected under ultraviolet light. The gel solution was then poured into the gel-running rig, the comb was placed at the end furthest away from the positive electrode and the gel was allowed to set. The rig was filled with 0.5 x TBE solution (just enough to cover the gel) and the comb was removed. 10µl of each sample was added to 10µl of loading buffer (50%v/v glycerol, 20 mM Tris at pH 7.5 and 0.25%w/v bromophenol blue) and the entire sample was loaded onto the gel. The gel was run at a voltage of 80V for 90 minutes using a Bio-rad 300 power pack and the DNA visualized using a UV light transilluminator.

2.8. Enzyme-linked immunosorbent assay (ELISA)

Antibody responses in serum of the immunized animals ware monitored using a microplate ELISA. The 96-well ELISA microtire plates (Dynatech, Sussex, U.K) were coated with 100µl per well of a 100µg /ml^{-1} BSA in PBS solution (pH 7.3) overnight at 4°C. The plates were washes three times with 0.05% v/v solution of Tween-20 in PBS (PBST) using an automatic plate washer. The serum samples were serially diluted (1 in 2) in PBS using round bottom microtiter plates (Fisions, Loughborough, Leics. U.K) and 50µl of each sample was added to each well of the coated ELISA plates. These

were covered and incubated for one hour at 37°C. The plates were again washed three times with the PBST solution. 50µl of goat anti-mouse horseradish peroxide conjugate (Sigma, Poole, Dorset, UK), diluted 1 in 1000 using PBS, was added to each well and the plates covered and incubated again for one hour at 37°C. The plates were again washed three times in PBST and 50µl of the substrate (2,2'azino-bis) 3-ethylbenzthiazoline-6-sulphonic acid (ABTS), at a concentration of 3 tablets per 50ml of a citrate buffer plus 5µl hydrogen peroxide, was added to each well and incubated at 37°C for 10 minutes for colour development. The citrate buffer was prepared in advance using 44% 0.1M citric acid 56% 0.2M disodium hydrogen orthophosphate. The optical densities of the plates were then read at 405nm using the Dynax MRX microplate reader (Dynax technologies, Billingshurst, W.Sussex, UK).

End-point titres were expressed as the last dilution, which gave an optical density (at OD 405) above the OD405 of negative controls after the 30-minute incubation. Serum samples from individual animals were studied to determine the titre range and the immune responses of the different groups of animals at each time point were compared. Antibody responses in serum to the V, F1 antigen, and diphtheria and tetanus toxoid immunised mice were also determined using the above method except with a few changes. In the case of V and F1 antigens, the plates were coated at the concentration of $1mg/100ml^{-1}$ and in the case of diphtheria and tetanus toxoid it was 1000 Lf units/$100ml^{-1}$. For the hepatitis B surface antigen, plates were coated with 3 $\mu g/ ml^{-1}$ antigen in carbonate buffer (pH 9.6). Otherwise, the ELISA procedure for the detection of antigen specific antibodies was carried out in the same way as outlined above.

2.9. Stastistical methods

Comparison between the immune responses of the different groups of animals were analysed for significance using Student's unpaired t-test. Differences with $p<0.05$ were considered significant.

CHAPTER: 3

3.0. Particulate formulations for nasal delivery

3.1. Introduction: The role of size, morphology (shape) and surface characteristics (charge)

3.1.1. The effect of particle size

Biodegradable microspheres, in particular polylactide (PLA) and poly (lactide-co-glycolide) (PLG) microspheres have been studied as candidates for vaccine delivery systems using parenteral administration. Efforts have been made to develop vaccine carriers for oral immunization (Eldridge *et al.*, 1991). Recently, the intranasal (i.n) administration of peptides and proteins have attracted particular interest (Chipps *et al.*, 1993; Abe *et al.*, 1995; Irie and Uekama 1999). Parameters that influence the efficacy of the vaccines delivered nasally have not been characterised fully. The efficacy of microparticles following mucosal delivery is a consequence of their uptake into the intestinal Peyer's patches following oral administration or the nasal associated lymphoid tissue (NALT) following i.n. administration (Beier and Gebert 1998). The intranasal route is probably the most efficient mucosal route for the induction of antibody responses in the serum, as well as local and distant mucosal secretions (Almeida and Alpar 1996; Velge-Roussel *et al.*, 2000; Liang *et al.*, 2001). It is thought that, whilst non-ciliated cuboidal cells have a particle sampling capacity, particulate antigens are preferentially taken up by M-cells, which overlay NALT (Wu and Russell 1997). However, the appropriate particle characteristics for optimal uptake into these sites and optimal stimulation of immune responses are poorly understood. Particle size-dependent uptake by gastro-intestinal epithelia is an area of some controversy. Previously, in our laboratories it was demonstrated that latex microparticles (mean size ~1μm) are also translocated from the nasal lumen into blood circulation resulting in high serum titres and protective immunity; using antigen-encapsulated PLA microspheres after i.n. administration (Almeida *et al.*, 1993; Alpar *et al.*, 1994). In the first part of the chapter, we aim to define the effect of particle size on the immune response to the encapsulated antigen, OVA by comparing PLA microspheres and nanospheres, at different loadings.

We will also address the effect of adsorption of TT to PLA lamellar particles and the effect of particle surface charge on the immune response to nasally delivered encapsulated antigen.

3.1.2. Adsorption of TT to PLA lamellar particles

Many purified, synthetic or inactivated antigens such as tetanus toxoid are poorly immunogenic and usually require several (booster) injections to confer adequate protection. Adsorption of vaccine antigens on to adjuvants such as aluminium hydroxide (Alum) is a common method for enhancing the immunogenicity. Other adjuvants such as mycobacteria, oil emulsions, liposomes and polymer microparticles (Chen 2000; Sheikh *et al.*, 2000) have also been used. The mechanism of action is complex but is known to involve factors such as antigen protection, stimulation of phagocytosis and the cells of the immune system as well as antigen retention at the site of deposition to provide a depot effect.

Microencapsulation of antigens using biodegradable polymers has been shown to potentiate the immune response (Bowersock and Martin 1999). In contrast to encapsulation, adsorption of labile antigens on to biodegradable polylactide particles may be advantageous; this avoids encapsulation procedures involving high shear rates and exposure to organic solvents. Sterilization of microspheres may damage the immunogenicity of loaded antigen. Antigen adsorption to sterilized particles may also provide a means of avoiding the loss of immunogenicity of vaccine due to irradiation. This is because the particles can be sterilized prior to adsorption of the antigen. This may be an important concern for the application of irradiation for sterilization of encapsulated antigens. Antigens adsorbed onto microparticles have already been shown to generate potent immune responses after subcutaneous (Coombes *et al.*, 1996; Coombes *et al.*, 1999) and nasal administration (Almeida and Alpar 1996). Concerns exist however, with respect to protection and desorption of adsorbed antigens *in vivo*.

Tetanus toxoid was used as a clinically relevant antigen. Close to 400,000 neonates die of tetanus annually, which could be prevented by immunizing women of childbearing age. Three consecutive injections of tetanus vaccine are required to achieve protection. Which is a problem in developing countries where cold chain storage is required. The availability of a single dose vaccine would therefore be highly beneficial. Improved

immune responses have been reported after injection of influenza virus adsorbed onto PLA lamellar particles (Coombes *et al.*, 1998). In the second part of this chapter we describe the immune response after intramuscular and nasal administration of vaccine adsorbed PLA lammelar particles.

3.1.3. Surface charge of particles: Effect on immune response to loaded antigen

It has been demonstrated that particle uptake by phagocytic cells may be largely dependent upon the surface properties of the particles (in addition to size). Researchers have (Tabata and Ikada 1988; Tabata *et al.*, 1996) demonstrated that microspheres with hydrophobic surfaces were more readily phagocytosed than those with hydrophilic surfaces. Others have also shown surface properties play, a major role in determining the interaction and uptake of particles by the phagocytic cells of the reticuloendothelial system (RES). Attempts have been made to avoid RES uptake by coating particles with hydrophilic surfactants (Rafati *et al.*, 1997) however, the desirability of such strategies largely depends upon their application. From the perspective of vaccine formulations it may be favorable to alter the surface characteristics to increase rather than suppress the cellular uptake of particulate delivery systems. Surface charge, hydrophobicity and steric hindrance are considered to be among the most important physicochemical characteristics of surfaces governing the interaction of particles with cells of immune system components (O'Hagan *et al.*, 1995). Surface-active agents have been used to modify the surface characteristics of, and immune response to, antigen loaded oil-in-water (o/w) emulsions. A significant increase in the magnitude of the immune response to a model protein antigen (BSA) was observed when more hydrophobic surfactants were used in the formulation (Conway *et al.*, 1998). More recently, the importance of the surface characteristics of polymeric colloidal carriers on their adjuvant activity towards adsorbed antigens has been highlighted (Alpar *et al.*, 1994). The use of mucoadhesive microparticles provides a potential strategy for improving the retention of drugs or vaccines within the nasal cavity, and thereby enhancing resultant immune responses to administered antigen and the bioavailability of drugs. In the final part of the chapter this issue will be addressed.

3.2. Methods and Materials

3.2.1. Materials

All materials in this chapter are listed in materials and methods (chapter 2).

3.2.2. Preparation of OVA encapsulating PLA micro/nanospheres for the investigation of effect of size on immune response to nasally delivered OVA loaded particles

Microspheres and nanospheres were prepared using a w/o/w double emulsion technique with some modifications. Briefly, a 5% w/v solution of the polymer (either PLA 2 kDa (Boehringer Ingelheim, Germany) or PLA 100 kDa (polysciences Inc. U.S.A) in dichloromethane containing 4% w/w pluronic F-68 was emulsified with ovalbumin (OVA) solution using a probe sonicator to produce the w/o emulsion. This w/o emulsion was further added to an aqueous solution of 2% w/v polyvinyl alcohol (PVA) 13-23 kDa (Aldrich U.K) and sonicated to produce a w/o/w emulsion. The double emulsion was then stirred at ambient temperature. The solidified micro/nanoparticles were collected by ultracentrifugation and washed three times with distilled water to remove any PVA residue and any free ovalbumin (OVA). The final suspension was freeze dried and stored at 4°C in a desiccator.

3.2.3. Characterization of OVA encapsulating PLA micro/nanospheres

The morphology (shape and surface characteristics) of the particles was investigated with a scanning electron microscope (Cambridge instruments, U.K). Microsphere size was determined by laser diffraction (Malvern Instruments Ltd, U.K). Determination of antigen loading was made using the bicinchoninic acid method following extraction with NaOH-SDS solution as outlined in chapter 2.

3.2.4. Immunization schedule for comparison of the effect of size

Four batches of microspheres or nanospheres were selected for this study. In all experiments female BALB/c mice, five *per* treatment group were used. Each animal

received intranasally 20μl of 20 μg of OVA (free or encapsulated) on day 1 and day 7. Periodically tail vein blood 100 μl samples were taken. The blood was allowed to clot overnight and the serum was collected by centrifugation and stored frozen at -20^0C. The magnitude of the titre of anti- OVA IgG in the serum was measured using an ELISA method as described in chapter 2.

3.2.5. Preparation of TT adsorbed PLA lamellar particles

Aqueous suspensions of PLA lamellar particles were produced by the addition of 10ml water to 5ml of stirred 2% w/v solution of PLA (Mw 2000, Polysciences) in acetone. The solvent was removed by evaporation by overnight stirring at room temperature.Suspensions of PLA lamellae (1ml, 10 mg) were incubated with tetanus toxoid (315 Lf units) overnight at room temperature. The particles were centrifuged and washed with PBS to remove unbound TT. The supernatants were collected and analysed for protein content using bicinchoninc acid (BCA) protein assay. The quantity of adsorbed antigen was obtained by subtraction and comparison to a standard TT run on the same plate.

3.2.6. Development and characterization of PLA microspheres in the presence of chitosan as emulsifying agent

Previously in our laboratories other emulsifying agents such as polyvinylpyrrolidone (PVP) instead of PVA was used (Conway *et al*, 1998). In order to modify the surface properties, other researchers have used agents such as cetyltrimethylammonium bromide (CTAB) (Singh *et al*, 2000), which has been shown to impart a positive charge to the microsphere formulation. Here, the mucoadhesive polymer chitosan was used in place of PVA in the preparation of positively charged PLA microspheres using both single and double emulsion preparation techniques.

3.2.6.1. Preparation of PLA microspheres by the single emulsion method in the presence of chitosan as emulsifying agent

A 5% (w/v) polymer was prepared in dichloromethane. This was added drop wise to 75ml of an aqueous solution containing various concentrations of chitosan (high

molecular weight) (0.05%-2% w/v) and homogenized using a Silverson homogeniser (Silverson Machines, Chesham, Bucks, UK) for 5-6 minutes at 16,000 rpm. Both solutions were previously pre-cooled in an ice bath for one hour. The solidified microparticles were collected by ultra centrifugation and washed three times with distilled water to remove any PVA residue and any free ovalbumin (OVA). The final suspension was freeze dried and stored at 4°C. Where PVA has been used the method is outlined in chapter 2.

3.2.6.2. Preparation of PLA microspheres by the double emulsion method in the presence of chitosan

The double emulsion method was used for the preparation of surface modified microspheres, the method chosen was the identical to outlined in chapter 2 for preparation of microspheres using PVA as the emulsifying agent, with the exception that various concentrations (0.05%-2% w/v) of chitosan base (high molecular weight) were for substituted for PVA.

3.2.7. Preparation of DT loaded PLA microspheres using chitosan or PVA as the emulsifying agent

A solution of diphtheria toxoid was emulsified with a 5% (w/v) solution of PLA kDa (10ml) in dichloromethane (DCM; oil phase) using a Silverson homogeniser (Silverson Machines, Chesham, Bucks, UK) for 5-6 minutes at 16000 rpm at high speed (8000 rpm) and room temperature. The resulting water-in-oil (w/o) emulsion (2.5ml) was then emulsified with a 0.5% (w/v) chitosan solution or 1.5% w/v PVA 13-23 kDa (50ml) in the same conditions as those for the first emulsion to produce a water-in-oil-in-water (w/o/w) emulsion. The latter mixer was then stirred magnetically at ~ 800 rpm overnight at room temperature, to allow the evaporation of the organic solvent and the formation of microparticles. Once the polymer wall was hardened, the microparticles were collected by centrifugation (10 min at 4000 × g), washed three times with double distilled water and freeze-dried. In these studies, the effects of the following formulation variables on microparticle size were investigated.

3.2.8. Particle characterization

The size, surface charge and morphology of the particles was determined as outlined in chapter 2. 'Materials and methods'.

3.2.8.1. Determination of diphtheria toxoid loading in microspheres

Determination of the antigen content was carried out after alkaline hydrolysis of the polymer using sodium hydroxide NaOH and extraction by SDS as previously described in chapter 2. Briefly, ~10-mg of microparticles, were admixed with 1.0ml of 1M NaOH containing 5% w/v SDS for 24 h at room temperature. The supernatant obtained by centrifugation (4,000×g for 10 min at room temperature), was analyzed by BCA method of protein assay. The percentage (w/w) of protein entrapped *per* dry weight of microparticles was determined. The percentage of entrapment efficiency was expressed by relating the actual protein entrapment to the theoretical protein entrapment as previously described by Jeffery *et al.* (1993). Each sample was assayed in triplicate. Assessments of antigen loading were made using the bicinchoninic acid method following extraction with NaOH-SDS solution.

3.2.8.2 The *in vitro* TT release from PLA lamellar particles

PLA lamellar particles (10 mg) with adsorbed TT were incubated in 2ml PBS containing 0.02% w/v sodium azide at 37^0C. The release medium was separated from the particles after day one and fresh medium was added to the sample tubes. This process was repeated at 3-day intervals up to 8 weeks. The release medium was analyzed for TT content using a BCA protein assay and the cumulative release amount of antigen (%) calculated.

3.2.8.3. Determination of antigen integrity

The determination of antigen structural integrity after the encapsulation procedure was investigated using SDS-PAGE as outlined in chapter 2. 'Materials and Methods'.

3.2.9. Immunization schedule

3.2.9.1. PLA microspheres and nanospheres

Four batches of micro and nanospheres were selected for this study. In all experiments female BALB/c mice, five *per* treatment group were used. Each mouse received intranasally 20μg of OVA (free or encapsulated) on day 1 and day 7. Periodically, tail vein blood samples were taken. The magnitude of the titre of anti-OVA IgG in the serum was measured using an ELISA method.

3.2.9.2. PLA lamellar particles with adsorbed TT

Immune response to adsorbed TT: PLA lamellar particles with adsorbed TT were dispersed in 0.1ml of PBS. Vaccine formulations were administered either intramuscularly or nasally to groups of 5 BALB/c mice. Each animal received intranasally 20μl and 50μl intramuscularly. Test bleeds were obtained at regular intervals. All sera were tested for antibody titre to TT by ELISA. Group 1 TT/PLA (7.5 Lf) single intramuscular injection, Group 2 TT/PLA (5 Lf) intramuscular injection followed by booster injection of TT/PLA (2.5 Lf) at day 28, Group 3 soluble TT (5 Lf) single intramuscular injection, Group 4 TT/PLA (7.5 Lf) single intranasal administration, Group 5 TT/PLA nasal administration followed by booster TT/PLA (2.5 Lf) at day 28, Group 6 soluble TT (5 Lf) nasal administration followed by a booster dose TT/PLA (2.5 Lf) at day 28.

3.2.9.3. PLA with PVA or Chitosan loaded with DT

The immunization schedule for these preparations is shown in the table below:

Group	Formulation	Dose/ Lf units	Dosing Schedule
1	PLA MS prepared with PVA	10	day 1 and day 7
2	PLA MS prepared with Chitosan	10	day 1 and day 7
3	Free Diphtheria toxoid	10	day 1 and day 7
4	PLA MS prepared with PVA	10	day 1 and day 67
5	PLA MS prepared with Chitosan	10	day 1 and day 67
6	Free Diphtheria toxoid	10	day 1 and day 67
7	PLA MS prepared with PVA	25	day 1 and day 7
8	PLA MS prepared with Chitosan	25	day 1 and day 7
9	Free Diphtheria toxoid	25	day 1 and day 7
10	PLA MS prepared with PVA	25	day 1 and day 67
11	PLA MS prepared with Chitosan	25	day 1 and day 67
12	Free Diphtheria toxoid	25	day 1 and day 67

Table 3.1: The immunization schedule for microspheres prepared with PVA or Chitosan. The experiment was designed to evaluate the effect of delayed boosting and the effect of increased antigen dose.

3.3. Results

3.3.1. Characterization of micro/nanospheres

OVA loaded nanoparticles were prepared using the double-emulsion method. A summary is shown in table 3.2. The particle size and distribution were significantly improved when the sonication power was increased from 30watts to 40watts. The yield for all batches is more than 70%. Various sizes (0.32 μm to 3.86 μm) of microspheres and different loading percentages of OVA (0.9% to 14% w/w) were prepared. The mean volume diameter of the PLA micropsheres with 0.9%, 5.0%, 10.1%, 14% w/w OVA loading was 0.32 (± 0.14), 0.32 (± 0.14), 3.86 (± 6.82), 0.45 (± 0.62) μm respectively.

Batch No	Polymer	Sonication Power watts	Loading w/w % ± s.d	Size μm mean ± s.d
IN1	PLA 2K	30	9.8 ±1.2	V: 1.35(2.11) N:0. 19(0.09)
HN-02	PLA 100K	30	10.2 ±0.8	V: 1.59(2.13) N: 0.20(0.10)
HN-03	PLA 100K	40	12.4 ±0.2	V:0. 36(0.18) N:0. 19(0.09)
HN-04	PLA 100K	30	13.0 ±1.6	V: 1.48(3.18) N:0. 19(0.10)
HN-05	PLA 100K	30	10.1±2.1	V: 3.86(6.82) N:0. 19(0.10)
HN-06	PLA 100K	40	14.4 ±1.1	V:0. 45(0.62) N:0. 19(0.09)
HN-07	PLA 100K	40	5.0 ± 0.5	V:0. 32(0.14) N:0. 18(0.08
HN-08	PLA 100K	40	0.9 ±0.2	V:0. 32(0.14) N:018(0.08)

Table 3.2: The preparation conditions of PLA nanospheres containing OVA showing loading and size of PLA micro/nanoparticles prepared by the double emulsion method. V= volume mean, N= number mean.

3.3.2. Characterization of PLA lamellar particles

The tendency for PLA to crystallize during precipitation from stirred solutions led to the production particles which were lamellar in form, having planar dimensions typically of 3 to 5μm (SEM) with a variable aspect ratio in the range 2.5:1 to 3:2. The thickness of isolated lamellae, determined from edge-on views in the SEM revealed a dimension of the order of tenths of a micron. The shape, dimensions and morphology of the PLA particles are shown in figure 3.1. The surface of the lamellae often exhibited a terraced or stepped topography, which is typical of polymer crystal growth. Sheave-like, structures formed by lamellae joined together along a common plane were also prevalent. The amount of TT, which could be adsorbed onto the lamellar particles, varied with the pH of the adsorption medium and in this case reached a maximum of 7.2 Lf/mg lamellar particles at pH 4.9. Approximately 14% of the adsorbed TT was released after 24 hrs incubation of PLA particles (7.2 Lf /mg) in PBS pH 7.4 at 37 ^{0}C.

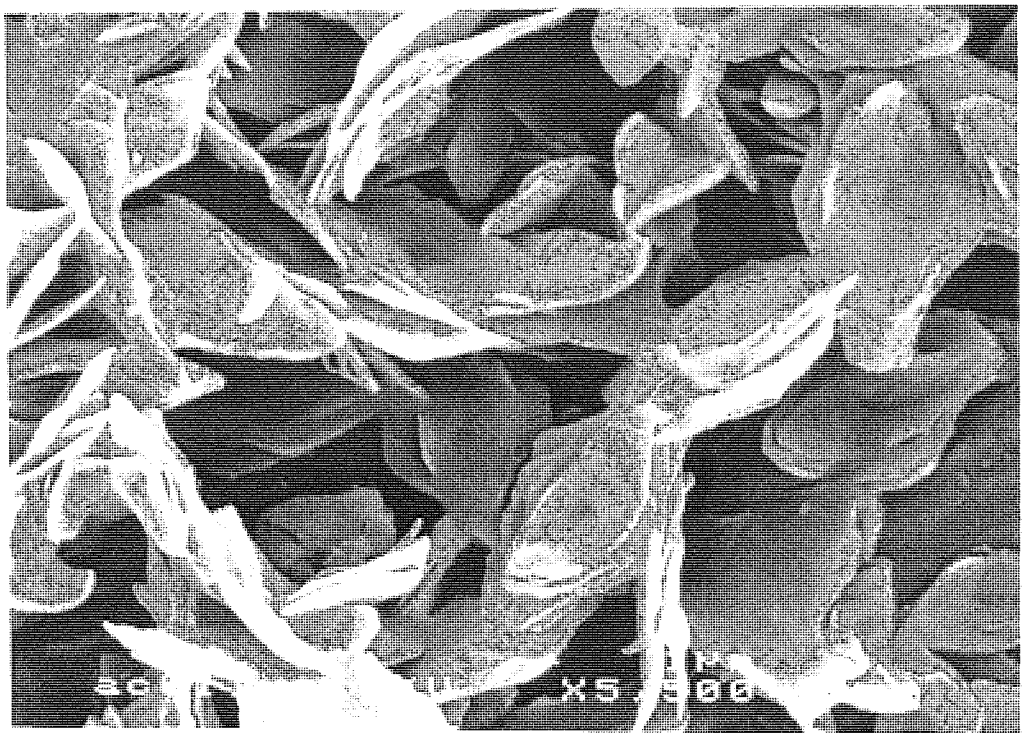


Figure 3.1: Scanning electron micrograph of PLA lamellar particles prepared by precipitation from a stirred acetone solution of the polymer, using water as the non-solvent.

3.3.3. Characterization of microspheres prepared in the presence of chitosan

Surface charge is a major characteristic responsible for mucosal absorption or transport of particles through mucosal surfaces (Tabata *et al.*, 1996). The main aim of this part of the work was to produce mucoadhesive, positively charged microspheres. If the chitosan is shown to be an effective emulsion stabilizer this offers a good opportunity for the incorporation of the chitosan into the formulation utilising the potentially advantageous properties of this agent for vaccine delivery.

These studies show that the use of chitosan as an emulsion stabilizer in both single and double emulsion preparation techniques enabled successful formation of microparticles suitable for entrapment of antigen or proteins. The concentration of chitosan was optimized and its effect on microsphere characteristics was analysed. At concentrations below 0.1% w/v chitosan (high molecular weight) the suspension was not stable and microspheres were not formed. At higher concentrations (above 2% w/v chitosan) the formation of the suspension was inadequate due to the higher viscosity of the solution.

Thus, the optimization of the microsphere preparations was evaluated using concentrations of chitosan between 0.1% and 1% w/v. The effect of concentrations of chitosan in the aqueous phase on the parameters of particle size and surface charge is shown in table 3.3 for the single emulsion method.

% chitosan (w/v)	**Particle diameter (volume mean, μm ± s.d.)**	**Surface charge (Zeta potential, mV ± s.d.)**
0 (1.5% PVA w/v)	5.23± 1.4	-29.8±9.5
0.10	4.11±1.6	-1.98±1.6
0.25	4.31±2.1	4.8±1.6
0.75	3.91±1.7	11.6±1.6
1.00	5.15±2.4	32.6±1.6

Table 3.3: Effect of chitosan concentration on the particle size and surface charge of the PLA microspheres prepared by the single emulsion method (n=3).

The concentration of chitosan was shown to influence the surface charge of the microspheres giving an increasing positive surface charge with increasing chitosan concentration. At lower chitosan (0.1 %w/v) concentrations, the particles had a negative surface charge. The size of the microspheres was not significantly affected by lower concentrations of chitosan, however, the higher concentration of chitosan used (1% w/v) appeared to increase the observed particle size. According to the electron micrographs, the particle size was generally between 4 and 5 μm (table 3.2) with a narrow size distribution. The morphology of representative unloaded batches is shown in figure 3.2. In both single and double emulsion methods the microspheres were shown to be of uniform size and have a smooth and spherical appearance. Similarly, the morphology of DT loaded microspheres was of uniform size and smooth, spherical appearance. This is shown in figure 3.3.

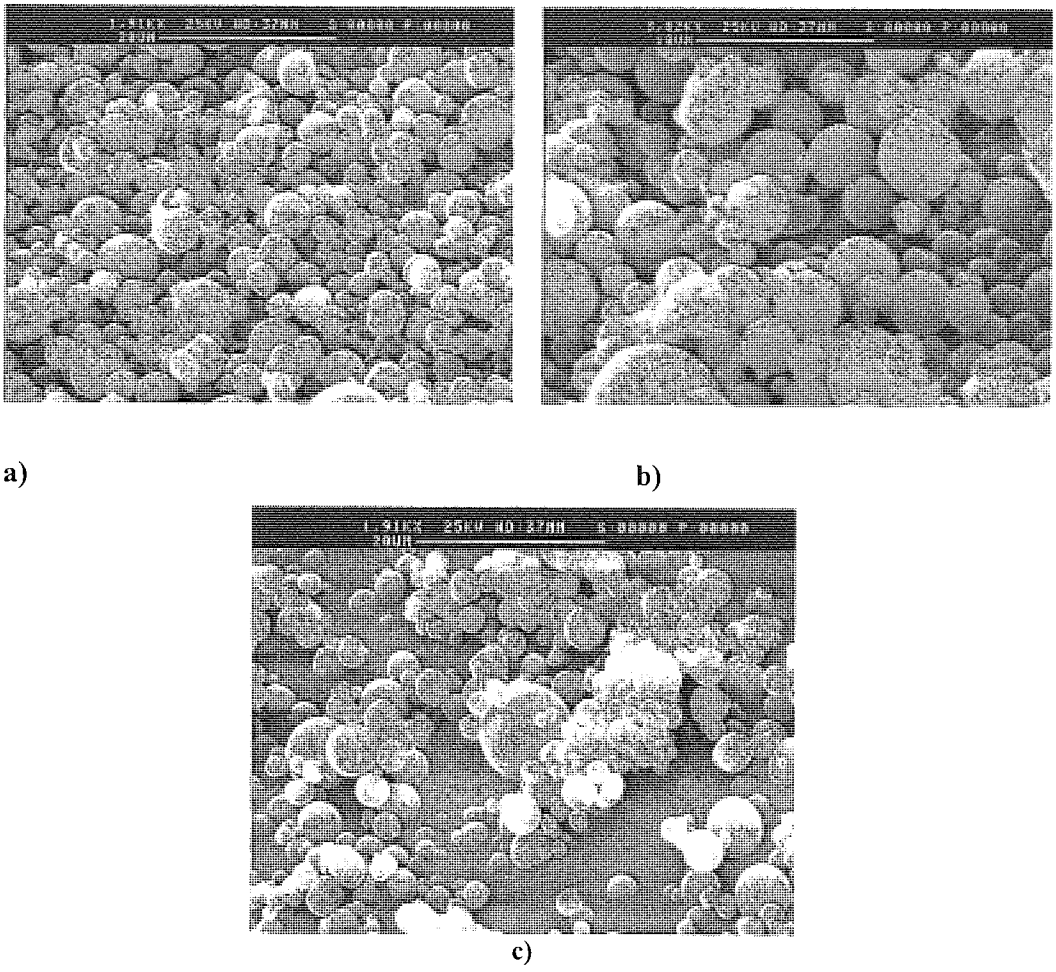
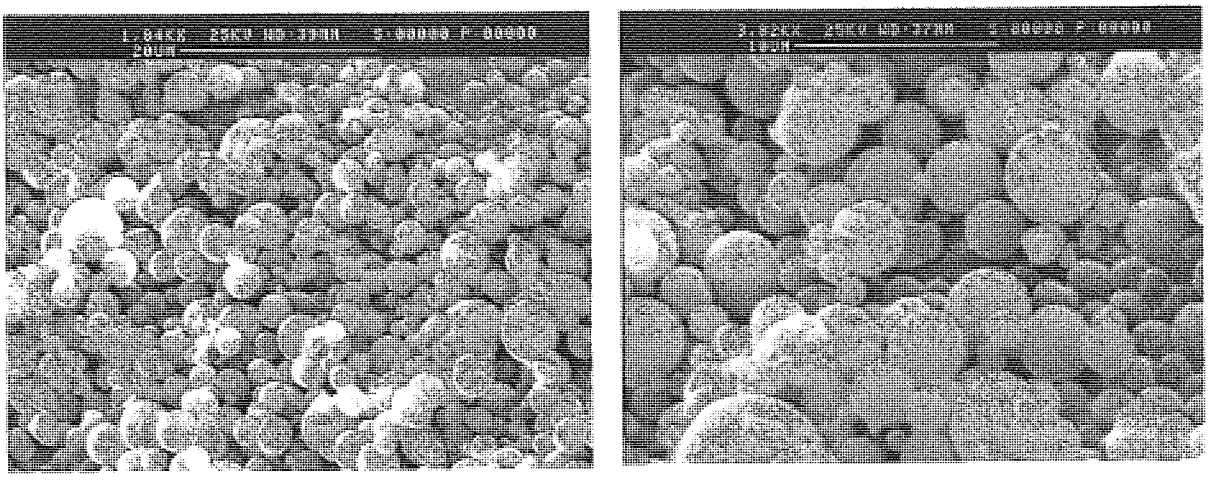


Figure 3.2: Scanning electron micrograph of plain PLA microspheres prepared by a) the single emulsion method with 0.75% w/v chitosan, b) the double emulsion method with 0.75% w/v chitosan (primary emulsion) and 0.5% w/v chitosan (secondary emulsion), c) the double emulsion method with 2.5% w/v PVA (primary emulsion) and 1.5% w/v PVA (secondary emulsion).



a) b)

Figure 3.3: Scanning electron micrograph of DT loaded PLA microspheres prepared by the double emulsion method using a) 0.75% w/v chitosan (primary emulsion) and 0.5% w/v chitosan (secondary emulsion), and b) 2.5% w/v PVA (primary emulsion) and 1.5% w/v PVA (secondary emulsion).

3.3.4. Loading and stability of DT in microspheres

The loading and size of the microspheres used in the immunization schedule are shown in table 3.4. The size range and loading was shown to be very similar for microspheres prepared using both chitosan and PVA as the emulsifying agent.

Stabilizing agent (concentration)	**DT loading (± s.d) lfunits/mg of Ms**	**Particle size volume mean, μm (± s.d)**	**Zeta potential mV (± s.d)**
Chitosan (0.75%w/v) internal (0.5% w/v) external	9.47 ±2.2	5.06 ±2.62	28 ±1.6
PVA (2.5% w/v) internal (1.5% w/v external	10.34 ±1.4	5.18 ±2.42	-23 ±1.6

Table 3.4: The size and loading of microspheres used in the immunization studies comparing formulations made with chitosan and PVA as the emulsifying agent.

The stability of the DT was confirmed using gel electrphoresis and is shown in figure 3.4. It can be seen that there is no difference between the unencapsulated and encapsulated DT for both the chitosan and PVA formulations.

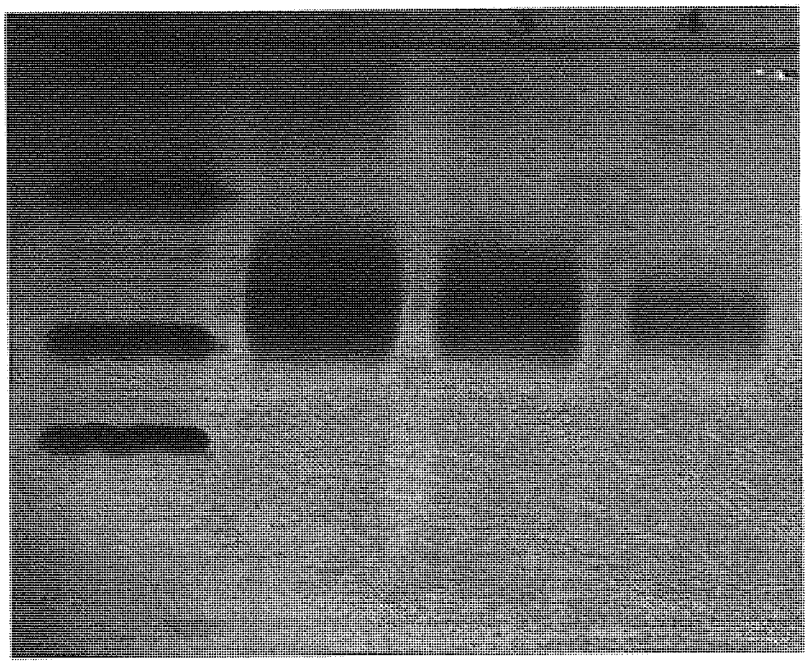


Figure 3.4: Stability of DT following microencapsulation shown by SDS PAGE following extraction of DT from microspheres as outlined in chapter 2. Lane descriptions: 1) Molecular weight markers; 2) Free diphtheria toxoid; 3) DT encapsulated in PLA MS prepared using PVA as stabilizer; 4) DT encapsulated in PLA MS prepared using chitosan as stabilizer.

3.3.5. Effect of size and antigen loading in immune response

The serum levels of anti-OVA IgG in the mice induced by the micro and nanospheres containing OVA were significantly ($P<0.05$) higher than antigen specific IgG induced by free OVA over the entire two months study period (figure 3.5). The exception to this being the microsphere preparation with a particle mean diameter of 3.86 μm. This may be due to poor uptake and elimination of the large particles. Low loaded (0.9% w/w) and the smallest (0.32μm) PLA nanoparticles resulted in the highest antibody titres, which were significantly higher than those of all the other groups (figure 3.5). The mean serum level of anti-OVA titres in the mice immunised intranasally increased with decreasing particle size (0.32 μm > 3.86 μm particle diameter)(figure 3.5). Since the number of microspheres increases with decreasing size and also with decreasing antigen loading the number of microspheres needed to deliver the same amount of antigen will also necessarily be increased, this facet may promote particle translocation elevated in an augmented immune response. Translocation of intact particles from the respiratory

lumen to systemic and mucosal inductive sites may enhance the magnitude of the immune response generated following intranasal delivery.

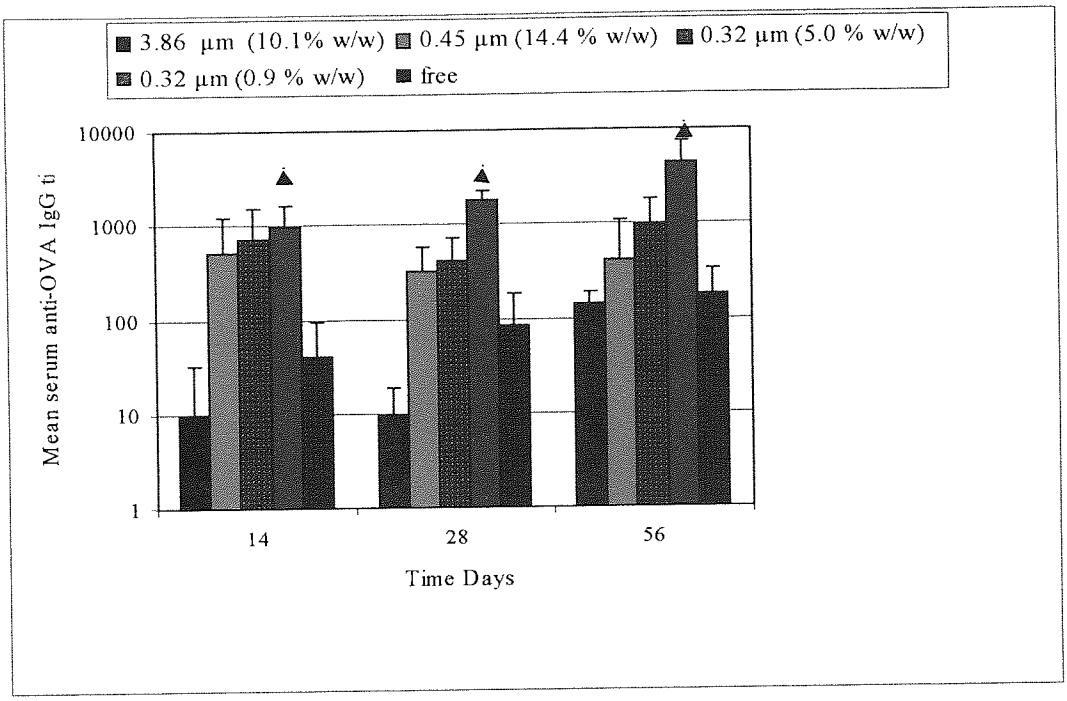


Figure 3.5: Serum immune response to nasally administered microencapsulated and free OVA. BALB/c mice were primed on day 1 with 20 µg free or microencapsulated OVA and boosted with the same dose on day 7. (n=5 *per* group; mean ±s.d).

3.3.6. The immune response to PLA lamellar particles with adsorbed TT

After i.m delivery TT adsorbed to PLA lamellar particles gave ~ 6 times higher primary antibody response and ~ 10 times higher secondary antibody response after boosting compared to soluble antigen (figure 3.6). A single i.m. administration of TT (7.5 Lf) adsorbed onto PLA lamellar particles resulted in a poorer immune response on day 35 compared to the same total dose delivered by two injections (figure 3.6).

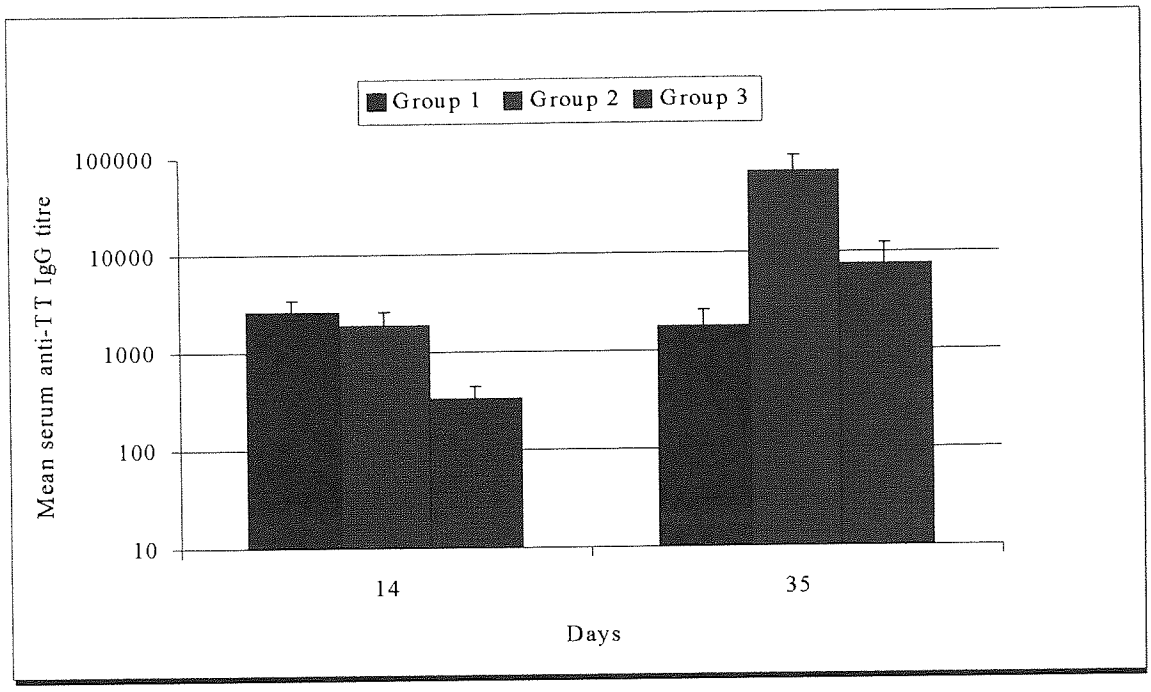


Figure 3.6: Serum immune responses to intramuscularly delivered tetanus toxoid. Soluble antigen or adsorbed onto PLA lamellar particles. Group 1; 7.5 Lf units TT adsorbed onto PLA lamellar particles, single administration on day 1. Group 2; 5 Lf units TT adsorbed onto PLA lamellar particles on day 1, followed by a booster dose of 2.5 Lf units adsorbed onto PLA lamellar particles on day 28. Group 3; Soluble TT, 5 Lf units on day 1 followed by 2.5 Lf units on day 28. (n=5 *per* group; mean ±s.d).

The levels of primary systemic IgG antibodies in mice that received TT/PLA intranasally were similar to those obtained after dosing with 5 Lf soluble TT (figure 3.7). However, the secondary immune response to TT/PLA was ~ 10 times higher than that induced by soluble antigen.

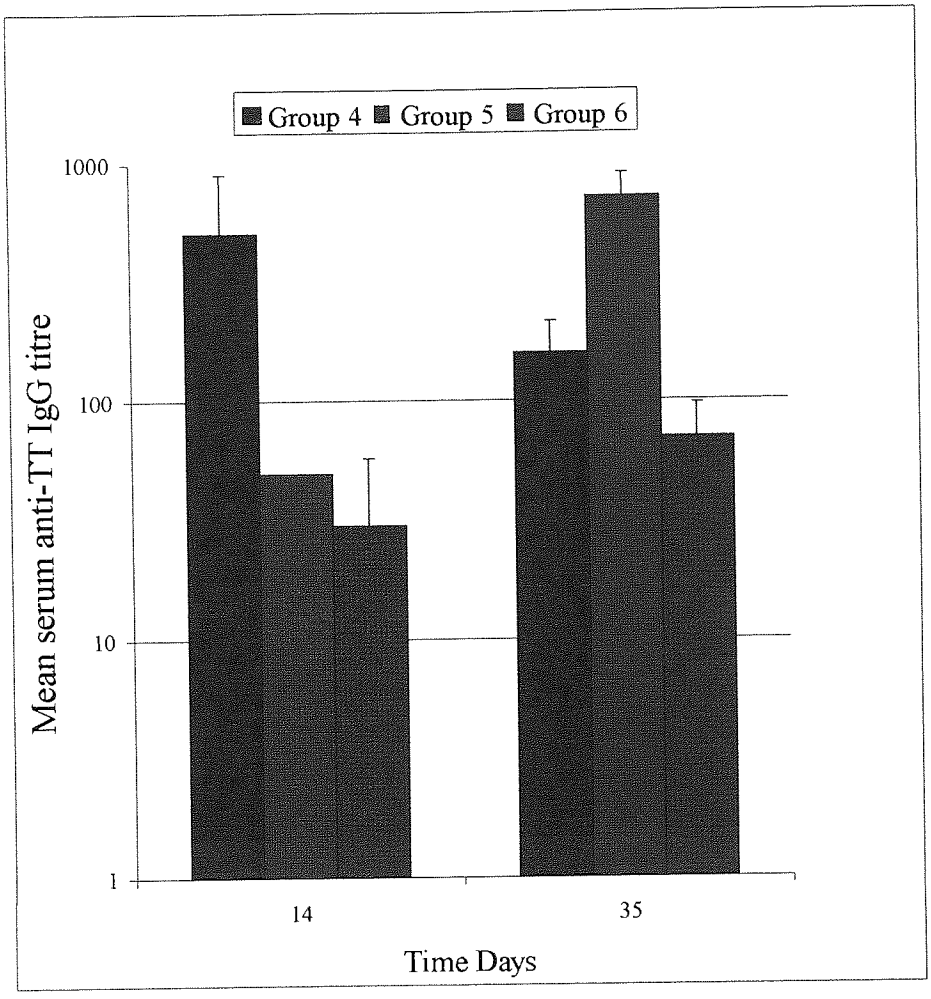


Figure 3.7: Serum immune responses to intranasally delivered tetanus toxoid. Soluble antigen or adsorbed antigen on PLA lamellar particles. Group 4; 7.5 Lf units TT adsorbed onto PLA lamellar particles, single administration on day 1. Group 5; 5 Lf units TT adsorbed onto PLA lamellar particles on day 1, followed by a booster dose of 2.5 Lf units adsorbed onto PLA lamellar particles on day 28. Group 6; Soluble TT, 5 Lf units on day 1 followed by 2.5 Lf units on day 28.

3.3.7. Effect of microspheres containing chitosan on antibody response elicited by DT after nasal administration

3.3.7.1. The effect of charge and mucoadhesive properties

The formulations administered on day 1 and day 7 comprising 10 Lf units showed the highest antigen specific antibody titres on day 14. Both of the emulsifying agents were effective in eliciting antibody responses, as might be expected the microencapsulated antigen was markedly more effective than free antigen in effecting the immune response. The use of chitosan, however, further increased the observed response. The

magnitude of the response was increased for the duration of the experiment in the case of the chitosan formulations in comparison to that observed from the free antigen and the PVA emulsified microsphere formulation (figure 3.x) although titres were observed to decrease significantly.

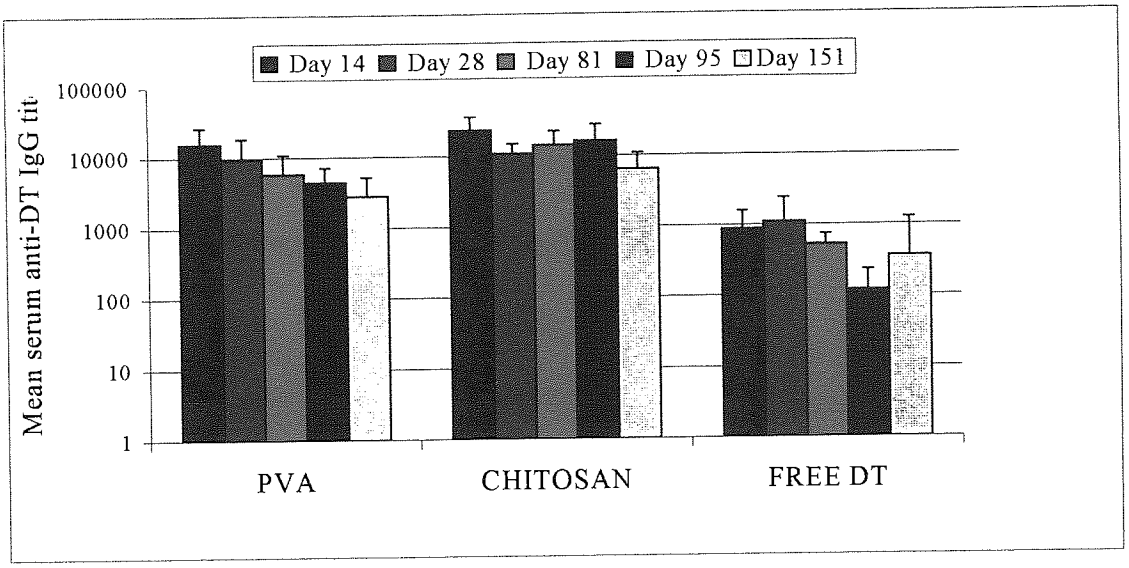


Figure 3.8: Serum immune response to nasally delivered diphtheria toxoid, either microencapsulated or in soluble form. BALB/c mice were dosed on day 1 with 10 Lf units and boosted on day 7, again with 10 Lf units. (n=5 *per* group; mean ±s.d).

3.3.7.2. The effect of delayed a boosting regime on the observed immune response

In this case, formulations were administered on day 1 and day 67. Formulations were identical as those administered in the previous section and the dose was the same (10 Lf units)(figure 3.9). Up to day 28 the immune response observed with a single dose of 10 Lf units was much lower than that observed when animals were boosted on day 7. On day 14 the response was an order of magnitude less than the boosted groups. Titres were still markedly increased in the case of the microparticulate (PLA chitosan MS) antigen in comparison to the free DT (figure 3.9). As before, the use of chitosan stimulated the highest response of the three relevant groups. After boosting on day 67, all formulations including the free antigen showed markedly elevated serum antigen specific antibody titres on day 81(figure 3.9). In the case of the microparticles, the full magnitude of the immune response was not realized until day 95. Antibody titres had continued to increase for the particulate delivery systems, whereas for the free antigen, titres had started to decrease. On day 95, the antibody titres conferred by the use of chitosan in the

microsphere formulation were five fold in excess of those observed for the microsphere formulation prepared with PVA, substantially enhancing an already potent antigen delivery system. The responses at day 151 were still markedly enhanced for the late boosted groups in comparison to the early boosting regime.

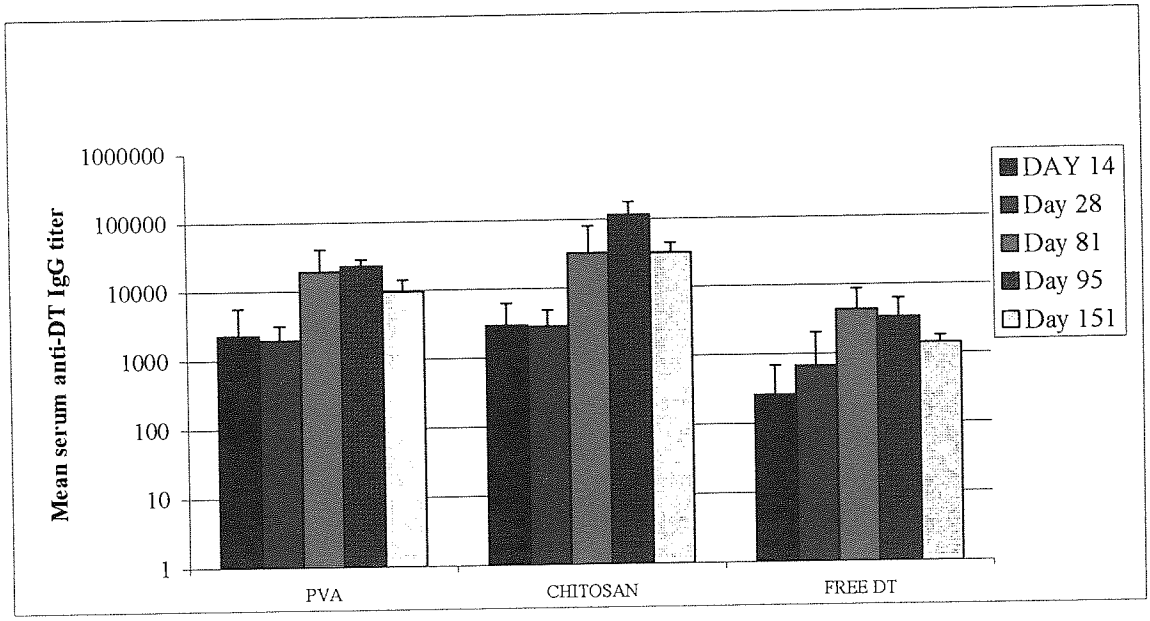


Figure 3.9: Serum immune response to intranasally delivered diphtheria toxoid, either microencapsulated or in soluble form. BALB/c mice were dosed on day 1 with 10 Lf units and boosted on day 67, again with 10 Lf units. (n=5 *per* group; mean ±s.d).

3.3.7.3. The effect of increased dose of antigen

The IgG antibody responses elicited by DT formulated in microspheres prepared using the chitosan stabilizer were greater than those for the microspheres prepared using PVA as the emulsifying agent(figure3.10). In addition, compared with free DT, both formulations resulted in significantly higher immune responses and the titres are higher up to day 151. When groups were given 25 Lf units DT, the initial primary antibody response was increased as compared to a 10 Lf unit dose. In both cases, the microspheres prepared with chitosan stimulated the highest immune responses. After boosting on day 67, the group, which was boosted with 10 Lf units, showed higher immune responses in comparison to the groups receiving 25 Lf units. In the case of the dosing regime incorporating boosting at day 7, the groups receiving either 10 or 25 Lf units in microspheres showed quite similar antibody titres when compared using Lf units as the comparative parameter. However, with the free DT, the increased Lf units enhanced the serum immune titres. This effect was also seen, and was markedly more

evident when boosting was at day 67. The results are summarized in figures 3.10 and 3.11

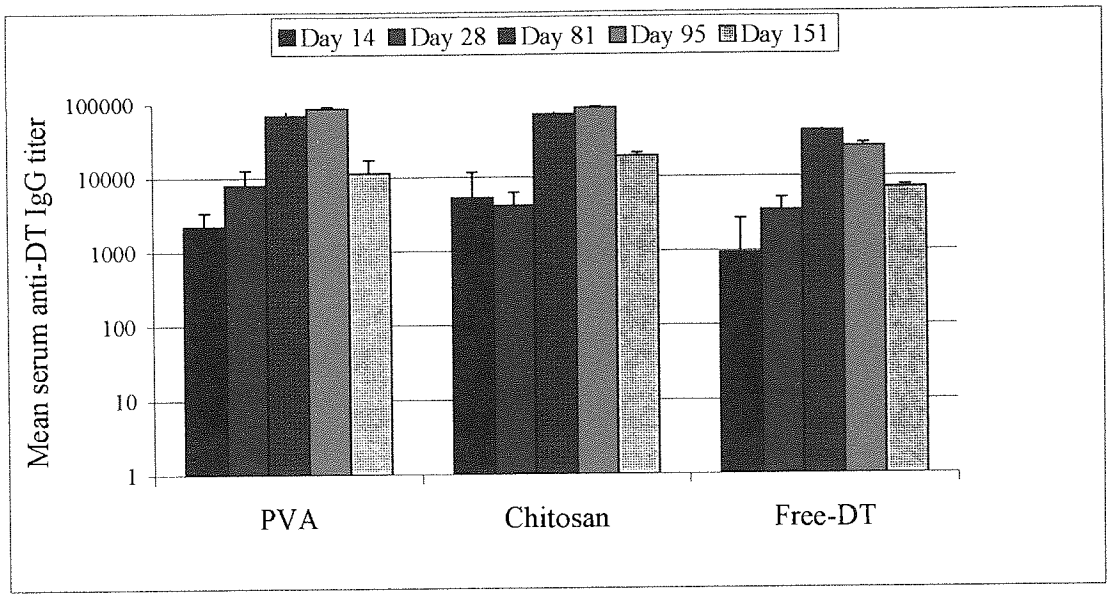


Figure 3.10: Serum immune response to nasally delivered diphtheria toxoid, either microencapsulated or in soluble form. BALB/c mice were dosed on day 1 with 25 Lf units and boosted on day 7, again with 25 Lf units. (n=5 *per* group; mean ±s.d).

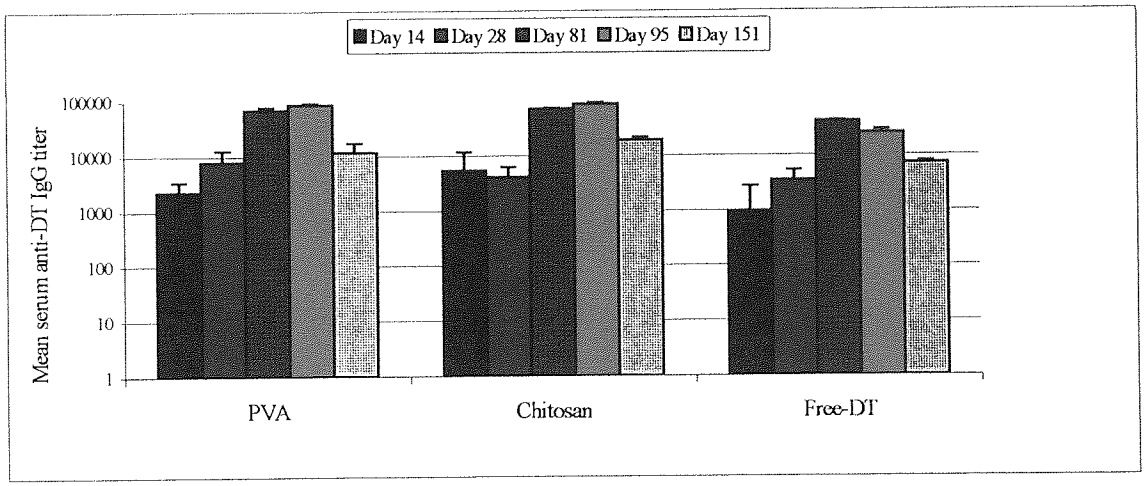


Figure 3.11: Serum immune response to nasally delivered diphtheria toxoid, either microencapsulated or in soluble form. BALB/c mice were dosed on day 1 with 25 Lf units and boosted on day 67, again with 25 Lf units. (n=5 *per* group; mean ±s.d).

3.4. Discussion and conclusions

3.4.1. Particle size

The potential of the nasal route as a mucosal site for drug and vaccine delivery has now been firmly established. The nasal mucosa possesses many advantages for drug and

vaccine delivery, including a highly vascularized epithelium of considerable surface area. Conditions in the nasal cavity may be less harsh than those present in the GI tract. This is due to the lower enzymatic activity and the reduced exposure to low pH. The existence of nasal-associated lymphoid tissue, which has a role that is analogous to that of the GALT, is thought to be of particular importance for the uptake of particulate carriers for the purposes of immunization. Several studies have demonstrated the potential of PLG microparticles as vaccine carriers for intranasal delivery. The intranasal immunization of microencapsulated *Bordetella pertussis* antigens has been shown to give protective immunity in mice (Shahin *et al.*, 1995). Intranasal immunization with microparticles has also induced protection in mice against aerosol challenge with ricin toxin (Yan *et al.*, 1996). In our laboratories, the intranasal delivery of tetanus toxoid in microparticles has been shown to induce markedly higher systemic and local immune responses than the free toxoid (Almeida *et al.*, 1993; Eyles *et al.*, 1999). Relatively few studies have been carried out to demonstrate the adjuvant effect of different sized microparticles after nasal administration. Particle size is thought to be a key factor in uptake following oral administration and it would appear that particles of certain compositions in the size range 50 to 3000 nm are capable of uptake and translocation (Eldridge *et al.*, 1989; Kofler *et al.*, 1997; Matsunaga *et al.*, 2000). There is considerable evidence to show that uptake increases with decreasing particle diameter within this range. Again following oral administration increased immune responses in addition to increased uptake with PLA particles of smaller size ranges has been shown (Alpar *et al.*, 1989; Tabata *et al.*, 1996). The results obtained here confirm a similar trend following intranasal delivery, with markedly higher immune responses following immunization with particles of smaller mean diameter.

3.4.2. Adsorption of antigens to particles

There are a number of issues, which are of concern and may need to be improved if biodegradable microsphere delivery systems can realise their potential as carriers for vaccines. These include the preservation of antigen integrity and immunogenicity during the encapsulation process with concomitant exposure to organic solvents and high shear stresses as well as post administration or during storage when the antigen can be exposed to a low pH caused by the degradation of the polymer. An alternative

approach is to adsorb antigens to the surface of biodegradable polymer particles. This is a simple procedure, which may help to ameliorate the effects of pH changes due to bulk polymer degradation and the use of solvents, and therefore should be less damaging to the vaccine. Furthermore, it should be much easier to scale up the production of vaccine for a clinical study and eventual full-scale manufacture. A recent study has shown that a potent immune response can be obtained after the intramuscular administration of influenza virus adsorbed onto polylactide lamellar particles (Coombes *et al.*, 1998) and this approach appears to show promise. The importance of the antigen location in microparticles on the immune response has been a matter of some conjecture. For instance, it has been reported that the levels of antibody elicited to influenza virus encapsulated in PMMA particles were considerably higher compared with the antigen adsorbed onto the particles (Kreuter *et al.*, 1976; Kreuter 1995) Similarly, It has been showen that encapsulated antigen performed better than adsorbed antigen (Eldridge *et al.*, 1993). Previously, in our labs it has been demonstrated that even surface adsorbed TT to microparticles elicited higher antibody responses after nasal or parentral administration compared with soluble TT (Almeida *et al.*, 1993). The study here corroborated that simple adsorption of antigen to particulate delivery microcarriers may be a useful strategy in vaccine delivery.

3.4.3. Positively charged PLA microspheres

Although biodegradable microspheres have been used extensively for systemic as well as mucosal immunization in animals the use of mucoadhesive PLA microspheres in the delivery for mucosal vaccines is limited. These experiments assessed the potential of utilizing agents with mucoadhesive properties in order to augment the immune response, facilitating uptake of microparticulate antigen by increasing the residence time of these formulations at mucosal surfaces.

Increasing the bioadhesion of PLGA microparticles has been shown to enhance particle uptake. The coating of microparticles with chitosan for the purposes of ocular drug delivery has been shown to enhance the bioavailability of indomethacin compared to uncoated particles (Calvo *et al.*, 1997). In this study, investigators used another cationic agent (poly-L- lysine) in order to coat microparticulate drug carriers. Poly-l-lysine coating was shown to be unable to enhance bioavailability of the drug, which may

indicate that it is not only the positive charge of chitosan, which is responsible for determining the observed enhanced bioavailability. Indeed, the mechanism of action may be independent of the positive charge. Recently, chitosan coating of PLGA nanospheres was shown to be effective for oral delivery of elcatonin (Kawashima *et al.*, 2000). For the application of vaccine formulations, bioadhesive PLGA microparticles have been shown to enhance mucosal immune responses to *Streptococcus sobrinus* glucosyltransferase following intranasal administration (Smith *et al.*, 2000) using gelatin as the mucoadhesive agent. In another study, the mucoadhesive additive, carboxy vinyl polymer, was used in order to enhance immune responses to orally delivered ovalbumin formulated in microparticulate carriers (Kunisawa *et al.*, 2000). The results shown by temporal variation in the boosting regime (i.e. boosting at day 7 or day 67 figures 3.8 and 3.9) are consistent with the accepted kinetics of the immune (and particularly antibody mediated) response. It is generally assumed that in the time between the initial and secondary exposure of the immune system to antigen, the antibody recognising such antigen develops an increased affinity for the antigen by processes of somatic hypermutation, subsequent restimulation of B cells, selection for higher affinity B cells for proliferation and the ensuing production of antibody with increased affinity for antigen. This process is referred to as affinity maturation. In addition, the boosting at day 7 is before the isotype switching of B cells and the ability of the immune system to mount a fully effective secondary immune response may be not yet fully elucidated.

Where the dose is concerned, a lack of potentiating effect at higher doses may not be wholly unexpected. In a study by Igartua *et al.* (Igartua *et al.*, 1998) when comparing the IgG immune responses of mice evoked by 1 or 10μg encapsulated into microspheres or 10 μg of free BSA, antibody levels obtained with microspheres at a dose of 10 μg BSA were observed to be greater than those after a dose of 1 μg BSA encapsulated for the duration of their experiment. However, no advantage was observed by administering a dose 10-times higher. It is generally thought that the immune system does not respond in a quantitative way and that there is not a proportional relationship between the dose administered and the subsequent observed immune response (Igartua *et al.*, 1998). The physico-chemical characteristics of microparticles are believed to regulate the targeting of microparticles through the efficiency of their absorption from

the gut lumen by the Peyer's patches and, in particular, the surface characteristics are thought to be important (Kim *et al.*, 1999; Li *et al.*, 2000). Therefore, alterations in the surface characteristics such as the surface charge and bio or muco-adhesive properties, in the form of chemical modifications of the polymer or in the form of coatings, may profoundly affect the efficiency with which the microparticles target the delivery of bioactive agents to mucosally associated lymphoid tissues and to APC. Many coatings have been used and examples include; chemicals, polymers, antibodies, bioadhesives, proteins, peptides, carbohydrates, lectins (Baras *et al.*, 1999; Delgado *et al.*, 1999; Kaiserlian and Etchart 1999). In our studies, the formation of chitosan-coated microparticles was achieved by a single step procedure that involved the incorporation of the positively charged polysaccharide in the microparticle formation medium. Here, the anti-DT serum antibody responses in mice given DT-alone were shown to be very weak. In contrast, chitosan-coated microparticles enhanced the serum antibody response to the intranasally administered DT. At all time points the mean serum IgG titres of chitosan-coated DT loaded microparticles immunized mice were significantly higher than those of the free DT immunised mice. The mechanism of action chitosan in enhancing serum antibody responses to i.n. applied diphtheria toxoid will require further investigation.

It may be that chitosan coated microparticles facilitate greater uptake of antigens across the nasal mucosa by slowing down mucociliary clearance, thus maintaining the contact of antigen with mucosa for a greater length of time (Lehr *et al.*, 1992; Aspden *et al.*, 1995; Lehr 2000).

It has also been shown that chitosan transiently affects the permeability of epithelia by modulating the gating properties of tight junctions. Chitosan transiently enhanced the nasal absorption of insulin (Illum *et al.*, 1994). However, the size of the antigen is much greater than that of insulin. It may be, therefore, that chitosan augments the immuonogenicity of antigens by a means other than merely increasing uptake across mucosal epithelia. Recently, chitosan was shown to enhance the systemic immune responses to several i.n. administered antigens (Jabbal-Gill *et al.*, 1998; Bacon *et al.*, 2000) Somavarapu *et al*1999, Bramwell *et al.*, 2000). Some studies have shown that chitosan can activate components of the non-specific immune system such as macrophages and can induce non-specific immunity to bacteria, fungi, and tumors.

In the studies outlined here, new microparticulate systems for nasal delivery of vaccines were developed and their capacity to enhance the immune response to nasally delivered diphtheria toxoid shown. Strategies for chitosan incorporation into PLA microparticles were based on approach that combines the potential of PLA microparticles as nasal carriers with the advantages conferred by the use of the cationic mucoadhesive agent, chitosan. The ability of the chitosan to enhance immune responses to an already potent adjuvant in PLA microspheres was clearly shown by these studies.

In summary, the main objective of this chapter has been to develop, optimize and improve particulate delivery systems for the intranasal administration of vaccines. It has been shown that improvements can be made by the careful manipulation of parameters such as size, antigen loading and surface charge and shape of particulate delivery systems. In particular, PLA microspheres prepared with chitosan may offer further potential for the enhancement of immune responses after mucosal delivery of vaccines.

CHAPTER: 4

4.0. Chitosan as a systemic and mucosal adjuvant

4.1. Introduction

The development of mucosal vaccines could be very useful for protection against pathogens that infect the host through mucosal surfaces. Mucosal vaccination offers a number of advantages over systemic vaccination (Chen 2000). It is a very efficient way to stimulate mucosal immunoglobulin A (IgA), which can prevent microbial adhesion and invasion of the host tissues. Moreover, mucosal delivery of antigens can induce secretion of antigen-specific IgA antibodies in mucosal areas distant from the site of immunization. Finally, in addition to secretory IgA, mucosal immunization potentially elicits high IgG and IgA responses at the systemic level (Russell *et al.*, 1999). These properties, together with the noninvasive nature of mucosal antigen delivery, make mucosal vaccination a potentially successful and flexible means to prevent both infection and disease. The administration of protein antigens directly to mucosal surfaces results in little or no response and can even lead to tolerance. Thus, mucosal adjuvants are essential for mediating mucosal as well as systemic responses to proteinaceous vaccine antigens. At this time, the lack of safe and effective mucosal adjuvants that augment secretory and systemic immunity is a limiting factor in the development of mucosal vaccines.

A number of adjuvants and/or carriers have been assessed as mucosal adjuvants including liposomes, microspheres, proteosomes, oligodeoxynucleotides and bacterial toxins (Freytag and Clements 1999; Pizza *et al.*, 2001). The most widely studied of these are cholera toxin (CT) from *Vibrio cholerae* and heat-labile enterotoxin (LT) from *Escherichia coli*. CT and LT efficiently promote secretory IgA in the mucosa but are too toxic for general use with vaccines for humans. Several groups are currently developing mutant forms with enhanced safety profiles (Park *et al.*, 2000). Ideally, an adjuvant should potentiate long-lasting expression of functionally active antibodies, elicit cell-mediated immunity (CMI), and enhance production of memory T- and B-lymphocytes with highly specific immunoreactivity against an invading antigen. In

addition to providing protection against immediate challenge with a foreign antigen, these responses should also provide protection against future antigen encounter by the host. An important consideration is the safety of the adjuvant concerning toxicity. The efficacy of an adjuvant is therefore described in terms of a balance between immune potentiation and toxicity.

4.1.1. The causative agents of tetanus and diphtheria and current strategies for vaccination

The antigens used in this chapter are tetanus, diphtheria toxoids, and subunits of plague vaccine V and F1. Tetanus is caused by *Clostridium tetani*, a Gram positive, anaerobic bacillus that forms spores found in soil and faeces. *Clostridium tetani* is a non-invasive opportunist that relies on spore introduction through damaged skin. Clinical symptoms of tetanus are mediated by the tetanospasmin neurotoxin. The toxin blocks inhibition of spinal cord reflex arcs, causing muscle rigidity and contraction, and interferes with release of transmitters in autonomic nerves. Autonomic dysfunction may manifest in the form of labile hypertension, tachycardia and other cardiac arrhythmias, pyrexia, peripheral vascular constriction, and sudden cardiac death.

The causative agent of diphtheria is *Corynebacterium diphtheriae*, nonsporulating gram-positive bacteria found only in humans. The bacillus is non-invasive, ordinarily residing in the superficial layers of the respiratory tract and sometimes in skin lesions. Transmission is achieved through contact with either respiratory secretions or infected lesion exudates. Symptoms include fever, malaise, sore throat and the development of a leathery membrane that covers the pharynx, including the tonsillar areas, soft palate and uvula. The virulence of *C. diphtheriae* results from the action of its potent exotoxin, which interferes with mammalian protein synthesis. In proportion to their severity, local symptoms of diphtheria may be accompanied by systemic complications, secondary to absorption and dissemination of exotoxin. Many infected individuals develop potentially fatal myocarditis, or peripheral neuritis resulting in paralysis (Duff 1999).

Current diphtheria and tetanus vaccines consist of formaldehyde treated (toxoid) toxins of molecular mass 65 and 150 kDa respectively. Protection from diphtheria and tetanus intoxication is mediated by appropriate humoral responses that must be of sufficient specificity and magnitude to facilitate neutralisation (Ourth 1974). Despite the existence

of these vaccines, tetanus and diphtheria still constitute major public health problems in many areas of the world (Duff 1999). Tetanus kills 400,000 children annually (Stanfield and Galazka 1984). The requirement for multiple injections of the currently licensed diphtheria and tetanus vaccines dictates that there is often comparatively poor coverage in countries where economic or logistical factors preclude this. For this reason, the WHO has indicated that the development of improved immunization strategies for these diseases is a priority (Galazka *et al.*, 1995; Chen *et al.*, 2000). In the case of diphtheria, this has been given added impetus following recent epidemics in Eastern Europe, which threaten to spread unchecked, exploiting the incomprehensive vaccine coverage that now exists in many areas.

The presence of toxin-neutralizing antibodies induced by immunization with diphtheria toxoid is very important for preventing this disease. The administration method for DT vaccination is generally *via* a subcutaneous or intramuscular injection although oral administration of toxoid incorporated into lozenges and inhalation of aerosolized toxoid has also been tried (Meyer *et al.*, 1974; Perry and Fetherston 1997). The latter of the two methods necessitated the administration of very high amounts of antigen or induced various allergic reactions. Recently, intranasal administration of DT with non-ionic excipients in mice (Gizurarson *et al.*, 1995) or with an enhancer mixture of caprylic/capric glycerides and polysorbate in humans (Aggerbeck *et al.*, 1997) has been reported. In this study, we have explored the potential for administration of DT both as a clinically relevant antigen in its own right and as a model antigen *via* the intranasal route. We have examined anti-DT-specific serum IgG and mucosal IgA antibody responses to DT given intranasally with chitosan as an adjuvant in mice.

4.1.2. *Yersinia pestis* and vaccination against plague

Plague is an extremely virulent and potentially lethal infection caused by the Gram-negative bacterium *Yersinia pestis*. The current vaccine used to immunize against plague often fails to engender 100 % protection against inhalational infection with *Y. pestis*. Similarly, logistical factors favor the development of non-parenteral immunization protocols to counter plague. Recently an improved parenteral vaccination strategy for plague, based on the recombinant subunit approach, has entered clinical trials. *Y. pestis* is typically transmitted to the human host *via* the bite of an infected flea,

the flea having previously fed on an infected rodent. Once in the host, the activity of the plasminogen activator protease complex secreted by *Y. pestis* promotes release and dissemination of the bacteria from the site of the bite to establish a new infection (Goguen *et al.*, 1995);(Sodeinde *et al.*, 1992). This route of transmission establishes bubonic plague, so-called because of the massive enlargement of lymph nodes or buboes that drain the site of the fleabite. Alternatively, septicaemic plague develops when there is bacteremia without the development of buboes. Septicaemic plague is characterised by fever, headache, and malaise with gastrointestinal involvement. If untreated, the bubonic form of the disease can progress to a generalised bacteremia with a secondary infection of the lung, to establish a secondary pneumonic plague. Plague can also be transmitted from an infected person (or animal) to a healthy one by the aerosol route, to establish a primary pneumonic infection (Perry and Fetherston 1997; Koornhof *et al.*, 1999). Following transmission to a susceptible host, the life cycle of *Y. pestis* is largely extracellular, after a brief, but essential, intracellular phase. The invading bacteria may be taken up by macrophages or polymorphonuclear cells such as neutrophils. Uptake of the bacteria by neutrophils is lethal for *Y. pestis*. Conversely, uptake by macrophages is advantageous to the bacteria because it can survive phagocytosis by these cells. Within macrophages, the bacteria exploit the conditions of optimum pH and low calcium to trigger the expression of a panel of virulence factors that contribute to anti-host activity. These include the pH6 antigen (thought to promote the spread of the organisms, during its intracellular phase, from infected to naïve macrophages), the F1 capsular protein, and the *Yersinia* outer proteins (Yop's) (Meyer *et al.*, 1974; Straley and Bowmer 1986). Subsequently, the fully virulent bacteria residing in macrophages may induce apoptosis of the macrophage. The latter phase is associated with a high-level of bacteremia such that blood is extravasated from major organs such as the liver, giving typical 'pale liver' morphology. Death results from an overwhelming septic shock. If exposure to infection is detected early enough, plague infection can be treated. The difficulty is that diagnosis of infection needs to be made before the onset of symptoms. Furthermore, antibiotic resistant strains have been isolated (Perry and Fetherston 1997). The most feared and virulent form of the disease is that of primary pneumonic plague, where infection can go undetected until the onset of symptoms. The mortality rate for individuals with primary septicaemic plague is also

high (30-50 %), probably because the antibiotics used to treat undifferentiated sepsis are not effective against *Y. pestis*. Similarly, if either septicaemic or bubonic plague progresses to a secondary pneumonic infection, the disease is very advanced and the fatality rate is high (Bolin *et al.*, 1988).

Y. pestis caused three major pandemics of disease in the 1st, 14th-17th and 19th centuries. Together these pandemics are thought to have caused 200 million deaths (Perry and Fetherston 1997). The second pandemic of plague (the Black Death) is estimated to have killed 30 % of Europe's population. Although *Y. pestis* no longer causes disease on this scale, it still presents a major public health problem in many parts of the world, notably Africa, Asia, China, Indonesia, South America and the southwestern United States. During the period 1967-1993, the WHO reported an average of 1666 plague cases *per* year world wide, with an average fatality rate of approximately 10 % (Perry and Fetherston 1997). These figures are in fact probably an underestimate, due to failure to diagnose and report all cases. Epidemics continue to occur, for example in Zaire in 1999, in Peru in 1992-1994 and in India in 1994. Whilst these epidemics are mostly of the bubonic form, a pneumonic plague outbreak in Indian city of Surat in Gujarat state in 1994 caused 6300 suspected cases and 54 fatalities (Ramalingaswami 1995).

Most vaccines, those currently used for immunization against plague were largely empirically developed. More recently, efforts have focused on the rational design of new approaches to plague immunization. In particular, development of a safe and effective subunit vaccine for plague based on the F1 and V proteins.

In light of the drawbacks and limitations associated with the killed whole cell (KWC) vaccines, there is a strong impetus to develop improved plague vaccines. The F1 (17.5 kDa) and V (35 kDa) subunits are natural virulence factors produced by *Y. pestis* (Perry and Fetherston 1997). F1 and V antigens were first demonstrated to be highly protective, conferring a degree of protection equivalent to that engendered by EV76 KWC vaccines, in 1995 (Williamson *et al.*, 1995). The fraction 1 (F1) antigen is a capsular protein that has anti-phagocytic properties (Meyer *et al.*, 1974). Virulence (V) antigen is a protein secreted by the *Y. pestis* bacterium under conditions of low calcium (Price *et al.*, 1989). V is thought to have an important role in the regulation of delivery

of other Yop's, that probably have an array of anti-host activity, into the host mammalian cell (Straley and Bowmer 1986). Recently, the F1 and V antigens have been produced as recombinant proteins (Liljeqvist and Stahl 1999) using genetically modified *E. coli* (Leary *et al.*, 1995). A recombinant fusion of F1 and V has also been developed (Anderson *et al.*, 1998). It has been shown that injection of aluminium hydroxide adjuvantised F1 and V into mice and guinea pigs is able to confer protection against aerosolised or injected challenges with high doses of virulent *Y. pestis* bacteria. Although the F1 and V subunits offer distinct advantages in comparison to previous plague vaccines, like most proteins, they are usually comparably weakly immunogenic when administered in the absence of an appropriate adjuvant (Mahon *et al.*, 1998). Currently licensed adjuvants provide limited relief in this respect, although there are still notable drawbacks associated with the use of aluminium salts for enhancing immunity to subunit vaccines (Gupta *et al.*, 1995). In particular, antigens adjuvanted with aluminium salts are not suitable for freezing/lyophilistaion or mucosal administration, and can induce specific IgE responses (Cox and Coulter 1999).

4.1.3. Chitosan: Properties and applications

Chitosan, β-(1-4)-2-amino-2-deoxy-D-glucan, is a linear polysaccharide derived by deacetylation of the natural polymer chitin, is a polycation at acidic pH values where most of the amino groups are protonated and has an apparent pKa of 5.5 (Muzzarelli *et al.*, 1999). Its biodegradability is facilitated by lysozyme, it has low toxicity in humans and these facets have resulted in increased interest in chitosan as an immunopotentiating agent. The mucoadhesive properties of Chitosan have attracted much of attention in recent years as a potential absorption enhancer across mucosal epithelia (Artursson *et al.*, 1994) (Illum *et al.*, 1994; Aspden *et al.*, 1996). It has been shown that chitosan is able to reduce the transepithelial electrical resistance *in vitro* of a cultured intestinal epithelial cell line Caco-2 (Artursson *et al.*, 1994). Chitosan was able to increase the transport of hydrophilic molecules such as [^{14}C]-mannitol and fluorescein-dextrans significantly in Caco-2 cell monolayers. Similarly, the transport of the peptide drug 9-desglycinamide, 8-arginine vasopressin (DGAVP, MW 1299.5) was increased markedly after co-administration with chitosan glutamate in a Caco-2 cell culture (Artursson *et al.*, 1994; Dodane *et al.*, 1999). Chitosan salts such as chitosan glutamate and chitosan

hydrochloride have been used *in vivo* as absorption enhancers for peptide drugs. The nasal application of insulin with chitosan glutamate at pH 4.4 led to a significant reduction in the blood glucose levels of rats and sheep (Illum *et al.*, 1994), while the intraduodenal application of buserelin and chitosan hydrochloride in a gel formulation at pH 6.7 increased the absolute bioavailability of buserelin from 0.1±0.1% to 5.1±1.5%. The increase in the transport of these compounds is believed to be a result of an interaction of the positively charged amino groups on the C-2 position of chitosan with negatively charged sites on the cell membranes and tight junctions, thereby altering the integrity of the tight junctions to allow for paracellular transport (Artursson *et al.*, 1994; Dodane *et al.*, 1999; Kotze *et al.*, 1999). Additionally, mucoadhesion may play an important role in this process by increasing the residence time of the drugs at the cell surface. However, the increased transport of all these compounds was obtained in acidic or neutral conditions, where (in most studies) the pH was less or in the order of the pKa (6.0 to 6.5) of chitosan. This suggests that the charge density of chitosan is an important factor for the enhancement of mucosal transport (Schipper *et al.*, 1997; Kotze *et al.*, 1999). Chitosan is a weak base and in neutral and basic environments the chitosan will relinquish its positive charge and precipitate from solution. Under these conditions chitosan could be ineffective as an absorption enhancer. Recent studies indicate that the binding of chitosan to epithelial membranes resulted in cellular F-actin depolymerization and disbandment of the tight junction protein ZO-1. This property is currently considered to be the major mechanism by which chitosan enhances drug adsorption (Schipper *et al.*, 1997). *In vivo*, chitosan is degraded by several glycosidases, primarily lyso-zyme and N-acetylglucosaminidase (Muzzarelli *et al.*, 1999). Chitosan also has stimulatory effects on macrophages and this activity was suggested as being mainly attributable to the N-acetyl-glucosamine groups (Peluso *et al.*, 1994). Trimethyl chitosan chloride (TMC) is a quaternised derivative of chitosan, but compared with chitosan, TMC is endowed with superior aqueous solubility over a broader pH range (Thanou *et al.*, 1999). Thus TMC retains penetration-enhancing properties under physiological conditions. TMC's, with different degrees of substitution, have been tested for penetration enhancing properties *in vitro* (Kotze *et al.*, 1999). *In vivo*, TMC derivatives can act as safe penetration enhancers for peptides administered by a variety of non-parenteral routes (Thanou *et al.*, 2000). Both chitosan and TMC are

mucoadhesive, and thus both polymers have the potential to reduce the effects of mucociliary clearance for nasally applied therapies. Because of their high molecular weight and hydophilicity, chitosan and TMC are unlikely to interact with cell membrane components or induce transcellular transport of hydrophilic compounds. To reinforce this, to date, no acute cellular toxicity has been reported following mucosal application of chitosan or TMC (Thanou *et al.*, 1999). Thus, in terms of the induction of deleterious effects on epithelial membranes, it seems likely that, if effective, this type of penetration enhancer may have good potential for clinical use as a mucosal immunological adjuvant. The aim of this investigation was to evaluate the effect and potential of different chitosan derivatives and chitosan salts (chitosan glutamate and chitosan hydrochloride) both alone and in combination with other adjuvants, specifically alum, saponin (Quil-A) and CTB in their ability as immunostimulants. In this chapter, a diversity of candidate, subunit and model antigens are used in order to more comprehensively elucidate the effectiveness of chitosans as immunoadjuvants.

4.2. Materials and Methods

4.2.1 Materials

Chitosan hydrochloride (113, low viscosity), Chitosan hydrochloride (213, high viscosity), Chitosan glutamate (113, low viscosity) and Chitosan glutamate (213, high viscosity) were obtained from Pronova Biopolymers, Norway. Carboxymethyl chitosan was from Carbomer, Canada. Cationic starch was from Sigma Chemical Co. Poole, Dorset, UK. Trimethyl chitosan (TMC-60, TMC-40 and TMC-20) was supplied for collaborative work by professor Junginger, Netherlands. All other chemicals were obtained from Sigma Chemical Co. Poole, Dorset, UK or as described in chapter 2 (materials and methods).

4.2.2. Methods

4.2.2.1. Preparation of chitosan microspheres by spray drying method

Chitosan chloride or chitosan base (high molecular weight) 0.1 to 0.4 grams were weighed into a 50 ml beaker, and 20ml of water or 1% v/v acetic acid aqueous solution

was added to dissolve the chitosan. 5% w/w BSA was added to the chitosan solution, the resulting solution was transferred into a 100 ml glass-stoppered conical flask, diluted to volume and kept overnight to allow full dissolution. 100ml of the chitosan was measured into a 250 ml beaker, and used for the spray dry process. Co-current spray drying was performed using a SD-04 spray drier (Lab Plant, England), with a standard 0.5 mm nozzle. The inlet temperature was controlled at 100°C. The spray rate was controlled at 6 ml/min^{-1}. The compressed spray air flow (represented as the volume of the air input) was set at 10 l/min^{-1}. The product was collected and stored in a dessicator at 4°C.

4.2.2.2. Characterization of spray–dried microparticles

The protein content of bovine serum albumin (BSA) loaded chitosan microspheres was determined by the BCA method. Samples were dissolved in 0.1% v/v acetic acid, precipitated with NaOH and then centrifuged to remove chitosan prior to BCA analysis. Microparticles were sized by a Malvern laser diffraction analyzer following suspension in isopropanol containing 0.1% v/v tween 20 and bath sonication for one min. Morphological assessment of microparticles was performed using scanning electron microscopy.

4.2.2.3. Immunization schedule for BSA loaded spray dried chitosan microspheres

Female BALB/c mice were vaccinated at 7-9 weeks of age. Four groups of mice (n=5) were used in this study. 5 μg of encapsulated BSA was delivered intranasally into the mouse nostrils with micropipette on day 1 and animals were boosted on day 14. Group B1 was dosed with chitosan (base) microspheres containing 5 μg equivalent of BSA in 20 μl of PBS. Group B2 was dosed with chitosan chloride microspheres containing 5μg equivalent of BSA. Group B3 was dosed with 20 μg of free BSA nasally. Untreated animals were used as a control. Blood samples were collected from the tail vein and the sera analysed for the presence of BSA specific antibodies using ELISA as outlined in chapter 2.

4.2.2.4. Immunization schedule for intranasal administration of DT with chitosan

Female BALB/c mice (25 g, 6-week-old) were lightly anaesthetized with an inhaled gaseous mixture of 3% (v/v) halothane (RMB Animal Health, UK) in oxygen (300 cm^3 min^{-1}) and nitrous oxide (100 cm^3 min^{-1}) for i.n. dosing procedures. 5 Lf DT in the presence and absence of 0.2 % w/v different chitosans or cationic starch was administered intranasally to BALB/c mice (n=5) using a micropipette. Each mouse received a 20 µl volume (10 µl *per* nostril), of particle suspension or protein solution as appropriate, administered with a micropipette. All experimentation strictly adhered to the 1986 Scientific Procedures Act. Blood samples were taken as described previously and antibody responses assessed using ELISA as outlined in chapter 2.

4.2.2.5. Immunization schedule for intramuscular administration of alum adsorbed TT with chitosan, saponin or P121

Four groups of female BALB/c were immunized intramuscularly with 50 µl comprising 1µg of alum adsorbed TT either in PBS or 0.2 % w/v chitosan chloride solution or 0.2% w/v P121 or PBS containing 10µg Quil-A. Animals received a priming dose on day 1 and a booster dose on day 35. Blood samples were collected one week before and after the booster dose for analysis by ELISA as described in chapter 2.

4.2.2.6. Immunization schedule for intranasal administration of DT with combinations of adjuvants

5 Lf DT in the presence and absence of 0.2 % w/v different chitosans or cationic starch was administered intranasally to mice using a micropipette. Each mouse received a 20 µl volume (10 µl per nostril), of particle suspension or protein solution as appropriate, administered with a micropipette. 5 groups of mice (n = 5) were dosed with 5 Lf units of DT with various adjuvants. At day 1: Group 1 received 5 Lf units DT in saline. Group 2 received 5 Lf units DT in 0.2% w/v chitosan chloride (CL113) + 10 µg of CTB. Group 3 received 5Lf units DT + 10µg of CTB in PBS. Group 4 received 5 Lf units DT in 0.2% w/v chitosan chloride (CL113) + 7.5µg of Quil-A. Group 5 received 5 Lf units DT in PBS + 7.5µg of Quil-A. On day 28, all groups received booster doses comprising the

same formulations except that DT was at 2.5 Lf units. Blood samples were collected for analysis by ELISA as described in chapter 2. Vaginal washes were taken by washing with 150 µl of PBS containing 0.02% w/v sodium azide. Samples were centrifuged and supernatants stored at -20°C for analysis by ELISA. Fresh faecal samples were collected from animals whilst collecting blood and were dissolved in 500 µl of PBS containing 0.02% sodium azide as a preservative and 0.1% trypsin inhibitor. The solution was centrifuged and supernatants were stored at -20°C for analysis by ELISA.

4.2.2.7. Immunization schedule for intranasal administration of V and F1 with chitosan

Five groups of female BALB/c were nasally instilled 20 µl with F1 (5µg) and V (1µg) in the presence and absence of three different TMC derivatives (with increasing degrees of quaternisation: 20, 40 & 60%), and humoral immune responses engendered by these treatments compared to those evoked by co-administration of chitosan chloride and free V and F1 antigens. Blood samples were collected 1 week before and after the booster dose for analysis by ELISA as described in chapter 2.

4.3. Results

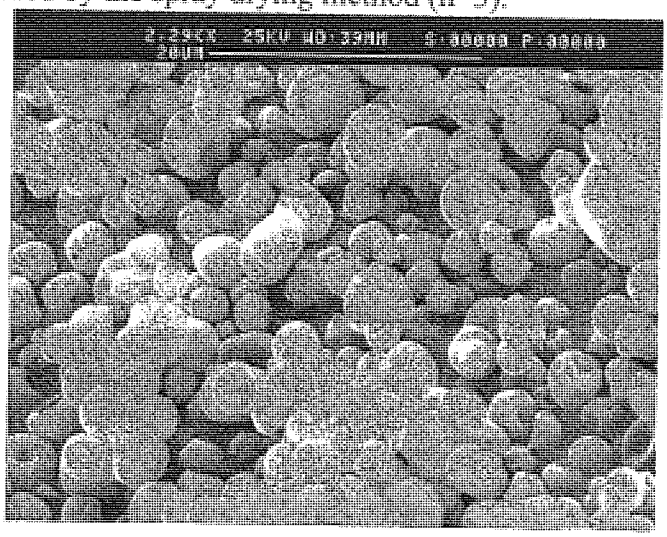
4.3.1. Characterization of BSA loaded microparticles

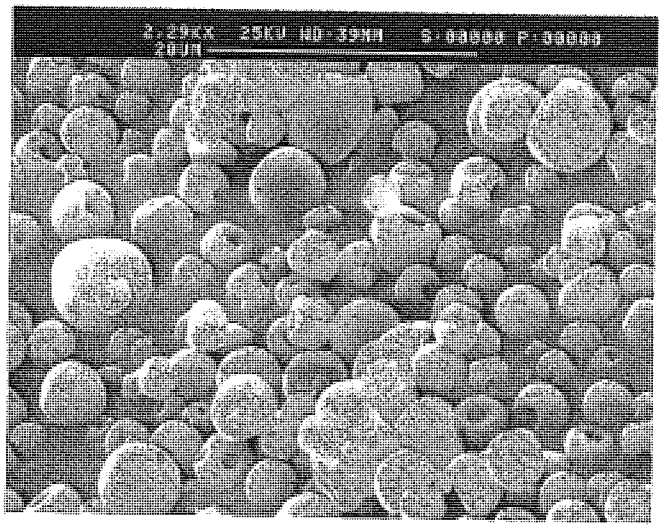
Properties of the spray-dried microparticles such as BSA loading and particle size are summarized in Table 4.1. Microspheres with an average diameter of ~ 5 µm and ~ 2 % w/w BSA loading were obtained using this method. Fig 4.1 shows the surface morphology of microspheres. Microspheres had depressions on the surface this may be due to fast evaporation of water from the microspheres, or due to high shear generated during the spray drying process.

Polymer	Theoretical load % w/w	Actual load % w/w	Efficiency of entrapment %	Particle size volume mean µm (±s.d)	Yield %
Chitosan (base)	2	1.96	98.0 (1.02)	3.18 (1.28)	34
Chitosan hydrochloride	2	1.94	97.4 (3.22)	3.26 (1.56)	36

Table 4.1: BSA loading, particle size and entrapment efficiency of chitosan microspheres prepared by the spray drying method (n=3).

a





b

Figure 4.1. Scanning electron micrograph of spray dried chitosan microspheres. a) Chitosan base. b) Chitosan chloride.

4.3.2. Stability of spray dried microencapsulated BSA

BSA is able to form oligomers, particularly under the conditions employed during spray drying, i.e.: higher temperatures. SDS-PAGE analysis of protein released from microparticles was performed and the results are shown in figure 4.2. No appreciable covalent BSA aggregates or degraded protein fragments were detected. This indicates that during the spray drying process, BSA remains stable. This result was in concordance with previous observations.

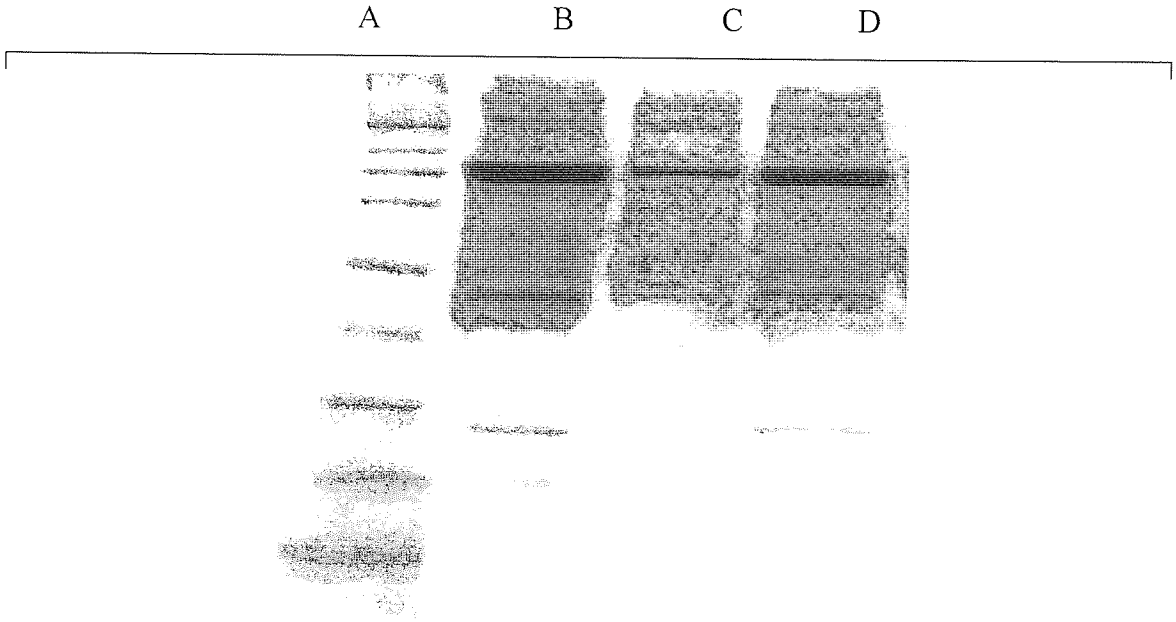


Figure 4.2: SDS-PAGE of BSA released from BSA loaded alginate microspheres. Lanes represent the molecular weight marker (A), native BSA (B), BSA released from chitosan base microspheres (C), BSA released from following liberation from spray-dried microspheres (D).

4.3.3. Immune response to BSA in spray dried chitosan microspheres

Intranasal administration of free BSA did not induce any detectable anti-BSA antibodies in plasma of mice analysed by ELISA(figure 4.3). In contrast, high titres of anti-BSA antibodies were induced in all mice when BSA was encapsulated in chitosan microspheres formulated using the spray drying method. Both chitosan chloride and chitosan base microspheres elicited a primary systemic immune response and considerably enhanced secondary immune responses (figure 4.3). Chitosan (base) gave a greater BSA specific IgG response than chitosan chloride microspheres, which was

approximately 40 times higher than those obtained for free BSA. This study shows that delivery of BSA in mucoadhesive chitosan microspheres enhances systemic immune response after nasal delivery, which is also dependent upon the nature of chitosan used in the preparation of microspheres.

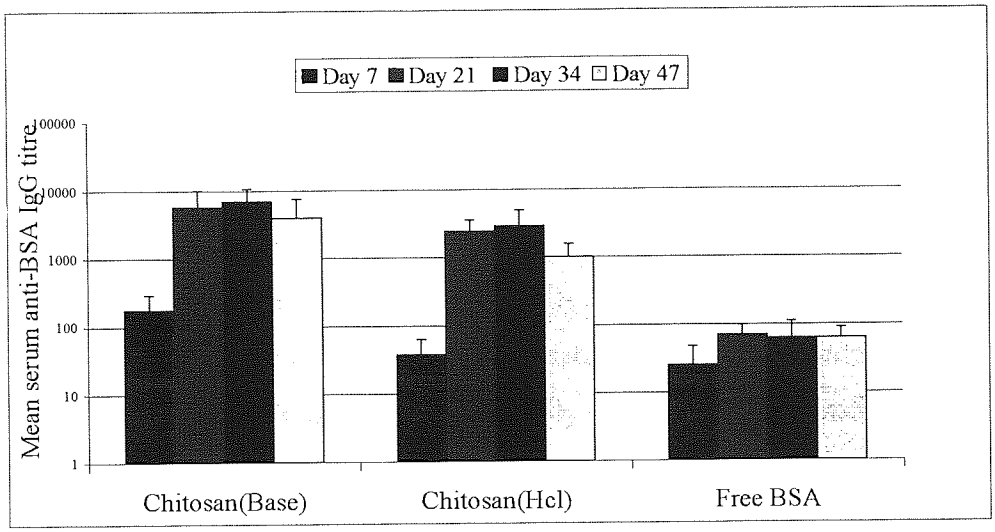


Figure 4.3: Anti-BSA specific IgG titres in serum of mice dosed intranasally with free BSA or BSA encapsulated in chitosan microspheres (chitosan chloride or chitosan base as indicated). On day 1, 5μg of microencapusalted BSA was delivered intranasally and boosted on day 14. In the case of free BSA 20μg of BSA was delivered nasally on day 1 and boosted on day 14 with same dose.

4.3.4. Effect of chitosan on the mucosal and systemic immunity to DT following intranasal immunization

4.3.4.1. Adjuvant effect of chitosan on antigen-specific serum IgG responses

To investigate whether intranasally administered chitosan, together with a soluble protein antigen, would act as an adjuvant for induction of systemic antigen-specific antibody responses, we performed a set of experiments with 5 chitosan derivatives which were chitosan chloride (low and high viscosity), chitosan glutamate (low and high viscosity), carboxymethylchitosan and cationic starch. Immunizations were carried out as indicated above and outlined in figure 4.4. When DT-specific serum IgG responses were measured following primary immunization, we observed that the titres in the group of mice that received DT with any of the chitosans were consistently higher than those of mice immunised with DT alone (figure 4.4). The group of mice which received DT with cationic starch, however failed to elicit antibody responses

significantly higher than the free antigen. The order of enhancement of serum antibody titres to DT with chitosans is: low viscosity chitosan chloride (180 times) >high viscosity chitosan chloride (135 times) > low viscosity chitosan glutamate (121 times) >carboxymethyl chitosan (CMC) (100 times) >high viscosity chitosan glutamate (65 times of that obtained with free DT which ware equivalent to preimmune mice). 0.2% w/v cationic starch failed to show any enhancement in immune response compared to free DT.After booster immunization, all groups elicited considerably higher immune responses. This observed secondary immune response to DT with chitosans was enhanced almost 100 fold in comparison to the primary immune response with the exception of the CMC derivative. Antibody titres to CMC formulations were enhanced in excess of 170 fold above the primary response. All formulations, with the exception of the cationic starch, elicited significantly higher antigen specific immune responses than those obtained for the free antigen (n = 5, P <0.05). The earlier trend prior to boosting, which showed a generalised trend towards increased immune responses with decreasing viscosity was not evident after the booster dose. The response obtained with the CMC formulation, however, was markedly increased in comparison to the other formulations. Taken together, these data clearly show that chitosan acts as an adjuvant for the induction of serum IgG responses to an intranasally co-delivered protein antigen. Enhancement of immune response depends upon the type of chitosan.

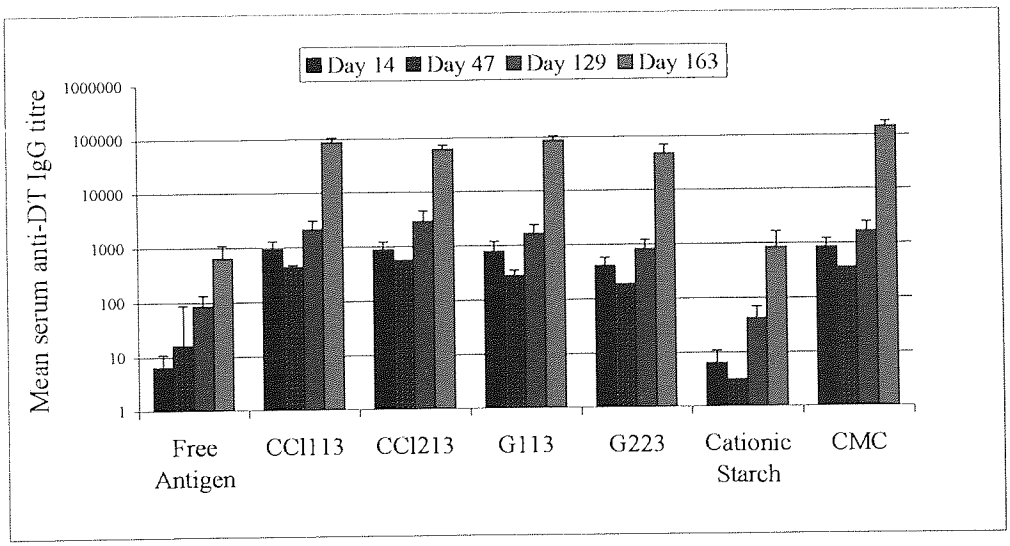


Figure 4.4: Serum IgG responses to DT delivered intranasally. Mice were immunised with 5 Lf DT on day 1 with or without adjuvant as indicated. Boosting was on day 153 with 2.5 Lf units (n = 5 *per* group; mean±s.d). CCl 113: Chitosan hydrochloride low viscosity, CCL213: Chitosan hydrochloride high viscosity, G113: Chitosan glutamate low viscosity, G213: Chitosan glutamate high viscosity, CMC: Carboxy methyl chitosan.

4.3.4.2. Mucosal immune response to intranasally administered DT with and without chitosans

The observation that intranasal administration of chitosan and DT resulted in high levels of antigen-specific serum IgG prompted us to investigate whether mucosal IgA responses were also induced. Lung washes and gut washes from the above mice were analysed for mucosal IgG and IgA antibody levels. Figure 4.5 shows that lung washes from mice receiving chitosan adjuvanted DT exhibited higher IgA and titres compared to lung washes of control mice, in which IgA responses were undetectable. Figure 4.6a and figure 4.6b shows that the gut washes of mice receiving chitosan exhibited high IgA and IgG titres compared to lung washes of control mice, where antigen specific IgA responses were very low.

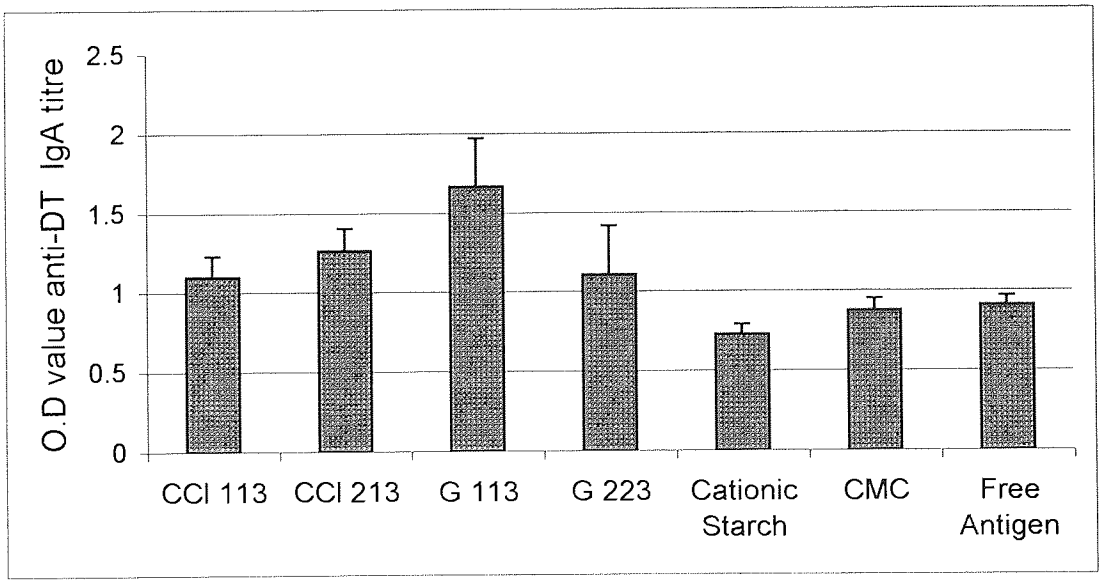


Figure 4.5: Mucosal immune responses in lung washes (IgA) to DT delivered intranasally. Mice were immunised with 5Lf DT on day 1 with or without adjuvant as indicated. Boosting was on day 153 with 2.5Lf units (n = 5 *per* group; mean±s.d).For all other information se Fig 4.4 legend.

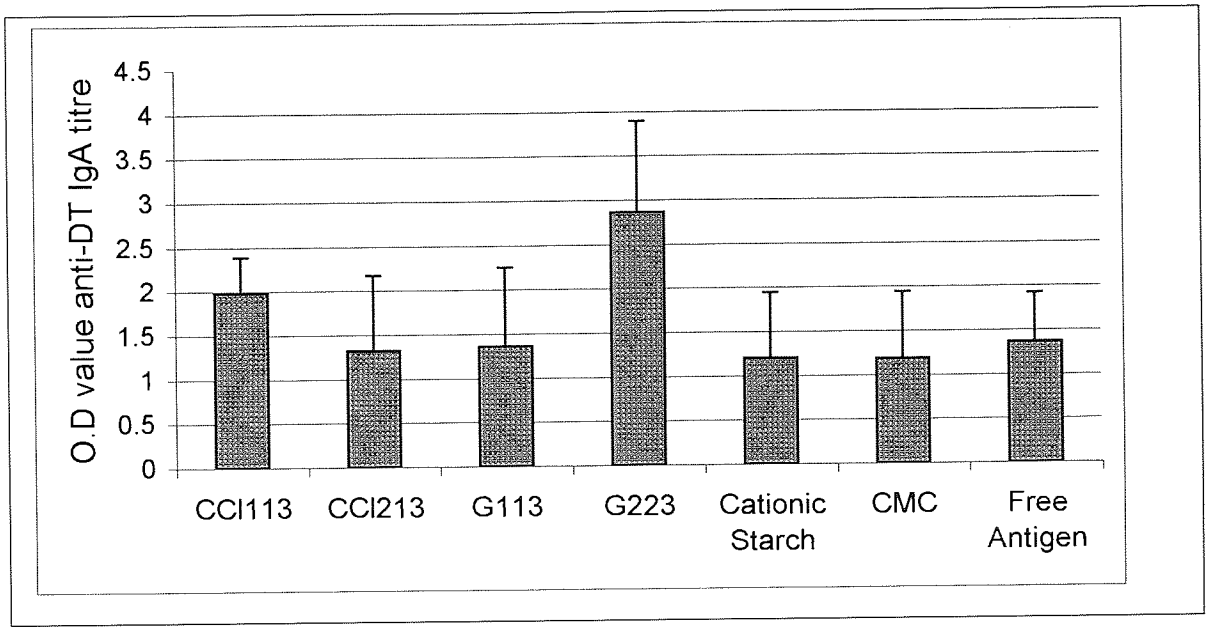


Figure 4.6a: Mucosal immune responses in gut washes (IgA) to DT delivered intranasally. Mice were immunised with 5 Lf DT on day 1 with or without adjuvant as indicated. Boosting was on day 153 with 2.5 Lf units (n = 5 *per* group; mean±s.d).

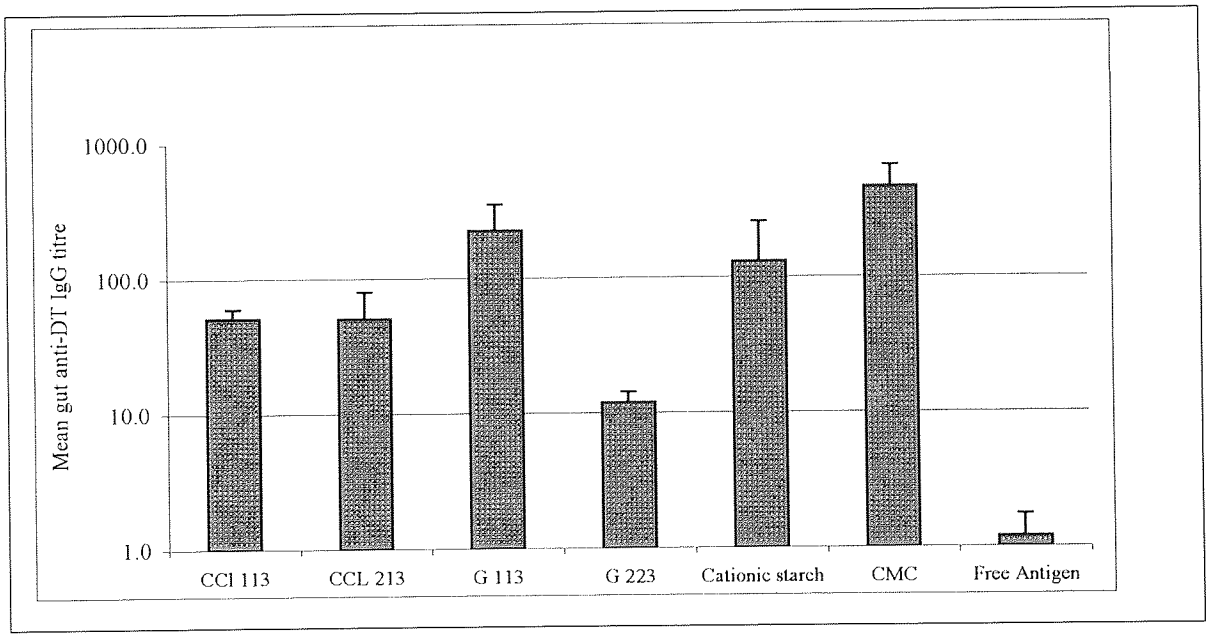
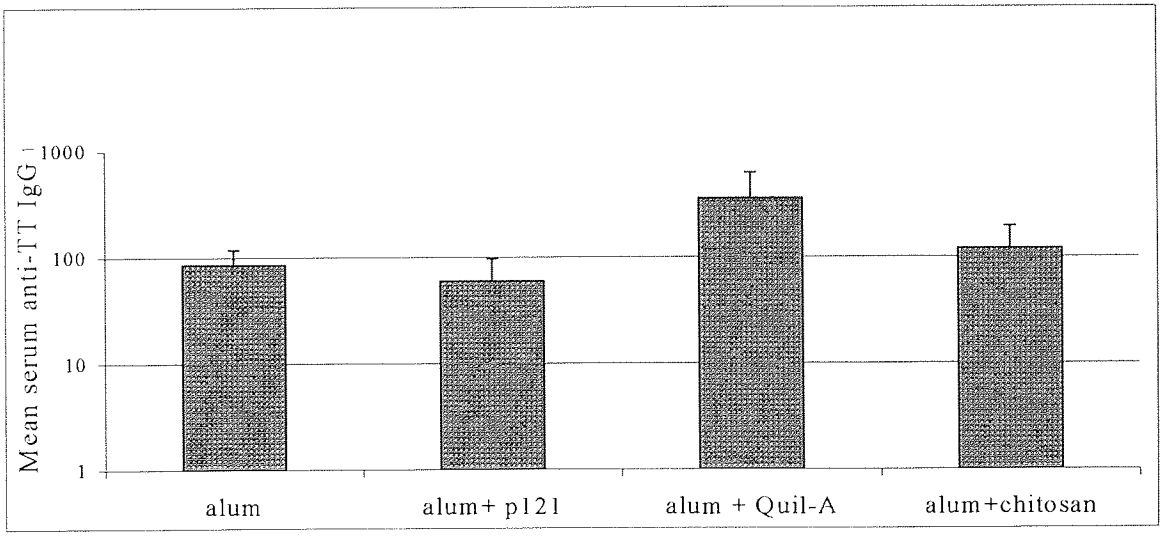


Figure 4.6b: Mucosal immune responses in gut washes (IgG) to DT delivered intranasally. Mice were immunised with 5 Lf DT on day 1 with or without adjuvant as indicated. Boosting was on day 153 with 2.5 Lf units (n = 5 *per* group; mean±s.d).

4.3.5. Effect of chitosan, block copolymer (P121) and Quil-A on immune responses to alum adsorbed TT after intramuscular administration

The addition of adjuvants to alum adsorbed TT administered intramuscularly was evaluated and the qualitative nature of the immune response examined. Groups of 5 mice were immunised intramuscularly as outlined in section 4.2.2.5. It has become clear that adjuvants can influence the IgG subclass profile (Allison and Byars, 1986). It has been noted in murine studies that aluminium hydroxide promotes primarily the production of IgG_1, whereas Qs-21 (saponin) promotes the generation of IgG_{2a}. In order to understand the influence of chitosan and other adjuvants on the type and degree of immune response to TT adsorbed alum, in these experiments, the efficacy of commercially available alum adsorbed TT vaccine was evaluated with the addition of different adjuvants and the antibody subclass profiles compared. Intramuscular administration of alum adsorbed TT showed (figure 4.7a), a primary immune response after one week. The serum specific TT IgG levels were highest in the group receiving Quil-A, followed by chitosan and P121, which elicited comparable responses. Compared to alum adsorbed TT, the addition of chitosan, P121 or Quil-A was found to further potentiate the immune response to TT. After boosting all groups showed enhanced serum immune responses and Quil-A augmented the immune response, achieving a 50-fold enhancement of TT specific IgG in comparison to alum adsorbed TT. These results are summarized in figure 4.7.

a



b)

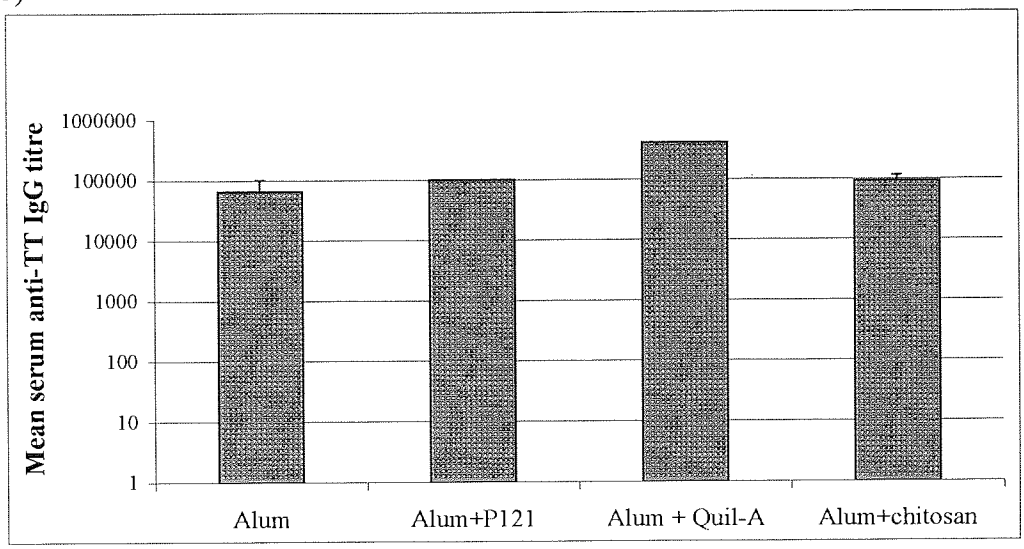
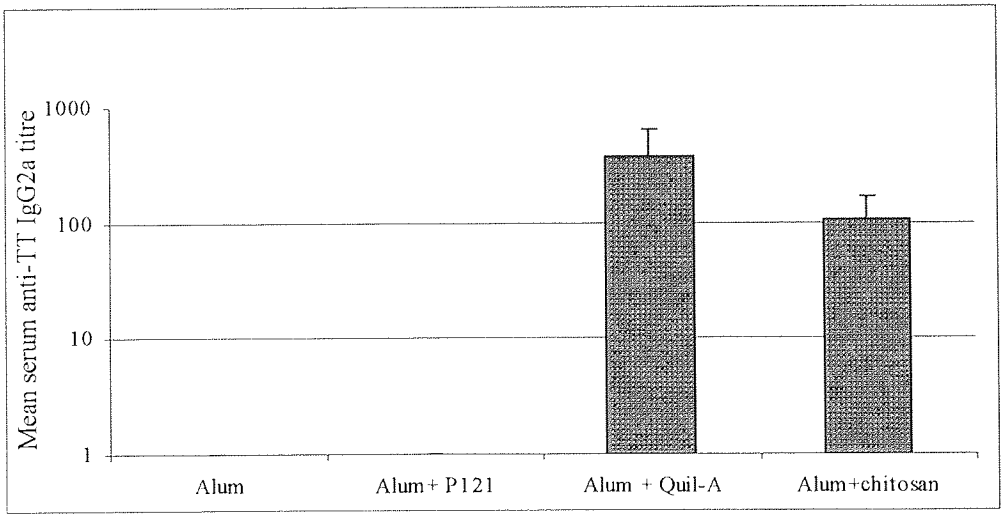


Figure 4.7: Serum immune response (IgG) to alum adsorbed TT after intramuscular administration in the presence and absence of additives. a) Primary response. b) Secondary immune response (n = 5 *per* group; mean±s.d).

After primary immunization, the groups of mice, which received alum, adsorbed TT in the presence of the saponin and chitosan were shown to generate significant titres of IgG2a. This was in contrast to the groups receiving alum adsorbed TT alone or in combination with the block copolymer, P121 where titres were negligible. Following boosting, the same trend was continued. These results are shown in figure 4.8.



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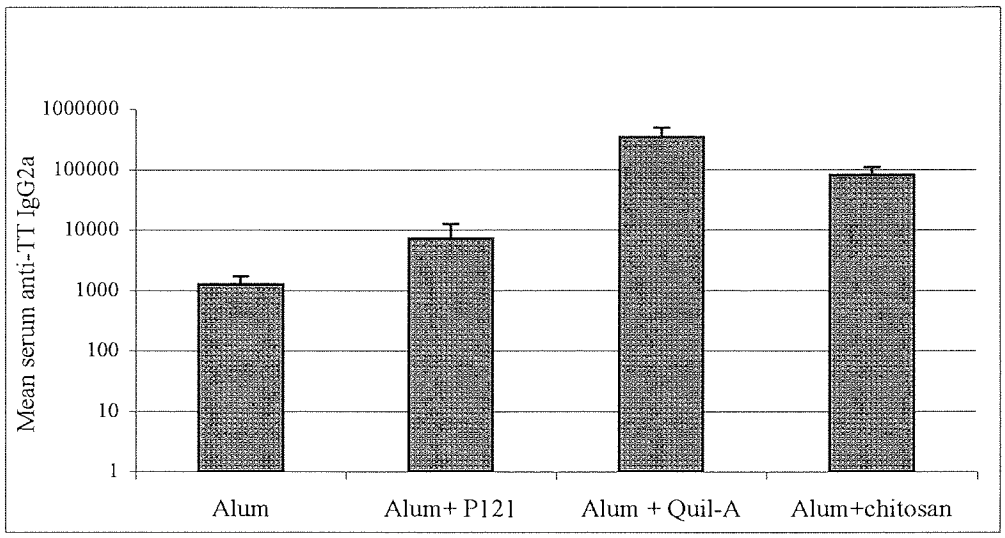


b

Figure 4.8: Serum immune response (IgG2a) to alum adsorbed TT after intramuscular administration in the presence and absence of additives. a) Primary response. b) Secondary immune response (n = 5 *per* group; mean ± s.d).

4.3.6. Effect of combination of adjuvants on the systemic immunity to DT following intranasal immunization

Cholera toxin (CT) has been shown to enhance the antigen specific mucosal immune response when applied together with antigen by the mucosal routes. Therefore CT and its subunit (CTB) are of interest as potential adjuvants for mucosal vaccines. The CT holotoxin consists of two subunits: the monomeric A (CTA) and the pentameric B (CTB) subunit. The basis for the adjuvanticity of CT holotoxin and the CTB subunit might be alternation of T cell regulation in mucosal lymphoid follicles. The toxicity of the CT holotoxin limits its usefulness as an adjuvant; therefore the CTB subunit offers more perspective as a component in mucosal vaccines (Goto *et al.*, 2000). Here, the potential adjuvant activity of the combination of chitosan with either saponin or CTB in the induction of the DT specific mucosal immune responses upon i.n. immunization was evaluated. The antibody response to DT was measured at various effector site of the mucosal immune system and in serum. Figure 4.9 shows serum immune responses to the i.n. application of DT with different adjuvants. Free DT elicited a low DT specific systemic IgG. In contrast significant serum specific IgG responses were measured upon i.n administration of DT with adjuvants. Among the formulations the addition of

chitosan to either CTB or Quil-A significantly enhanced the serum specific DT IgG response compared to the groups of mice, which received DT either with Quil-A or CTB. The combination of chitosan and Quil-A showed a significantly greater systemic immune response compared to that elicited by the free DT. It appeared that chitosan and Quil-A exerted a synergistic effect on the immunlogical response to DT. This data clearly indicates the combination of adjuvants results in higher serum immune response. Among the combination of adjuvants chitosan plus Quil-A resulted in the highest serum specific DT IgG response.

Free DT after nasal administration failed to produce any detectable serum specific IgA response to DT (or a negligible response). Intranasal administration of DT with adjuvants influenced significantly the production of serum specific IgA response to DT. The group of mice, which received DT plus CTB, showed highest serum specific IgA response. All the formulations elicited significantly higher serum specific IgA responses ($p<0.05$) than the free DT. The group of mice, which received DT with chitosan plus CTB, failed to show any enhancement in the serum specific IgA response to DT compared to CTB alone. This result is in contrast to serum specific IgG to DT with chitosan plus CTB where combination of these two adjuvants produced a higher immune response.

The group of mice which received DT with Quil-A showed an enhanced the serum IgA response but of a lower magnitude in comparison to the group of mice receiving DT with CTB. The addition of chitosan to Quil-A formulations enhanced serum IgA responses to twice that of the Quil-A alone.

We also measured the serum specific IgG to CTB. It is known that CT and CTB produces antibody towards themselves when given with other antigen. In this experiment the influence of chitosan on serum the immune response to CTB was evaluated. The addition of chitosan to CTB enhanced serum specific IgG response compared to CTB alone. The results are summarized in figure 4.9.

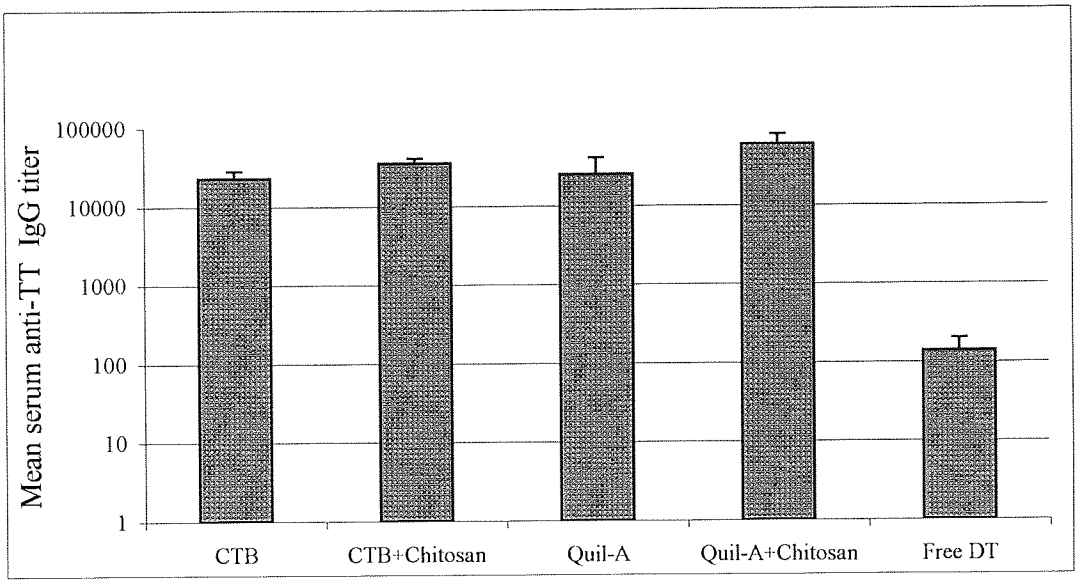


Figure 4.9: Serum immune responses to i.n. application of DT with different adjuvants (n = 5 *per* group; mean±s.d).

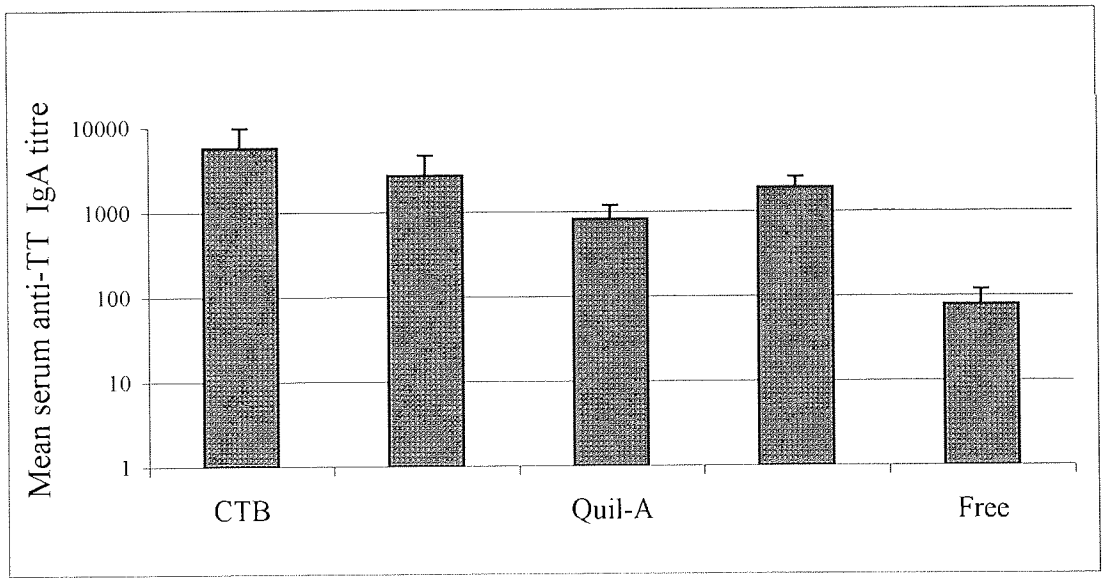


Figure 4.10: Serum immune responses (IgA) to nasally delivered diphtheria toxoid with different adjuvants (n = 5 *per* group; mean±s.d).

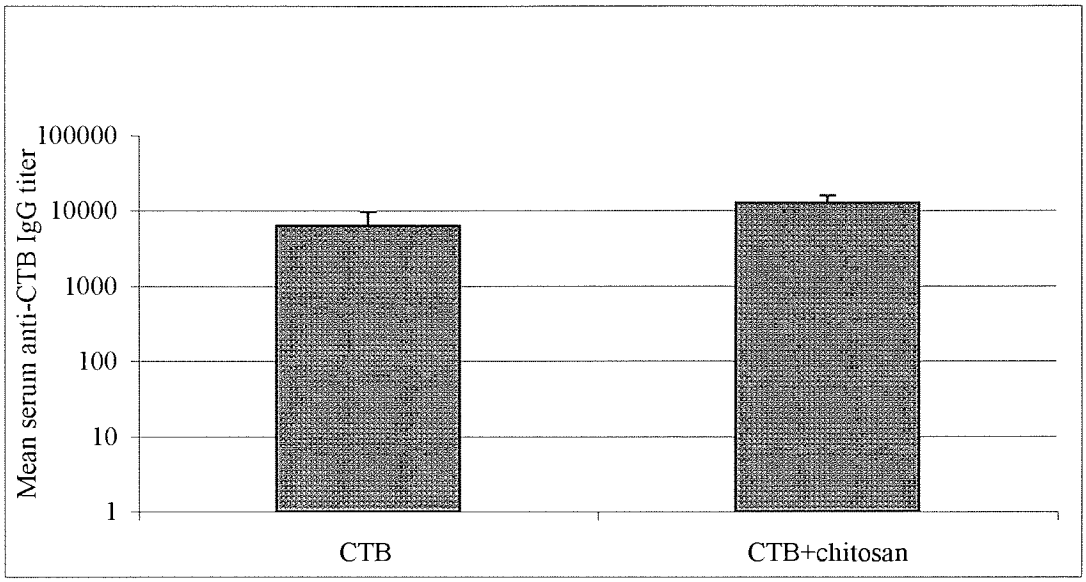
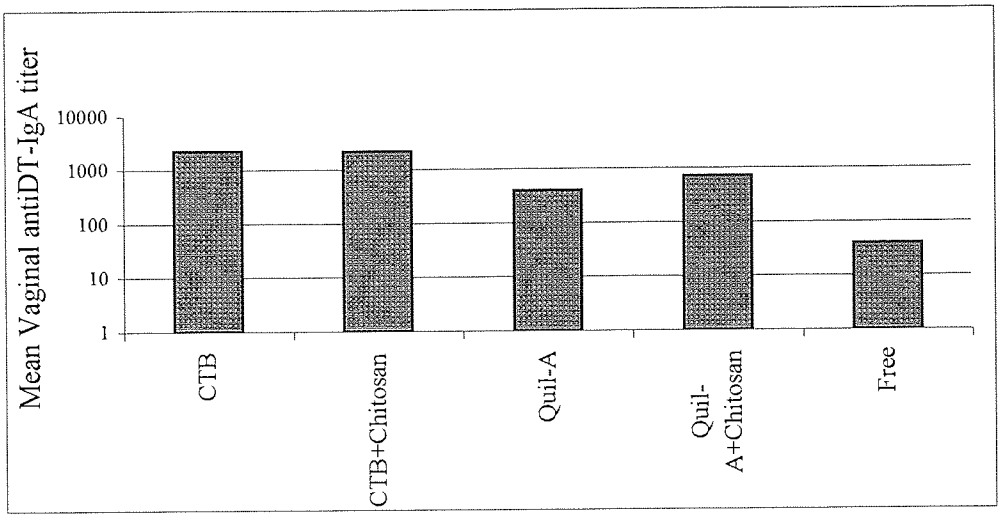


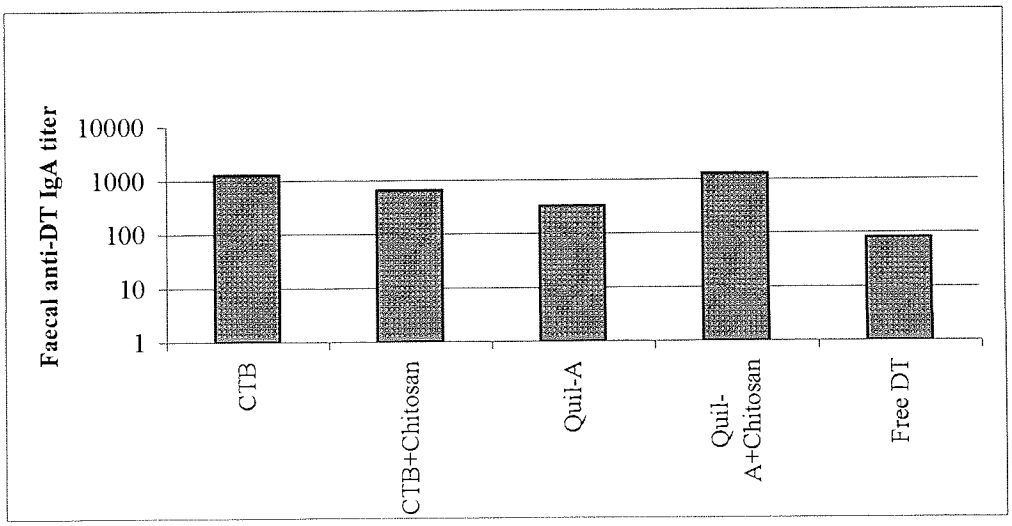
Figure 4.11: Serum immune responses to nasally delivered CTB in the presence of chitosan or Quil-A (n = 5 *per* group; mean±s.d).

4.3.7. Effect of combination of adjuvants on the mucosal immunity to DT following intranasal immunization

Nasal immunization with soluble antigen resulted in negligible IgA responses in vaginal washes and feacal matter. However, nasal administration of DT with adjuvants resulted in significant antibodies in vaginal washing and fecal matter. The group of mice, which received DT with CTB, produced the highest IgA response in vaginal washing. This result is inconsistent with the serum IgA response, where the group of mice, which received the same combination, produced the highest serum IgA. The group of mice which received DT with Quil-A or DT with Quil-A plus chitosan produced enhanced IgA response in vaginal washings among these groups the combination of adjuvants resulted in higher antibodies. However, compared to the group of mice received CTB the antibody titres were in a lower magnitude. The above trend in IgA response in fecal matter was also observed. Free DT produced negligible IgA response in fecal washes. All formulations resulted in higher IgA response in fecal washings compared to free DT. The group of mice that received DT with CTB or with chitosan plus Quil-A resulted in highest IgA response in fecal washings followed by the group of mice received CTB plus chitosan or Quil-A. The results are showed in figure 4.10

a





b

Figure 4.12: Mucosal immune responses (pooled samples) a) vaginal b) faecal washes to nasally delivered DT with CTB or Quil-A alone or with combination with chitosan (n = 5 *per* group; mean ±s.d)

4.3.8. N-trimethyl chitosan chloride (TMC) acts to enhance immunological responses to intranasally administered subunit vaccines

N-trimethyl chitosan chloride (TMC) has been referred to as a potential absorption enhancer of peptide therapies across mucosal membranes. In contrast to chitosan, TMC is water soluble through at neutral and basic pH. Furthermore, it retains the ability to temporarily open tight junctions. An important parameter appears to be the degree of trimethylation. In the current study we have nasally instilled F1 (5 μg) and V (1 μg) in

the presence and absence of three different TMC derivatives (with increasing degrees of quaternization: 20, 40 & 60%), and compared humoral immune responses engendered by these treatments to those evoked by co-administration of chitosan chloride and free V and F1 antigens.

The results indicate that mucosal co-administration of TMC60 or TMC40 augments the humoral response to F1 and V above and beyond that generated by i.n. instillation of F1 and V in phosphate buffered saline or chitosan hydrochloride. TMC20 failed to improve titre to V, although the effect on immunity to F1 was comparable with that of the more substituted chitosan derivatives (TMC40 & 60) (figure4.13). Such findings are consistent with previous observations (Kotze *et al.*, 1999) in which TMCs of high quaternization were found to be more effective in enhancing the permeability of intestinal epithelial (Caco-2 cells) to mannitol than was chitosan itself. These data suggest that, even quite small, modifications in the chemical structure of the chitosan can influence mucosal responses. It is also apparent that the different TMC derivatives may exert different adjuvant effects on different types of protein: immunity to V was highly dependent on the degree of quaternisation of the co-administered enhancer, although titres to F1 were comparatively similar with TMC 60, 40 and 20.

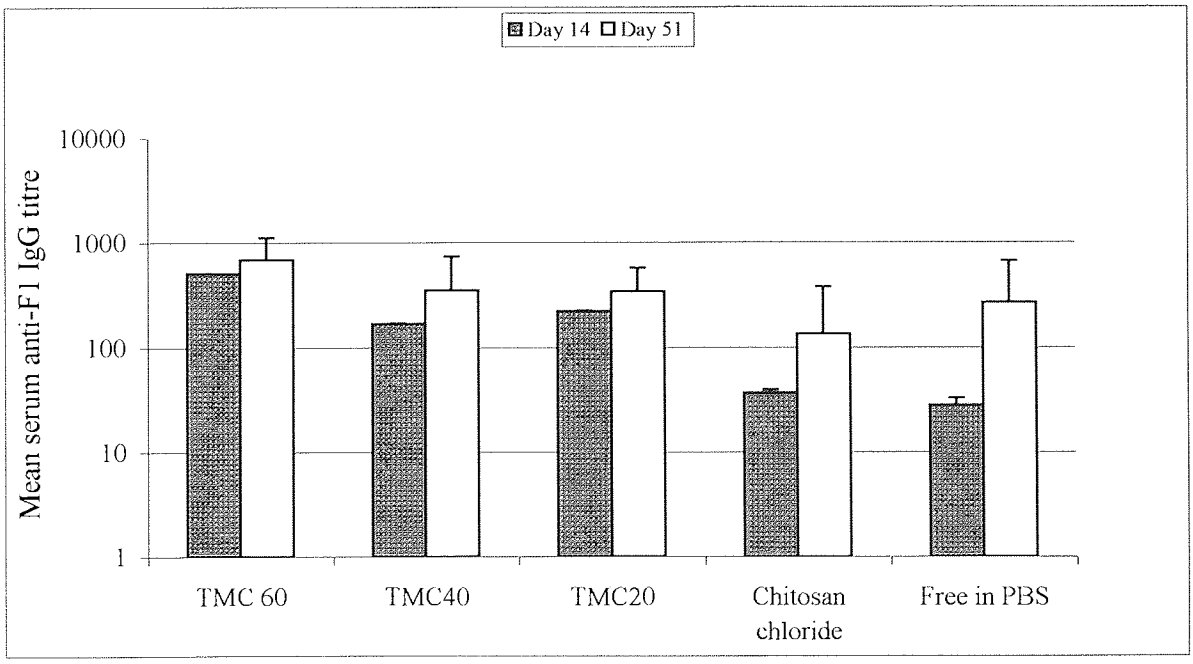


Figure 4.13: Effect of trimethyl chitosan on immune responses to nasally delivered V subunit vaccine (n = 5 *per* group; mean±s.d).

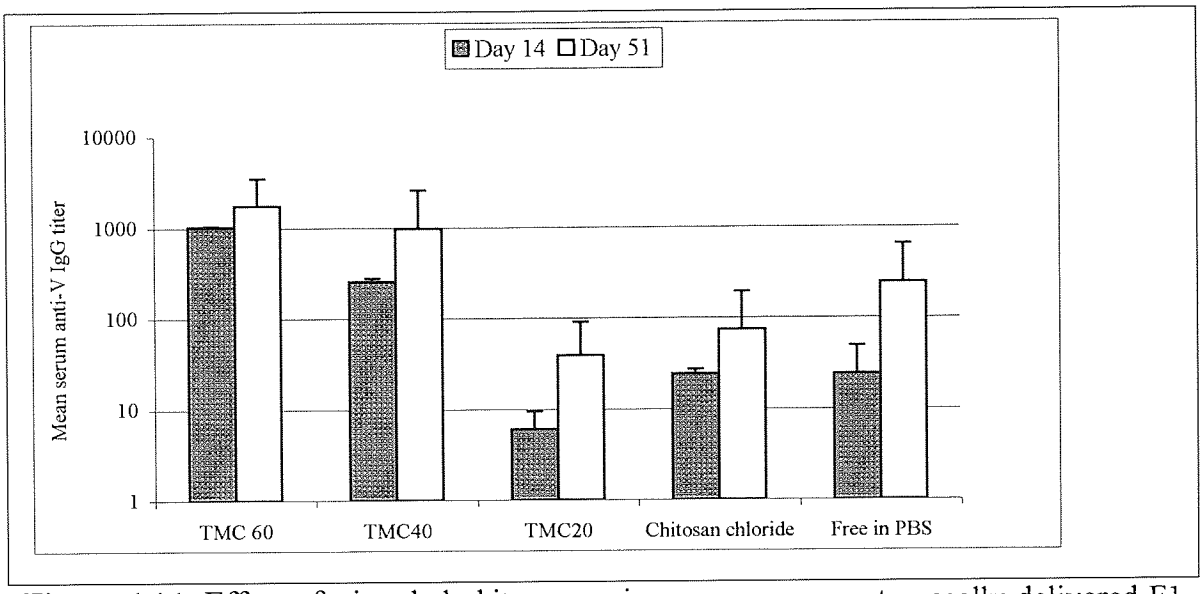


Figure 4.14: Effect of trimethyl chitosan on immune responses to nasally delivered F1 subunit vaccine (n = 5 *per* group; mean±s.d).

4.4. Discussion and conclusions

4.4.1. Spray dried chitosan microspheres

Chitosan microspheres were successfully prepared using spray the drying method. The surface of the microspheres appears to be pitted (figure 4.1). This may be due to the fast evaporation of water from the microspheres and also due to high shear generating during spray drying process. The yield of microspheres was around 40 % when the chitosan solution was spray-dried; product yield was lower, because more product deposited in the spraying chamber, this is in accordance with previous studies. Stability of encapsulated BSA was preserved in the microspheres indicates that the spray drying method can be used to encapsulate antigen in microspheres. *In vivo* results indicate that mucoadhesive chitosan microspheres containing antigen can be used to deliver effectively antigens via nasal mucosal route to elicit immune responses. The immune response generated by microencapsulated BSA was significantly ($p<0.05$) higher than free BSA. The enhancement of immune response depends upon the type of chitosan. Tengamnuay, *et al* 2000 compared the nasal absorption of kyotorphin, an enzymatically stable opioid dipeptide in the presence of free amine chitosan or chitosan salts and showed that free amine chitosan enhanced absorption of peptide higher than the

chitosan salts at lower pH. We also showed BSA encapsulated in chitosan microspheres produced an enhanced immune response higher than BSA encapsulated in chitosan chloride microspheres. This is in accordance with the degree of permeation enhancement of chitosan through the nasal mucosa.

4.4.2. Effect of type of chitosan on the mucosal and systemic immunity to DT following intranasal immunization

Intranasal immunization of DT with mucoadhesive polymer chitosan induced high levels of DT specific serum IgG and mucosal IgG and IgA responses. It was observed that the titres in the group of mice that received DT with chitosan were consistently higher than those of mice immunized with DT alone. Taken together, these data clearly show that chitosan acts as an adjuvant for the induction of serum IgG responses to an intranasally co-delivered protein antigen. We have shown that chitosan a mucoadhesive polymer that reversibly affects the structure of tight junctions (Shipper *et al.*, 1997) acts as a mucosal adjuvant for antigens delivered through the marine nasal mucosa. We have used different chitosans:chitosan chloride and chitosan glutamate. When intranasally delivered with DT to mice, chitosan chloride 113 (low molecular viscosity) gave the highest serum IgG response (primary response). In general low molecular viscosity chitosans salts acted as better mucosal adjuvants. Kotze *et al.*, 1999 evaluated the effects of two chitosan salts, namely chitosan hydrochloride and chitosan glutamate (0.5 and 1.5% w/v), on the transepithelial electrical resistance (TEER) and permeability of Caco-2 cell monolayers, using the radioactive marker [^{14}C]-mannitol. Both salts are caused a pronounced lowering in the TEER of Caco-2 cell monolayers in the order of 70 +/-1.0 % (chitosan glutamate) and 77 +/- 3.0 % (chitosan hydrochloride), 20 minutes after incubation started. In agreement with the TEER results the transport of the radioactive marker, [^{14}C]-mannitol, was increased 25-fold (chitosan glutamate) and 36-fold (chitosan hydrochloride) respectively. Our results with different salts as mucosal adjuvants also depend upon type of chitosan salt used. Effect of type of chitosan salt however is only pronounced before boosting.

The ability of intranasally delivered chitosan to induce antigen-specific IgA antibodies in mucosal sites distant from the immunization site confirms the common mucosal immune response. This is particularly useful when a local response is required in

mucosae that are not easily accessible to direct immunization or in mucosae where it is generally difficult to elicit an immune response, such as that of the female reproductive tract (Kozlowski *et al.*, 1999). It has been shown that chitosan can enhance antibody responses to subunit influenza virus vaccines delivered to the respiratory tracts of mice (Bacon *et al.*, 2000).

Ohlsson-Wilhelm *et al* (Ohlsson-Wilhelm *et al.*, 1996) explored the use of bioadhesive polymers for delivery of influenza vaccine *via* the mucosal route and showed enhanced responses to orally delivered vaccine. The mechanism by which mucoadhesive polymers bind to and/or penetrate into the mucin is the subject of active research. Mucoadhesives appear to adhere to the mucous layer through forces, which differ from those involved in mucin-mucin interactions. This binding appears to facilitate the interaction of antigen with lymphoid tissues of the mucosal tissue, enhancing mucosal responses to antigen delivered nasally in a mucoadhesive polymer. The mucoadhesive polymer binds to mucin maintains the antigen closer to the lymphoid tissue of the nasal mucosa. In addition, such binding may also increase the local concentration of the antigen, which would facilitate interaction with NALT.

The effects of chitosan on tight junctions had been shown *in vitro* and *in vivo* for rat and rabbit intestinal epithelium (Thanou *et al.*, 2000). Chitosan may exert an effect on tight junctions similar to that shown for the intestinal epithelium, resulting in increased permeabilization of the nasal mucosal barrier. Thus, the mucosal adjuvanticity of chitosan could be the result of an increased delivery of antigen into the nasal lymphoid tissue. In this regard, it has been shown that the increased permeability observed in the intestinal and nasal mucosae exposed to CT contributes to its adjuvant activity (Holmgren *et al.*, 1993). However, it is possible that besides acting as a permeabilizing agent, chitosan may exert some immunomodulatory effects on immune cells, such as those reported for LT and CT (Hornquist and Lycke 1995; Martin *et al.*, 2000; Pizza *et al.*, 2001). The mechanism by which chitosan enhances the immune responses to mucosally administered antigens is presently under investigation. Studies in our laboratories have shown that the residence time of microspheres in the presence of chitosan is prolonged in the murine model. It is interesting that the most effective mucosal adjuvants studied so far are toxins mainly produced by enterobacteria or by

other bacteria that infect mucosal surfaces. These include LT, CT, and pertussis toxin [Rappuoli, 1999 #381], and chitosan can now be considered a novel non-toxic biopolymer with mucosal adjuvant activity. This study clearly shows that mucosal adjuvanticity of chitosan depends upon type of the chitosan.

4.4.3. Combination of adjuvants

The addition of block copolymer P121, chitosan or Quil-A to alum adsorbed TT significantly enhanced serum IgG after intramuscular administration compared to alum adsorbed TT. This study clearly shows addition of chitosan to alum adsorbed TT influenced the antibody response to TT. The addition of an effective adjuvant to a vaccine is often critical to the generation of the most beneficial immune responses. Optimization and characterization of adjuvant formulations is necessary. The adjuvanticity of alum (aluminium hydroxide) has been attributed partly to its ability to act as a short-term antigen depot so that it can present antigen to the immune system over a period of time. Alum is the only adjuvant widely licensed for human use. The addition of saponin was one of the earliest strategies to increase the immunogenicity of alum-adsorbed antigens (Kensil 1996).

QS-21 can act as a co-adjuvant with aluminium hydroxide adsorbed antigens, thereby stimulating higher antibody titres than those induced by alum alone. This was demonstrated in mice with recombinant feline leukemia virus (FeLV) subunit vaccine based on the viral envelope protein gp70 (Wu *et al.*, 1992). A vaccine formulation consisting of gp70 adsorbed to alum elicited lower titres both to gp70 and to FeLV than the same formulations with added QS-21. The recombinant gp160/QS-21/alum combination induced titres in BALB/c mice that were 25 to 125 fold higher than those induced by the gp160/alum formulations alone (Wu *et al.*, 1992). Quil- A is a partially purified form of saponin, a crude extract from the bark of the tree Quillaia saponaria. Purified saponins have been shown to induce Th1-like responses including the induction of cytotoxic T-lymphocytes to soluble antigens (Kensil *et al.*, 1998; Mowat *et al.*, 1999). Saponins are also able to induce effective TH2-like responses.

In our studies also the addition of Quil-A to alum adsorbed TT influenced the immune response. This result is in accordance with the previous findings. Subclass analysis of serum IgG revealed that only the addition of chitosan and Quil-A to alum adsorbed TT

produced IgG2a response, which indicates that like Quil-A, chitosan can also influence the IgG subclass profile.

Seferian and Martinez (Seferian and Martinez 2000) showed in their studies that the addition of chitosan to an emulsion influenced the antibody subclass profile to recombinant beta-human chorionic gonadotropin (hcG) after parenteral immunization. Isotype analysis of these antibodies revealed an IgG1 response in mice immunized with zinc-chitosan particles and a mixed IgG1, IgG2a and IgG2b response with the emulsion. The adjuvant activity of chitosan-alum formulations compared favorably to alum-Quil-A formulations.

Chitosan suspensions or microparticles have been reported to have immune stimulating activity such as; i) increasing accumulation and activation of macrophage and polymorphonuclear cells, ii) suppressing tumor growth, iii) promoting resistance to infections by microorganisms, iv) inducing cytokines, v) augmenting antibody responses and vi) enhancing delayed type hypersensitivity (DTH) and cytotoxic T lymphocyte (CTL) responses (Ishihara *et al.*, 1993; Tokumitsu *et al.*, 1999). The mode of enhancement of immune response to alum adsorbed TT by chitosan may be due to the macrophage activation properties of chitosan or the combination of the depo nature of alum and activation of immune system by chitosan.

In another experiment we showed that the addition of chitosan to Quil-A or CTB enhanced adjuvanticity of these adjuvants after nasal delivery of DT. This may due to mucoadhesive property of chitosan, which may enhance the residence time of mucosal adjuvants. In a similar study, Singh *et al.*, 2001 evaluated a bioadhesive delivery system for intranasal administration of a flu vaccine, in combination with a mucosal adjuvant (LTK63). A commercially available influenza vaccine, containing hem agglutinin (HA) administered intranasally in a bioadhesive delivery system, which comprised esterified hyaluronic acid (HYAFF) microspheres, to various *in vivo* models. Their results indicated that the addition of bioadhesive systems significantly enhanced serum IgG responses and engender higher hemagglutination inhibition (HI) titres in comparison to the other groups. In addition, the bioadhesive formulation also showed a significantly

enhanced nasal wash IgA response. In addition to mucoadhesive properties chitosan can also act as immunostimulant after mucosal administration.

4.4.4. Effect of trimethylchitosan on nasally delivered *Yersinia pestis* subunits

The enhancement of the immunogenicity of nasally administered vaccines by chitosan was not restricted to diphtheria toxoid, TT, BSA but also to other antigens such as i.n. delivery of V and F1 was also significantly enhanced by chitosan. In an other study we evaluated the effect of the degree of methylation of chitosan on immune response to nasally delivered V and F1 subunit antigen response to F1 and V beyond that generated by i.n. instillation of the antigens in PBS or chitosan. TMC20 failed to improve titre to V, although the effect on anti-F1 antibody levels was comparable with that of the more substituted chitosan derivatives. Such findings were consistent with previous studies in which TMCs of high quaternisation were found more effective in enhancing the permeability of Caco-2 monolayers to macromolecules (Kotze *et al.*, 1999). These preliminary data suggest that TMC, and potentially other absorption enhancing compounds, may prove useful for i.n. administration of proteinaceous subunit vaccines. It is interesting that even quite small modifications in the chemical structure of the polymer can impact on its effectiveness to act as a mucosal adjuvant. Also apparent is that the different TMC derivatives may exert dissimilar effects on different types of antigen: the degree of immunopotentiation of V was highly dependent on the degree of quaternisation of the administered TMC, although F1 was adjuvanted by TMC60, 40 and 20. We speculate that this may be because, in comparison to V, F1 is a very hydrophobic aggregative macromolecule (Perry and Fetherston 1997), absorption of which might not be substantially enhanced by transient opening of aqueous intracellular pores. Such findings highlight the complicity of the mechanics of mucosal adjuvant action, and add further weight to the idea that data generated following *in vivo* testing of 'model' antigens will not be universally applicable for all immunogens. Further work is needed to ascertain if the magnitude and longevity of the immunological responses engendered by nasal administration of F1 and V in the presence of TMC are adequate to confer protection.

In conclusion, chitosan can be used as mucosal adjuvant and systemic adjuvant. The immunoadjuvant activity of chitosan strongly depends upon the type of chitosan. Chitosan also influences the type of immune response after intramuscular administration with alum-adsorbed antigen. The facts that the effect of chitosan on tight junctions is reversible and that chitosan does not cause tissue damage at the epithelial level or induce acute systemic side effects upon mucosal delivery (Illum 1998), in conjunction with the evidence that it acts as a potent adjuvant when intranasally delivered to mice, make chitosan a promising tool for the mucosal delivery of therapeutic agents (Senel *et al.*, 2000) and for the development of mucosal vaccines against pathogens of the respiratory, gastrointestinal, and urogenital systems.

CHAPTER: 5

5.0. Effect of chitosan on immune response to BSA encapsulated in blends of different molecular weight PLA microspheres: Potential for single dose vaccines

5.1. Introduction

Particular aspects of antigen delivery from biodegradable microspheres and its relevance for the immune response have been investigated (Eldridge *et al.*, 1989). Currently it is recommended that children are immunized against a variety of diseases over the course of several years, many of these vaccines require repeated immunization to assure complete protection. Unfortunately children in developing countries, children from transient populations present in urban areas and children in rural areas often do not receive the required booster immunizations. The main reasons for this failure to receive the complete immunisation include poor or limited access to medical care, lack of patent education, regarding the importance of booster vaccinations and cultural or social misconceptions about vaccines. One potential solution to this problem might be the development of single administration vaccines (SAV).

5.1.1.Single dose vaccines (SDVs)

Several approaches have been tried to develop SDVs, including liposomes, niosomes, emulsions and microspheres. Eldridge *et al.* (Eldridge *et al.*, 1993) demonstrated that primary and secondary responses could be achieved with SDVs either by blending batches of vaccine microspheres prepared with different co-polymer ratios, or by blending batches of vaccine microspheres having two distinct size distributions. Their studies showed that microspheres less than 10 μm in diameter apparently were phagocytised by macrophages, released antigen rapidly as a result and produced an immune response faster than microspheres of the same co-polymer ratio that were too large to be phagocytosed. In this study, we have investigated the use of combining different molecular weight L-PLA in microsphere preparations, through which the possibility of designing tailor-made release profiles could be evaluated. The rate of degradation of different molecular weight L-PLA polymers has been documented

previously (Morris *et al.*, 1994), with lower molecular weight species exhibiting a higher degradation rate and a subsequently faster rate of hydration and antigen release (Spiers *et al.*, 2000).

Ovalbumin-loaded poly (D, L-lactide-co-glycolide) microparticles, produced by emulsion/solvent evaporation stimulated the production of moderate serum IgG antibody levels after a single subcutaneous (s.c.) administration in mice and the duration of the immune response paralleled the degradation rate of the carrier (Coombes *et al.*, 1996). Formulations based on slow resorbing PLGA (75:25) maintained relatively constant peak antibody levels for 26 weeks and high titres for over 1 year at a level approximating the peak response to the faster resorbing PLGA (50:50), OVA-loaded particles, which was of lower duration. Vaccine formulations prepared by simple mixing of blank PLGA microparticles and OVA exhibited low primary immune responses, which were only elevated by boosting. Similar findings have been interpreted to suggest that sustained presentation of surface protein to the immune system was a major factor in the induction and long-term maintenance of high antibody titres following a single subcutaneous administration of OVA-loaded microparticles (Coombes *et al.*, 1996).

Singh *et al.* (Singh *et al.*, 1997) encapsulated hepatitis B surface antigen (HBsAg) in microparticles prepared from polylactide-co-glycolide (PLG) and polylactide (PLA) polymers using a solvent evaporation process. The immunoreactivity of the entrapped antigen was investigated by SDS-PAGE and Western blot. Various combinations of small and large microparticles with controlled release characteristics were investigated in CD-1 mice. Groups of animals were immunized with 30 μg equivalent of HBsAg in microparticles *per* animal. The control group received three injections of 10 μg of HBsAg on alum at 0, 1 and 6 months. Results indicated that a single injection of HBsAg in microparticles could maintain the antibody response at a level comparable to the three-injection alum schedule for at least 1 year. An *in vitro* inhibition assay was developed to demonstrate that antigen-antibody reactivity was comparable for the microparticle-immunised mice and the alum immunized mice. A competition assay with a monoclonal antibody specific for the neutralizing epitope of HBsAg demonstrated comparable binding for the sera from the microparticle and alum immunized mice.

It was demonstrated in our laboratories by Spiers *et al.* (Spiers *et al.*, 2000) that L-PLA microspheres comprising mixtures of molecular weight fractions 2kDa, 100kDa and 300kDa gave release profiles which did not appear optimal for single-dose delivery, although it was observed that an initial burst effect was then followed by the release of remaining material over a longer period. The highest burst effect was observed with the 2kDa polymer, which subsequently released the small amount of remaining BSA relatively rapidly, while the higher Mw polymers showed a less significant burst effect which was then followed by a considerably slower antigen release.

It may be hypothesized that increased immunogenicity *in vivo* is likely to be achieved by a more moderate initial burst effect followed by a moderate rate of release of the remaining antigen. Towards this goal, it is hypothesized that the use of the 50kDa L-PLA polymer might enable such a release profile to be achieved. The ultimate aim of the study described in this chapter is to develop a vaccine antigen delivery system, which elicits more effective immunity to mucosally and parenterally administered antigens *in vivo*.

5.1.2. Co-administration of microspheres and chitosan

The co-administration of antigens together with mucoadhesive polymers such as chitosan is one approach to increasing nasal residence time and improving nasal uptake of vaccine (Illum *et al.*, 1994). This was the premise for the inclusion of chitosan in these formulations designed as potential SDVs. Chitosan has been shown to be a suitable vaccine carrier *via* the intranasal route (Somavarapu *et al.*, 1998) and also is a potent adjuvant via parenteral routes (chapter 4) and (Seferian and Martinez 2000). The ability of microparticulate delivery systems to enhance immune responses significantly has been shown in previous chapters and is well known. In chapter 3, chitosan has been used in the formulation of microspheres in place of PVA as a stabilizing agent. The work in this chapter was designed to evaluate the potential of microparticulate delivery systems and the immune enhancing properties of chitosan as additive or synergistic adjuvants.

Previously we demonstrated that microparticulates could transgress the nasal epithelial barrier and appear in the blood circulatory system of experimental animals following

intranasal (i.n.) administration (Almeida *et al.*, 1993). We have also reported that i.n. administration of microparticulates with entrapped, or adsorbed, antigenic material serves to engender robust local and systemic humoral and cellular immunological responses (Almeida *et al.*, 1993) . A key factor reducing the efficacy of intranasally administered microparticulates is likely to be their short nasal residence time, due to the high efficiency of the mucociliary clearance mechanism in the upper respiratory tract (Abdel-Hameed *et al.*, 1996). Chitosan is a bioadhesive polymer with absorption enhancing properties (Illum *et al.*, 1994; Aspden *et al.*, 1996) and can be a useful vehicle for increasing their bioavalability of small and large molecules (Miyazaki *et al.*, 1994; Thanou *et al.*, 2000). In this study we have tested the hypothesis that i.n. administration of microparticulates in conjunction with chitosan and other polycations may extend their nasal residence. We speculate that extending the nasal residence time will result in increased particle-epithelial barrier interaction, thus improving the likelihood of drug/microparticle absorption.

5.2. Materials and methods

5.2.1. Materials

L-PLA polymer (2kDa) was obtained from Boehringer Ingelheim, Germany, while 50kDa and 100kDa molecular weights were purchased from Polysciences Inc. (Warrington, PA, USA). BSA (Fraction V) and other laboratory chemicals were obtained from Sigma Chem. Co. (Poole, UK). Gamma ray emitting scandium-46 labeled styrene-divinyl benzene 7 μm diameter microspheres (NEN-TRAC; NEN Life Science Products, UK), with a specific activity of 0.19MBq mg-1 were used. Chitosan (high molecular weight) was obtained from Sigma Chem. Co. (Poole, UK) and chitosan hydrochloride from Pronova biopolymers (Pronova, Drammen Norway). Fluorescein isothiocyanate (FITC) labelled Fluoresbrite™ polystyrene microspheres (Polysciences, PA, USA), of mean diameter 10 μm, were used in the cell culture experiments.

5.2.2. Microsphere preparation

Microspheres were prepared using the double-emulsion solvent evaporation method described in chapter 2. Briefly, polymer (250 mg per batch) was dissolved in the organic phase (DCM, 5ml) before primary emulsification (where two polymers were used in a preparation, these were combined at this stage by co-dissolution of 125 mg of each polymer into the organic solvent). The primary aqueous phase (0.5ml) containing BSA (6mg) and PVA (13-23kDa, 87-89% hydrolysed, 2.5% w/w) was emulsified with the organic phase by probe sonication at 60mW for 2 minutes, with cooling using an ice bath. The resulting primary emulsion was added in a rapid but drop wise manner to a chilled solution of PVA (13-23kDa, 87-89% hydrolysed, 5%w/v) (75ml) whilst being homogenised at 18,000 rpm (Silverson SL-2). Homogenisation was maintained for a total of 5 minutes, before the resulting suspension was stirred overnight at 300 rpm using a magnetic stirrer at room temperature to encourage evaporation of residual organic solvent. Microspheres were then harvested by centrifugation and washing three times with double-distilled water (Beckman Ultra, fixed angle rotor, 31000 rpm) and lyophilization at 10mTorr for 48 hours with shelf temperature of 20°C applied for the final 2 hours.

5.2.3. Analysis of microspheres

5.2.3.1. Particle size distributions

Particle size determination for resulting microspheres in aqueous suspension was carried out using a Malvern Mastersizer/E (Malvern Instruments, Malvern, UK).

5.2.3.2. Morphology and surface topography

Representative samples of microsphere batches were examined using scanning electron microscopy (SEM). Samples were mounted onto adhesive carbon pads and subjected to gold sputter coating under argon for 1.5 minutes at 10mW. Images were obtained on a Philips Instrument at 100kV with zero tilt.

5.2.3.3. Antigen loading characteristics

The extent of BSA loading into microspheres was determined using the BCA assay as described in chapter 2.

5.2.3.4. BSA release profiles *in vitro*

Triplicate samples of microsphere batches (~5mg) were added to release medium (1.00ml) comprising of phosphate buffered saline (PBS) containing 5mM sodium azide and 0.01% sodium dodecyl sulphate (SDS) in 1.5ml Eppendorf tubes. These tubes were incubated at 37°C with agitation, and removed at appropriate time intervals. Six timepoints were used, with three samples of each batch being analysed at each time point, using the BCA assay.

5.2.4. *In vivo* dosing and blood sampling schedule

For *in vivo* studies, BALB/c mice (5 *per* treatment group) were immunised initially with 15μg of BSA either as free antigen or as equivalent dose in PLA microspheres, intranasally or intramuscularly. All dosing volumes were 50 μl. Blood was collected by tail vein bleed on day 31 and 63. The groups were boosted orally with 100 μg of BSA as free antigen (or equivalent dose encapsulated in PLA microspheres, typically less than 1 mg *per* dose) in sterile PBS to subgroups of mice (3 of 5, in each treatment group). Blood samples were obtained further to this on days 120 and 134 after initial dosing.

To evaluate the effect of chitosan on the immune response to these formulations, a selection of the BSA formulations were co-administered with a 0.2% w/v chitosan solution, as detailed in tables 5.1 and 5.2.

Group no.	Group Name	Formulation		
		BSA	PLA Mw (kDa)	Chitosan Mw (0.2%w/v solution)
1	Nfree	15 μg (in saline)	--	--
2	NFHC	15 μg (in saline)	--	High
3	NFLC	15 μg (in saline)	--	Low
4	NFE2	15 μg (5μg in saline, 10 μg in microsphere)	50	--
5	NFE2HC	15 μg (5μg in saline, 10 μg in microsphere)	50	High
6	NE2HC	15 μg in microsphere	50	High
7	NFE3	15 μg (5μg in saline, 10 μg in microsphere)	100	--
8	NFE3HC	15 μg (5μg in saline, 10 μg in microsphere)	100	High
9	NE3HC	15 μg (in microspheres)	100	High
10	NFE5	15μg (5μg in saline, 10 μg in microsphere)	2+100 (50:50 blend)	--
11	NFE5HC	15 μg (5μg in saline, 10 μg in microsphere)	2+100 (50:50 blend)	High
12	NE5HC	15 μg (in microspheres)	2+100 (50:50 blend)	High
13	NFE5LC	15 μg (5μg in saline, 10 μg in microsphere)	2+100 (50:50 blend)	Low
14	NE5LC	15 μg (in microspheres)	2+100 (50:50 blend)	Low

Table 5.1: Formulations and corresponding group no. for intranasal administration of BSA in BALB/c mice. Groups were dosed on day 1; subgroups (3 of 5) were boosted on day 65 Blood samples were obtained on days 28, 42 56, 72, and 86.
Key: N = intranasal route, F = free BSA (administered in sterile saline), E2 = encapsulation in 50kDa PLA, E3 = encapsulation in 100kDa PLA, E5 = encapsulation in a 2kDa + 100kDa blend (50:50 ratio) of PLA, LC = low Mw chitosan (hydrochloride), HC = high Mw chitosan (chitosan base).

Group no.	Group Name	Formulation		
		BSA	PLA Mw (kDa)	Chitosan Mw (0.2%w/v solution)
1	Mfree	15 μg (in saline)	--	--
2	MFHC	15 μg (in saline)	--	High
3	MFLC	15 μg (in saline)	--	Low
4	MFE2	15 μg (5 μg in saline, 10 μg in microsphere)	50	--
5	MFE2HC	15 μg (5 μg in saline, 10 μg in microsphere)	50	High
6	ME2HC	15μg (in microsphere)	50	High
7	MFE3	15 μg (5 μg in saline, 10 μg in microsphere)	100	--
8	MFE3HC	15μg (5μg in saline, 10 μg in microsphere)	100	High
9	ME3HC	15 μg (in microspheres)	100	High
10	MFE5	15 μg (5 μg in saline, 10 μg in microsphere),	2+100 (50:50 blend)	--
11	MFE5HC	15 μg (5 μg in saline, 10 μg in microsphere),	2+100 (50:50 blend)	High
12	ME5HC	15 μg (in microspheres)	2+100 (50:50 blend)	High
13	MFE5LC	15 μg (5 μg in saline, 10 μg in microsphere),	2+100 (50:50 blend)	Low
14	ME5LC	15 μg (in microspheres)	2+100 (50:50 blend)	Low

Table 5.2: Formulations and corresponding group no. for intranasal administration of BSA in BALB/c mice. Groups were dosed on day 1; Blood samples were obtained on days 28, 42 56, 72, and 86.
Key: M = intramuscular route, F = free BSA (administered in sterile saline), E2 = encapsulation in 50kDa PLA, E3 = encapsulation in 100kDa PLA, E5 = encapsulation in a 2kDa + 100kDa blend (50:50 ratio) of PLA, LC = low Mw chitosan (hydrochloride), HC = high Mw chitosan (chitosan base).

Mock challenge consisting of 1 μg of free BSA administered subcutaneously in 400μl PBS was carried out on groups dosed intramuscularly on day 210 and blood samples collected after 7 days for ELISA.

The serum from all blood samples were obtained, and stored at –20°C until antibody titre assessment by ELISA.

5.2.5. Assessment of BSA stability to encapsulation process

The structural integrity of the BSA protein was assessed before and after microencapsulation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as previously described in chapter 2.

5.2.6. Analysis of antibody titres to i.n. and i.m. vaccination

Serum anti-BSA whole IgG titres were determined using the ELISA method described in chapter 2. All samples were run in parallel with negative serum from naïve animals, and results interpreted in terms of the highest serum dilution factor giving greater optical density readings than the highest value obtained for the negative serum.

5.2.7. Viscosity measurements

The viscosity of the test solutions was measured using a Brookfield (Stoughton, MA, USA) Digital viscometer model DV-I+ operated at ambient temperature.

5.2.8. *In vivo* biodistribution studies

Experimentation strictly adhered to the 1986 Scientific Procedures Act. Female BALB/c mice (25g, 6-week-old) were used for *in vivo* studies. To ensure identical microsphere deposition within the nasal cavity, mice were lightly anaesthetised with an inhaled gaseous mixture of 3 % (v/v) halothane (RMB Animal Health Ltd., UK) in oxygen (300 cm^3 min^{-1}) and nitrous oxide (100 cm^3 min^{-1}) for i.n. dosing procedures.

Groups of 16 mice were intranasally dosed with 50 μg of NEN-TRAC microspheres suspended in 10 μl volumes of three test solutions (chitosan, polyornithine and saline). Prior to nasal administration the radioactivity associated with the various nasal dosage

forms was ascertained using a 1282 compugamma universal gamma counter (LKB WALLAC (Finland)). Within the three nasal treatment groups, four (n=4) animals were humanely killed by cervical dislocation at 1 minute, 15 minutes, 5 hours and 24 hours. Snouts, both lungs, and entire gastrointestinal tract (from oesophageal sphincter to rectum) were then removed from freshly culled mice and placed into mini-scintillation tubes. The radioactivity of the various body compartments was measured and represented as the percentage of the administered radioactive dose that was detected in each region of the respiratory/alimentary canals.

5.2.9. *In vitro* investigation of microparticle bioadhesion in the presence and absence of chitosan

Caco-2 cells of passage 29, seeded at a density of 500, 000 cells/cm^2, were cultured on plastic 24-well plates for 7 days. Confluent cell monolayers were treated in one of four ways: (i) Cells were preincubated with Hank's balanced salt solution (HBSS) (pH 7.4) for 15 minutes at 37°C prior to addition of a 0.01 mg ml^{-1} suspension of FITC labelled latex particles (10 mm) in HBSS. (ii) Cells were preincubated with aqueous chitosan HCl (0.2% w/v, pH 7.43) for 15 minutes at 37°C prior to addition of a 0.01 mg ml^{-1} suspension of FITC labelled latex particles (10 mm) in HBSS. (iii) Cells were pre incubated with aqueous poly-L-ornithine (0.05% w/v) for 15 minutes at 37°C before addition of a 0.01 mg ml^{-1} suspension of FITC labelled latex particles (10μm) in HBSS. (iv) Cells were preincubated with Hank's balanced salt solution (HBSS) (pH 7.4) for 15 minutes at 37°C prior to addition of an aqueous solution of FITC in HBSS (0.01 mg ml^{-1}). After addition of the various fluorescent materials, the cells were incubated for 15 minutes at 37°C before the donor solution was aspirated and the cells were washed twice (0.5 ml x 2) with ice-cold phosphate buffered-saline (PBS)/azide (0.05 % w/v). After the two wash steps, the cells were solubilised with Triton-X 100 (1% v/v) for at least two hours at 37°C. Fluorescence intensity associated with the solubilised cells was quantified using a Wallac (Finland) Victor2 1420 Multilabel counter (set to a 485/535 nm wavelength) and expressed as the percentage of the fluorescence of the donor phase/ 106 cells. These experiments were repeated three times.

5.2.10. Statistics

Differences between treatment groups were compared with Students t-tests.

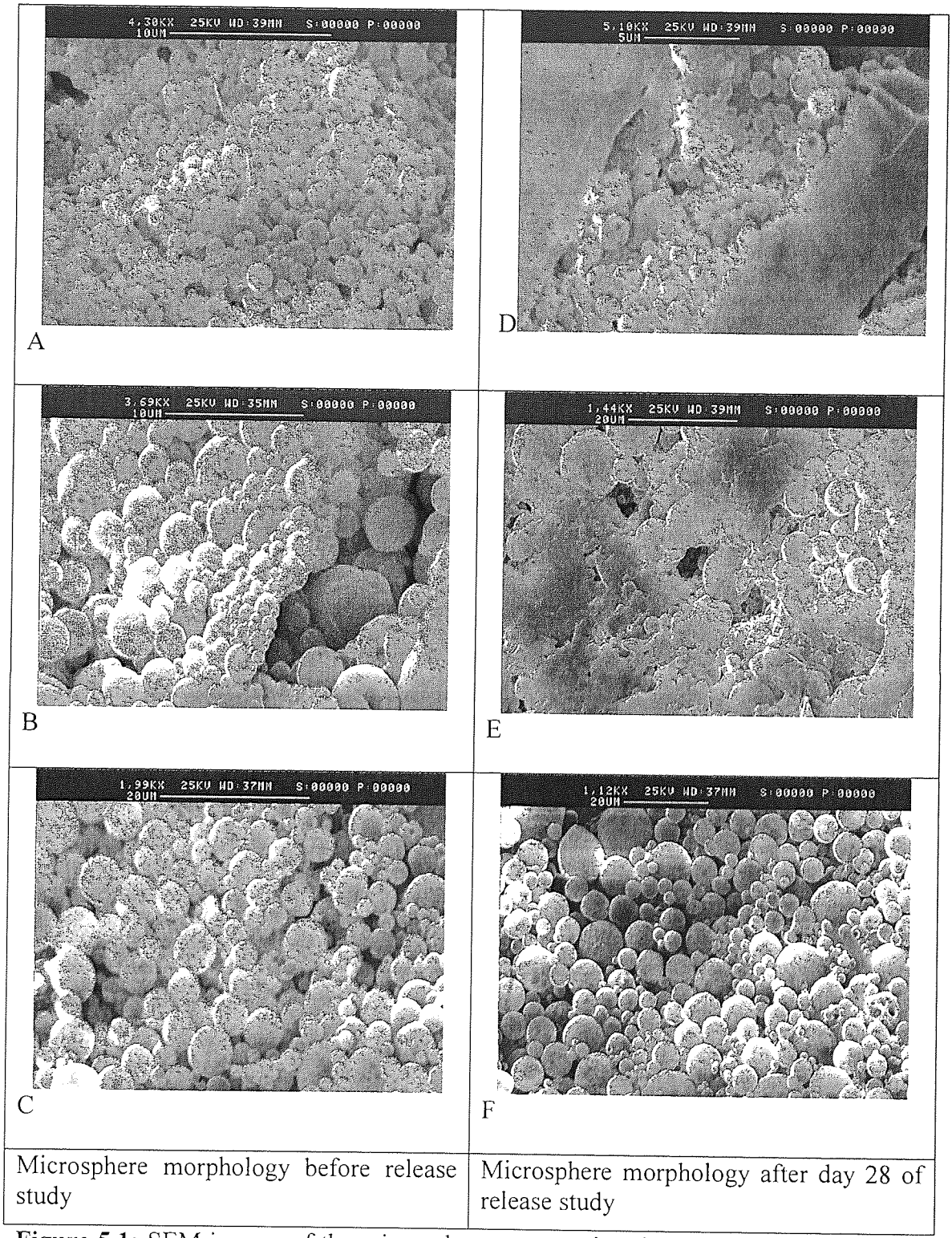
5.3. Results

5.3.1. Microsphere characterization

The physico-chemical characteristics of the microsphere batches prepared are given in table 5.3. The microspheres prepared for this comparative study demonstrated good reproducibility between batches in terms of particle size and protein loading. In addition, high yields (~80%) and encapsulation efficiencies (~75%)were attained using the given formulation and method of preparation.

5.3.2. Microsphere morphology

SEM analysis of particle morphology and surface morphology (figure 5.1) demonstrated that spherical particles were formed consistently in each formulation and that the particle sizes observed using this visual technique were in keeping with those given by laser scattering (see methods section). Microspheres appeared to exhibit mainly smooth surface characteristics. All preparations in this study demonstrated similar mean particle diameters (number mean <4 μm; volume mean 7-16 μm). The fact that there was no visual difference in morphology of the microspheres regardless of the molecular weight or blends indicated that the physico-chemical parameters influencing the formation of particles were not affected by the polymer properties. This bodes well for the use of this formulation method for the reproducible production microspheric vaccine formulations with varying molecular weights.



Microsphere morphology before release study	Microsphere morphology after day 28 of release study

Figure 5.1: SEM images of the microspheres, comparing the morphology before (A-F) and after day 28 of the release of BSA. A and D microspheres prepared with 2kDa PLA polymer, B and E microspheres prepared with 50kDa PLA polymer, C and F microspheres prepared with 100kDA polymer.

5.3.3. Effect of PLA molecular weight on microsphere size

The size distributions of the microsphere formulations were determined by laser diffraction, and expressed as the mean number diameter. Generally, low molecular weight polymer (PLA 2kDa) resulted in the smallest diameters, while the higher Mw polymers resulted in larger microspheres (see table 5.3). This trend was present within the polymer-blend formulations; the inclusion of PLA 2kDa reduced the microsphere diameters, while addition of 100kDa polymer generally caused an increase. This is in agreement with the tenet that increasing the viscosity of the primary emulsion during the double emulsion method increases the particle size. Increasing Mw of polymers increases their viscosities.

5.3.4. Effect of PLA polymer molecular weight on BSA loading

From the assessment of the loadings of BSA in the formulations (Table 5.3), it would appear that the PLA 2kDa effects an increase in the entrapment of the protein, whereas the higher molecular weight polymers resulted in lower loadings. Blending of the polymers resulted in generally higher loadings, and the enhancing effect of the lower molecular weight polymers on the entrapment efficiencies was reduced (see table 5.3).

Prep^{n}	Polymer(s)	Number mean diameter (μm)(±s.d)	BSA loading (% w/w) ±s.d
1	2kDa only	1.4 ± 0.77	2.31 ± 0.45
2	50kDa only	3.5 ± 2.28	1.54 ± 0.23
3	100kDa only	3.6 ± 2.54	1.80 ± 0.78
4	2kDa + 50kDa (1:1)	1.8 ± 0.63	2.32 ± 0.24
5	2kDa + 100kDa (1:1)	2.5 ± 1.09	2.40 ± 0.8
6	50kDa + 100kDa (1:1)	3.7 ± 2.06	2.26 ± 0.18

Table 5.3: Microsphere batches prepared using 2kDa, 50kDa and 100kDa L-PLA individually and in combination for comparative study.

5.3.5. Effect of polymer molecular weight on profiles of BSA release from microspheres

BSA release profiles (figure 5.2) demonstrated that the lower molecular weight polymer provided a high degree of burst, compared to the higher molecular weight polymer, while mixtures of Mw gave moderate degrees of burst, as predicted. The post-burst release of BSA was slow for all preparations during the entire 28-day period of analysis. SEM analysis of microspheres after this time showed little change in morphology or surface characteristics, even for those composed solely from 2kDa polymer (preparation MR1), which had exhibited the highest cumulative BSA release over the 28 days. These findings support the hypothesis that the burst effect observed is due mainly to the loss of adsorbed protein from the microsphere surface rather than the release of encapsulated material. Previous studies have also demonstrated this trend of burst effect with polymer molecular weight, which may be attributed to the fact that the lower Mw polymers are lower in viscosity than higher Mw species. Therefore, in a mixed solvent system such as an emulsion, hydrophilic proteins such as BSA may migrate toward the microsphere surface *i.e.* toward the aqueous phase) more rapidly through organic solvent droplets containing lower Mw polymers than through higher Mw polymers. Thus, it is likely that as a result of such protein migration, microspheres composed from lower Mw L-PLA may have contained a higher proportion of surface-associated BSA than those composed from higher Mw polymer, leading to a greater burst effect.

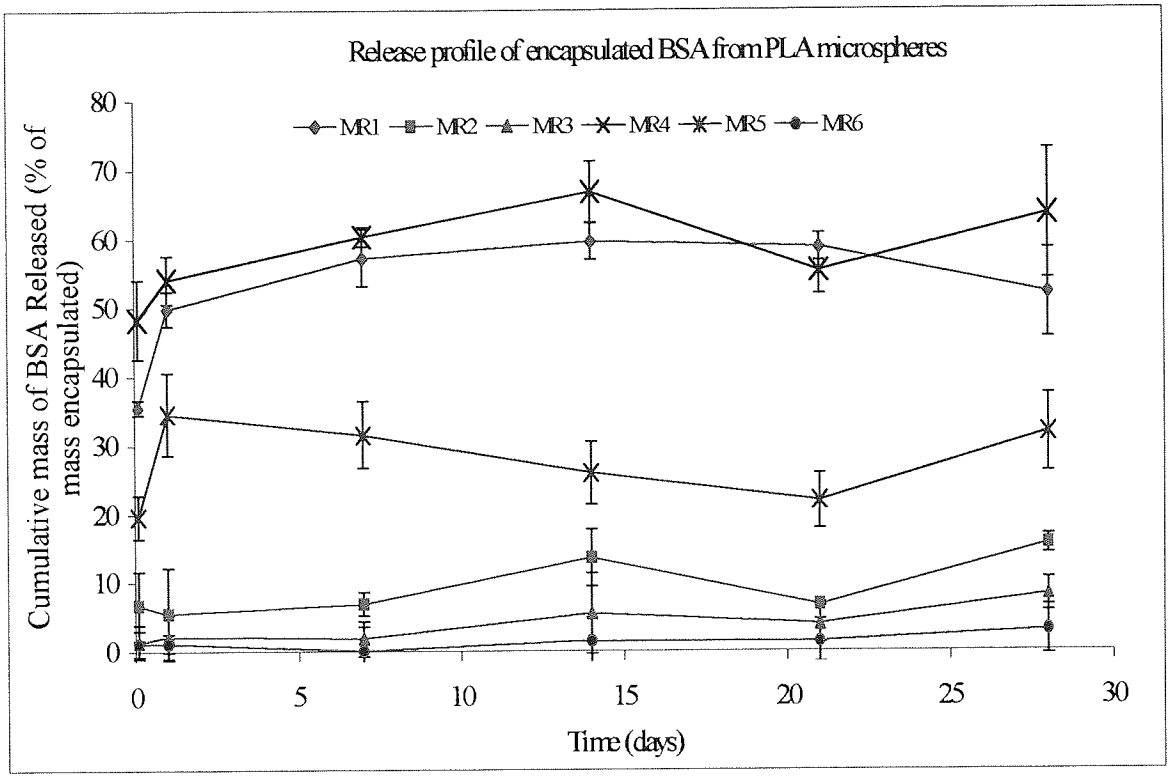


Figure 5.2: Release profile of BSA from microspheres prepared form PLA of different molecular weights and blends.
100% PLA 2kDa (MR1), 100% PLA 50kDa (MR2), 100% PLA 100kDa (MR3), 50% PLA 2kDa and 50% PLA 50kDa (MR4), 50% PLA 2kDa and 50% PLA 100kDa (MR5), 50% PLA 50kDa and 50% PLA 100kDa (MR6).

5.3.6. Structural integrity of BSA after microsphere encapsulation

The figure below shows an SDS-PAGE of BSA samples before and after encapsulation into microspheres. It can be seen that the migration of the protein bands are identical, indicating retention of the structural integrity of the BSA extracted from the microspheres.

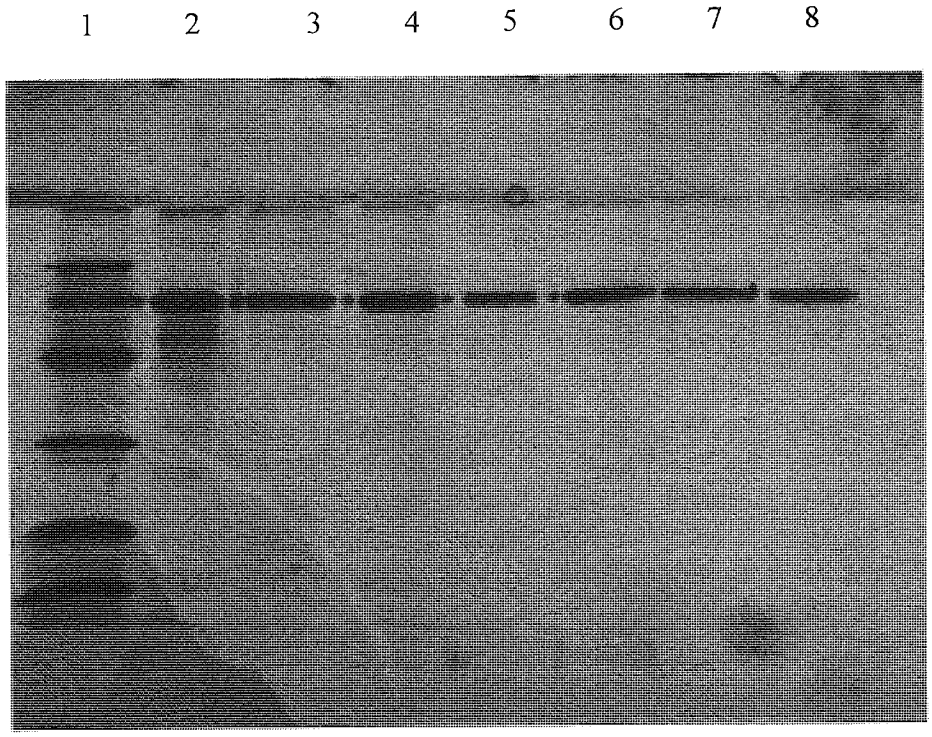


Figure 5.3: SDS-PAGE analysis of BSA samples. Samples were obtained before microencapsulation and after extraction from microspheres. Lane descriptions: 1) Molecular weight marker, 2) Free BSA, 3) BSA extracted from 2kDa PLA MS, 4) BSA extracted from 50kDa PLA MS, 5) BSA extracted from 100kDa PLA MS, 6) BSA extracted from 2kDa + 50kDa PLA MS, 7) BSA extracted from 2kDa + 100kDa PLA MS 8) BSA extracted from 100kDa + 50kDa PLA MS.

5.3.7. Immune response to nasally delivered BSA formulations given in free form or encapsulated in microspheres and response to oral boosting

Serum anti-BSA IgG titres obtained from the ELISA assays are shown in Figure 5.4 below. Significant responses were observed one month after initial, single dosing with all formulations of encapsulated BSA, administered intranasally (day 31) compared to free antigen. The levels of antigen specific IgG observed at this stage appeared to remain consistently high after a further month (day 63). This indicates that the entrapment of BSA in microcapsules elicits strong immune response *via* this route. On day 31, a difference between the groups receiving 2kDa (N1) and groups receiving 50kDa (N2) or 100kDa (N3) polymer entrapped antigen was observed in that the 2kDa formulation elicited the highest antibody response of these three formulations. This difference, however, was not thought to be significant. The addition of the 2kDa polymer to the 100kDa formulations was however shown to significantly enhance

antigen specific antibody titres as compared to the 100kDa polymer alone on day 31. On day 63, the differences among the groups were diminished although antibody titres in general were increased. The higher Mw formulations appear to show improved antibody titres at this time point. At the end of the study on day 134, antigen specific antibody titres were reduced but very high in comparison to free antigen, which had shown short lived immune responses barely detectable after day 63 (at days 120 and 134).

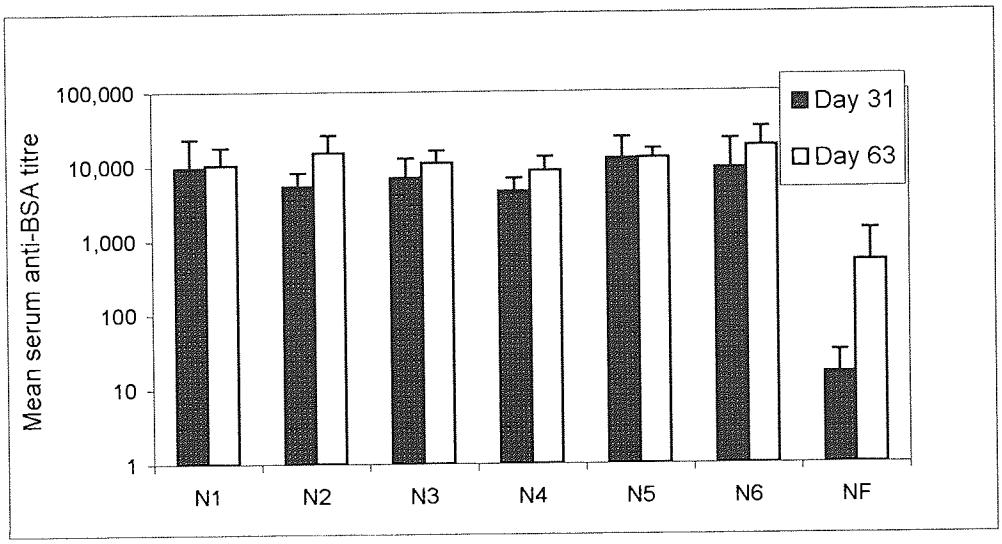
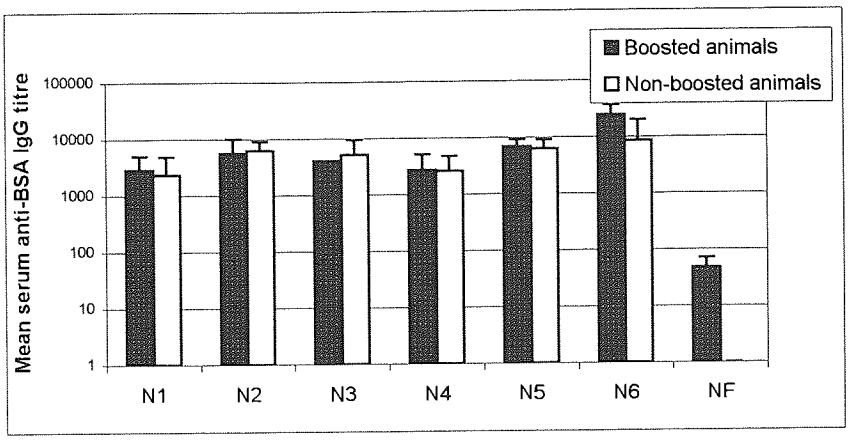


Figure 5.4: Serum immune response to nasally delivered BSA formulations given in free form or encapsulated in microspheres BALB/c mice (5 *per* treatment group) were immunized with 15 μg of BSA in 20μl PBS either as free antigen or as equivalent dose in PLA microspheres.
N1) MS prepared with 2kDa, N2) MS prepared with 50kDa PLA, N3) MS prepared with 100kDa PLA, N4) MS prepared with 2kDa PLA+50kDa PLA, N5) MS prepared with 2kDa PLA+100kDa PLA, N6) MS prepared with 50kDa PLA+100kDa PLA, NF) Free BSA

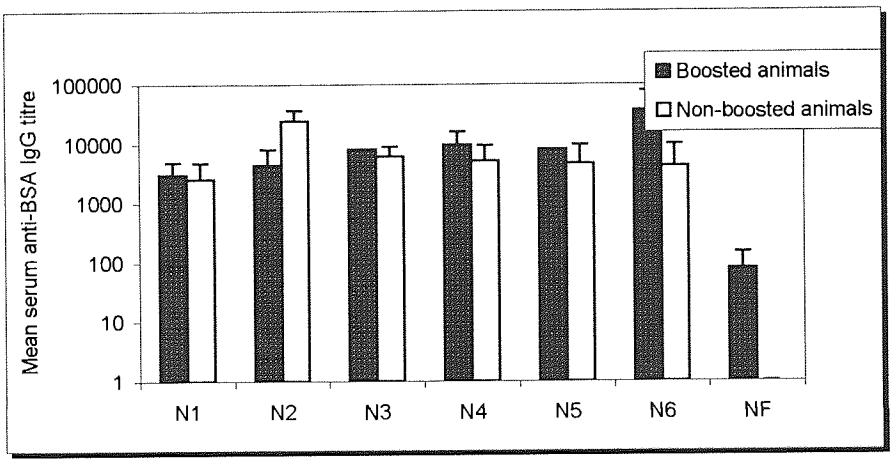
Analysis of sub-groups of boosted and non-boosted animals (figure 5.5) (which had received the initial priming dose intranasally) at later time points demonstrated that the regime of single oral boosting with 100μg of BSA (or the equivalent dose of encapsulated BSA) led to increased serum anti-BSA IgG titres in selected treatment groups. In particular, group N6 may be identified as having displayed a noticeable increase in serum IgG titres just 7 days following oral boosting (day 120, titres 24576 vs. 8448), an effect which became even more marked 21 days after boosting (day 134, titres 36864 vs. 4224). These results are summarized in figure 5.5A and 5.5B.

a)



Day120

b)



Day134

Figure 5.5: Serum immune response to nasally delivered BSA formulations given in free form or encapsulated in microspheres BALB/c mice (5 *per* treatment group) were immunized with 15μg of BSA on day 1 and boosted orally (in 100μl PBS) on day 113 with 100 μg of BSA either as free antigen or as equivalent dose in PLA microspheres. N1) MS prepared with 2kDa, N2) MS prepared with 50kDa PLA, N3) MS prepared with 100kDa PLA, N4) MS prepared with 2kDa PLA+50kDa PLA, N5) MS prepared with 2kDa PLA+100kDa PLA, N6) MS prepared with 50kDa PLA+100kDa PLA, NF) Free BSA.

5.3.8. Immune response to intramuscularly delivered BSA either given in free form or encapsulated in microspheres

Serum anti-BSA IgG titres obtained from the ELISA assays are shown in Figure 5.6 below. Significant responses were observed one month after initial, single dosing of all formulations of encapsulated BSA, administered intramuscularly (day 31). This indicates that the entrapment of BSA in microcapsules elicits strong immune response similar to that observed following intranasal administration at day 31. The levels of antigen specific IgG observed at this stage appeared to remain consistently high after a

further month (day 63), where the 100kDa formulation appeared to show increased antibody titres in comparison to the other formulations. On day 134, however, there is not much difference observed between the formulations. Titres are reduced at this time point but still significantly higher than the free antigen.

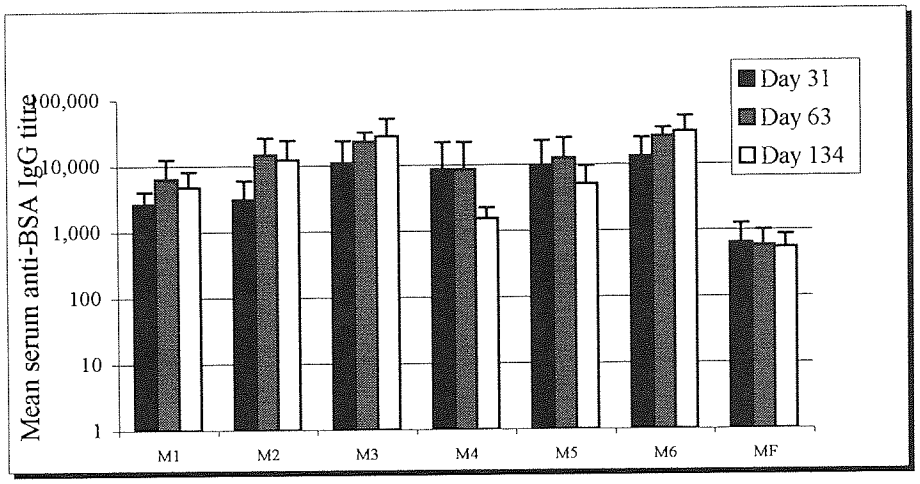


Figure.5.6: Serum immune response to intramuscular delivered BSA formulations given in free form or encapsulated in microspheres BALB/c mice (5 *per* treatment group) were immunized with 15 µg of BSA either as free antigen or as equivalent dose in PLA microspheres. M1) MS prepared with 2kDa, M2) MS prepared with 50kDa PLA, M3) MS prepared with 100kDa PLA, M4) MS prepared with 2kDa PLA+50kDa PLA, M5) MS prepared with 2kDa PLA+100kDa PLA, M6) MS prepared with 50kDa PLA+100kDa PLA, MF) Free BSA.

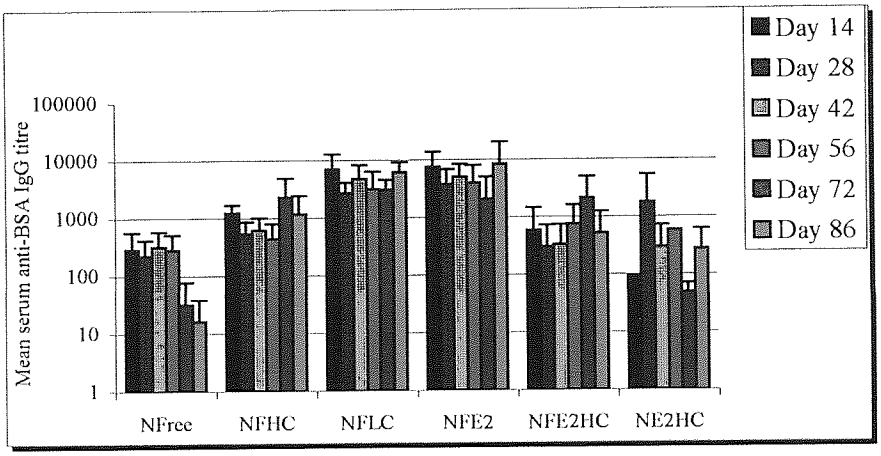
5.3.9. Immune response to nasally delivered BSA given in free form or encapsulated in microspheres with and without chitosan and response to oral boosting

Selected formulations from the above studies were tested for immunogenicity with the addition of free antigen and in the presence of chitosan. On day 14, all of the formulations generated immune responses significantly higher than the free antigen (Figure 5.7). The addition of either low or high Mw chitosan to the microsphere formulations failed to show a significant enhancement of antigen specific antibody titres. In fact, *via* the nasal route, titres are decreased in the presence of chitosan. However, in accordance with earlier studies, the simple addition of chitosan to antigen was shown to have effective adjuvant action. The strategy of adding free antigen to the microsphere formulation was not shown to enhance immune responses and responses

were lower in comparison to the administration of antigen wholly entrapped in the microsphere formulation.

ELISA assays performed on serum taken 7 days and 21 days after oral boosting demonstrated an appreciable increase in antibody titres for boosted animals in many of the treatment groups (Figure 5.8). For animals receiving chitosan in the initial dose, the observed primary response was not significantly better than those not receiving chitosan (Figure. 5.7). However, following oral boosting, serum IgG titres in mice that had initially received chitosan were appreciably higher than the groups, which had not been given chitosan initially (Figure. 5.8). These data suggest that chitosan may have affect on the memory response.

a



b

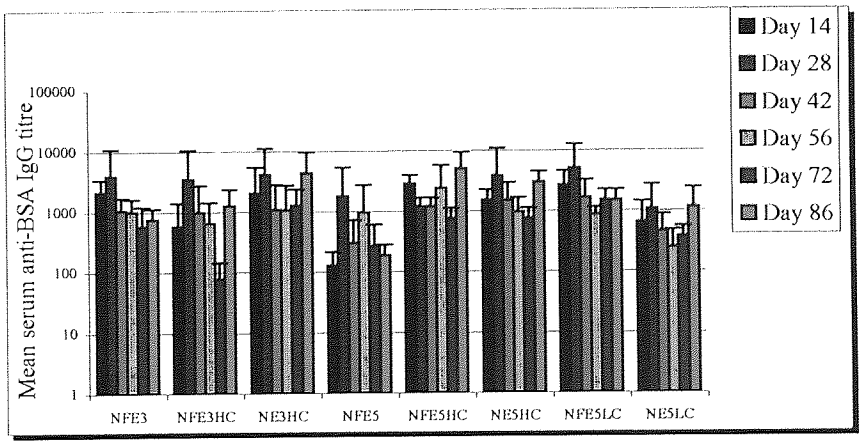


Figure 5.7(a&b): Immune response to nasally delivered BSA given in free form or encapsulated in microspheres with and without chitosan. Descriptions of groups given in table 5.1.

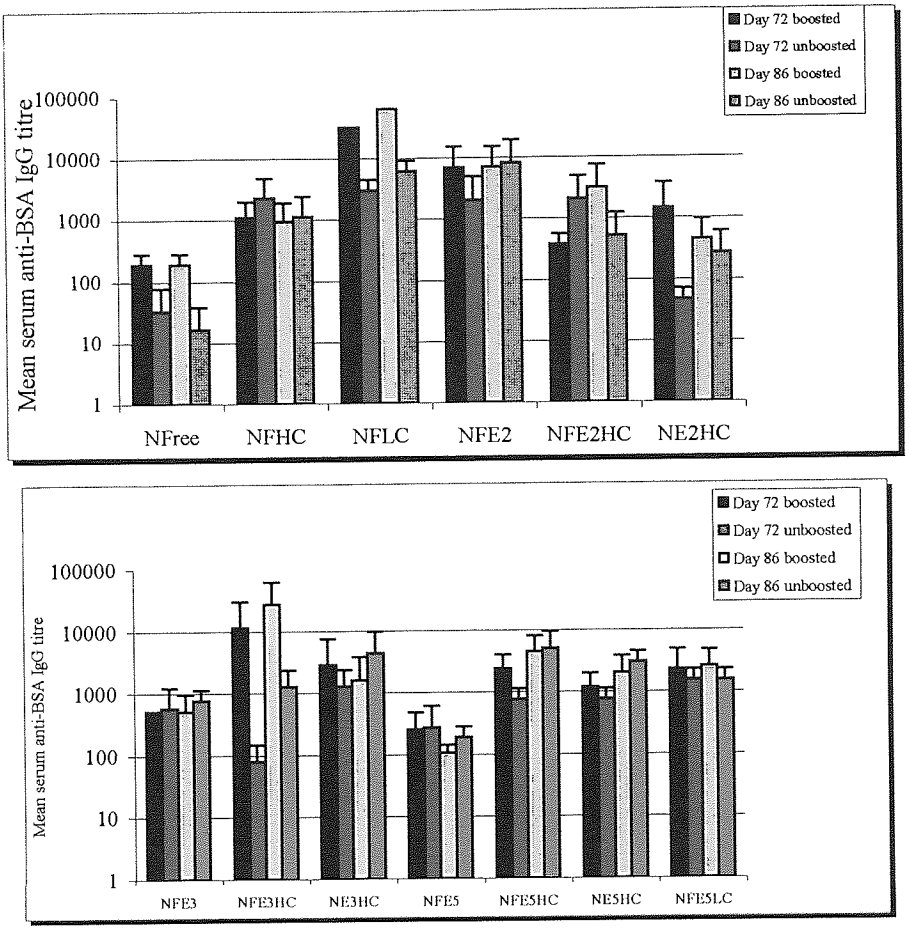


Figure 5.8: Immune responses to nasally delivered BSA given in free form or encapsulated in microspheres with and without chitosan on day 1 and boosted orally with 100 μg of BSA. Descriptions of groups given in table 5.1.

5.3.10. Immune response to intramuscularly delivered BSA given in free form or encapsulated in microspheres with and without chitosan and response to mock challenge

As above, selected formulations were tested intramuscularly for immunogenicity with the addition of free antigen and in the presence of chitosan. Again, all formulations elicited antigen specific antibody responses significantly higher that those observed with free antigen (figure 5.9). On day 14, among the microsphere formulations, the group of mice, which received the 50kDa, PLA encapsulated antigen showed a higher serum specific IgG response followed by the microspheres from the blends of 100kDa and

50kDa whereas 100kDa alone gave a lower immune responses. However, the difference between the formulations is not significant. The response to all formulations decreased over time.

In contrast to intranasal administration, the addition of chitosan in all formulations enhanced significantly the antigen specific immune response compared to antigen in microspheres given in saline. The simple addition of chitosan to the free antigen was shown to enhance antibody titres by this route of administration but the addition of chitosan to the microsphere formulation was shown to be markedly superior. In fact the response to these formulations showed synergy of the two-adjuvant systems. In addition, the inclusion of chitosan in the free antigen and microsphere formulations not only enhanced the immune response but also influenced the duration of the immune response. Even after 3 months, titres were still increasing in the case of microsphere formulations dispersed in the chitosan solution. Perhaps surprisingly, the microsphere entrapped antigen without the presence of free antigen but in the presence of chitosan gave the highest immune responses. Similarly to the results obtained following intranasal administration, the presence of the free antigen did not confer any enhanced immunogenicity in comparison to the wholly entrapped antigen. In addition, this did not appear to be affected by the molecular weight or blend of the polymers used. Both high and low Mw chitosans potentiated immune responses to microencapsulated BSA. However, the low Mw chitosan was found to be superior to the high Mw chitosan when administered with antigen alone by the intramuscular route. In summary, data obtained for these experiments suggest that the effect of chitosan inclusion on serum antibody titres is much less pronounced than the increase observed due to the encapsulation of BSA, which gave responses more than 20 fold higher than the free antigen in the case of intranasal administration ($P<0.05$). A striking increase in responses to formulations administered intramuscularly with chitosan was observed, with some preparations evoking titres more than 10 fold higher than microencapsulatd BSA given without chitosan, and 80 fold higher than the free antigen ($P<0.001$).

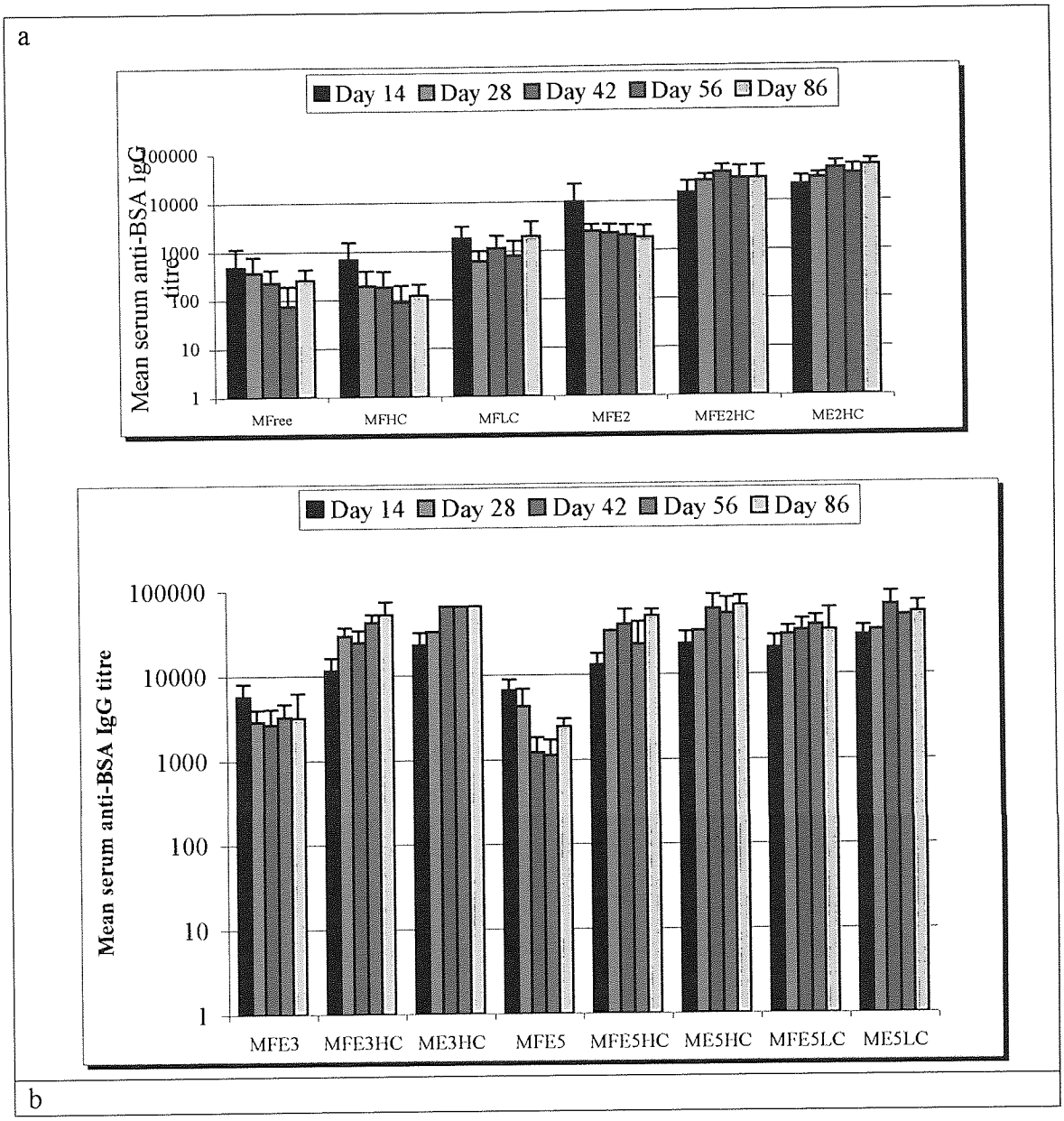


Figure 5.9(a, b): Immune responses to intramusculary delivered BSA given in free form or encapsulated in microspheres with and without chitosan Descriptions of groups given in table 5.2.

Following mock challenge, in comparison to the free antigen all formulations generated a superior antigen specific response (with the exception of the free antigen with high molecular weight chitosan). Results here showed the same trend as the responses following intramuscular immunisation (Figure 5.9).

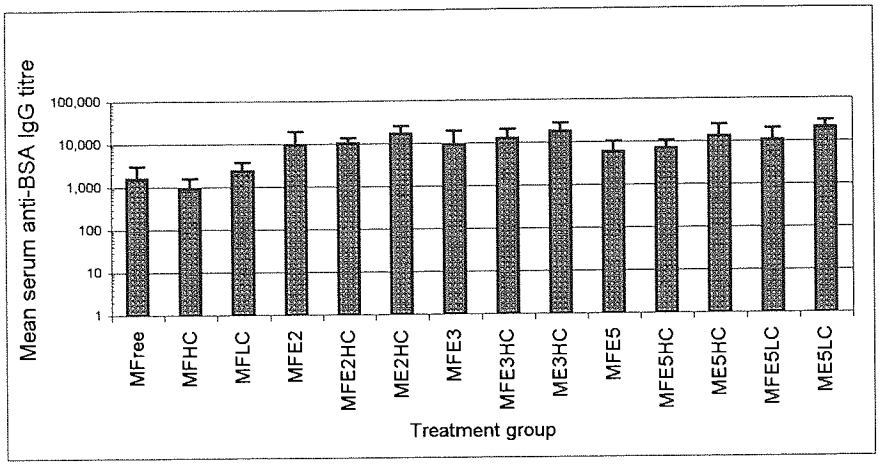


Figure.5.10: Serum Ig G response 7 days following mock challenge with 1µg of BSA subcutaneously in PBS. Treatment group description is given in table 5.2

5.3.11. Effect of chitosan and polyornithine on nasal residence time of intranasally administered microspheres

0.10±0.01 % of the radioactivity associated with the NEN-TRAC® microspheres was attributable to un-bound 'free' scandium-46 after agitated incubation of the microspheres at 37°C in PBS over a period of 24hr. This was not significantly different from the amount of free scandium-46 associated with the particles at the 1h incubation time point (0.09±0.02 %). From these data we surmised that, over the 24 h experimental period, the particle-radiolabel complex was satisfactorily stable for the purposes of these investigations.

Viscosity measurements showed 0.2 % w/v chitosan hydrochloride to be the most viscous bioadhesive tested (2.47 cp), followed by 0.2 % w/v poly-L-ornithine hydrobromide (0.98 cp).

The biodistribution of NEN-TRAC microspheres following nasal administration in the presence and absence of different absorption enhancing compounds is shown in Table 5.4. Consistent with previous findings (Eyles *et al.*, 2001) negligible amounts of radioactivity were detected in the lungs of mice dosed intranasally in the manner outlined in the methods section.

Nasal administration of microspheres in a chitosan hydrochloride vehicle served to significantly prolong nasal residence time of the particles relative to the saline control

(beyond the 1 minute time point). At the 5 hour and 24 hour time point, snouts from mice treated with chitosan hydrochloride contained more radioactive microspheres than animals dosed with microspheres suspended in poly-L-ornithine ($P<0.040$). Nasal administration of microspheres in a poly-L-ornithine vehicle did not significantly alter the biodistribution of microspheres relative to the saline treated controls.

1 minute post intranasal administration			
Vehicle	Nasopharangeal region %± s.d	Lungs %± s.d	Gastrointestinal tract %± s.d
Saline	58.30±25.06	0.82±0.73	14.16±19.42
Polyornithine	86.48±9.75	0.38±0.68	0.06±0.02
Chitosan Hcl	94.81±6.9	0.05±0.02	0.44±0.64
15 minutes post intranasal administration			
Vehicle	Nasopharangeal region %± s.d	Lungs %± s.d	Gastrointestinal tract %± s.d
Saline	34.52±6.33	0.82±0.730.	43.53±7.95
Polyornithine	51.66±16.06	0.05±0.01	31.88±7.31
Chitosan Hcl	55.27±15.42	0.04±0.01	15.76±8.19
5 hours post intranasal administration			
Vehicle	Nasopharangeal region %± s.d	Lungs %± s.d	Gastrointestinal tract %± s.d
Saline	8.32±4.56	0.30±0.52	41.03±16.35
Polyornithine	13.83±6.89	0.05±0.02	29.36±13.08
Chitosan Hcl	55.28±9.69	0.04±0.01	22.98±6.06
24 hours post intranasal administration			
Vehicle	Nasopharangeal region %± s.d	Lungs %± s.d	Gastrointestinal tract %± s.d
Saline	8.32±4.56	0.04±0.01	1.41±0.76
Polyornithine	3.06±1.09	0.04±0.01	1.76±1.05
Chitosan Hcl	14.12±6.91	0.04±0.01	3.08±1.75

Table 5.4: Percent of nasally administered radioactive dose (following intranasal administration of NEN-TRAC® microspheres suspended in either saline, chitosan Hcl or polyornithine) detected in the snouts, lungs and GIT of BALB/c mice.

5.4. Discussion and conclusions

5.4.1. Single dose vaccines

In conventional protein based vaccines, multiple dose administration of an antigen is usually required to induce a lasting immunity. The preparation of formulations capable of releasing the antigen in a pulsatile or a controlled manner for a time that is sufficient to induce such immunity may be a promising improvement in immunization coverage and individual compliance (Bittner *et al.,* 1999) (Benoit *et al.,* 1999; Baras *et al.,* 2000). Immense efforts have been made to utilize micoparticles containing an antigen as a single shot vaccine (Kersten *et al.,* 1996) (Cleland *et al.,* 1997) Microparticles have been found to release antigen *in vitro* and *in vivo* in a pre-programmed manner such as a pulsatile or a continuous mode for several months, potentially eliminating the necessity of multiple injections. However, obstacles still exist and need to be overcome for its successful practical application. Hilbert (Hilbert *et al.,* 1999) described microencapsulation of antigens within biodegradable polymers and suggested that a dosage form consisting of a vaccine antigen partly in solution form and partly in microencapsulated form in various types of microcapsules could be used for single shot immunization. In contrast to their results, in our studies, we were not able to show any enhancement in immune responses when antigen in solution was added to microspheres. This may be due to the fact that we have utilized a different route of administration (intramuscular and intranasal in comparison to subcutaneous) and different polymers. Eldridge (Eldridge *et al.,* 1993) reported that a combination of two microspheric preparations could release a vaccine antigen at different times, providing discrete primary and booster doses following a single administration. A mixture of 1 to 10 and 20 to 125 μm sized microspheres, made of copolymer DL PLGA containing SEB toxoid, induced both primary and a secondary anti-SEB response following a single dose administration in mice. The authors suggest that microspheres of 10μm in diameter were phagocytosed and released the antigen at a substantially accelerated rate compared to the other microspheres contributing to the primary response, while the microspheres with larger dimensions, being slowly phagocytosed, released the antigen at a slower rate and that these resulted in the stimulation of the secondary response in the mice. However, strategies employing different molecular weight polymers and different

polymer blends showing markedly different *in vitro* release profiles did not appear to correlate with *in vivo* immunological data (Spiers *et al.*, 2000). The results obtained here also corroborate these findings, as we observed different release kinetics *in vitro* which were not translated into *in vivo* immune responses in accordance with these release profiles. In addition, the ideal antigen delivery profile may still be somewhat unclear for effective vaccination (Walduck *et al.*, 1998). It is thought that continuous delivery of antigen, even by parenteral routes, may lead to immunological tolerance but this may be dependent upon the particular antigen and our results are in accordance with others (Walduck and Opdebeeck 1996).

5.4.2. Effect of addition of chitosan to microspheres on immune response

Chitosan has been shown as an effective adjuvant for liposomal tetanus toxoid delivered *via* the intramuscular and, in particular, the intranasal route (Bramwell, 1999). In these studies chitosan has been used as a novel adjuvant formulation in conjunction with PLA microparticles with entrapped BSA, as a potential single dose vaccine carrier. Microencapsulated antigens can elicit humoral and cellular immune responses, possibly as a result of the effective targeting of particulate antigens to professional APC. However, the mechanisms by which the particles are taken up and processed, and how the encapsulated antigen is directed towards MHC class I or II presentation pathways, is not entirely clear (Johansen *et al.*, 2000). Chitosan has been reported to have immune stimulating activity, including enhancing the accumulation and activation of macrophages and polymorphonuclear cells, suppressing tumour growth, promoting resistance to infections by microorganisms, induction of cytokines, augmenting antibody responses and enhancing delayed type hypersensitivity and CTL responses (Seferian and Martinez 2000). We have seen, here, the adjuvant properties of in particular the low molecular weight chitosan, but the ability of chitosan to enhance the adjuvant effect of polymer microspheres is a new phenomenon. In particular, the synergistic effect of the chitosan and persistent nature of the immune response achieved may be of considerable interest.

In other studies, the use of microspheres dispersed in mucoadhesive polymer has been shown to enhance the immune response to ovalbumin delivered orally (Singh and O'Hagan 1998). Despite the mucoadhesive nature of chitosan, the results obtained

following intranasal administration of microparticulate antigen in the presence of chitosan were less than those obtained for free antigen with the simple addition of chitosan. One of the mechanisms by which chitosan may exert its adjuvant effects *via* mucosal administration is by the opening of tight junctions (Aspden *et al.,* 1996) and the enhancement of antigen uptake (Somavarapu and Bramwell, 2000). This may have been more effective for the free antigen than for the microspheres and the particle size may be of some importance. Long-term immunity against microorganisms, whose elimination is mediated by antibodies, can only be achieved by boosting the effector and memory B-cell pool and neutralizing antibodies. Microsphere based vaccines can induce long-term immunity by providing an extra cellular depot of antigens, which are recognized by B-cell receptors and circulating antibodies.

The parameters of microsphere processing and trafficking inside antigen-presenting cells and throughout lymphoid tissues,have not yet been fully elucidated with respect to microsphere-vaccine efficacy. It has been criticized that neither continuous nor pulsatile antigen release takes into account the phenomenon of affinity maturation (Johansen *et al.,* 2000), which occurs during the secondary response and is inversely related to the antigen dose that is administered; high doses of antigen produce poor maturation, compared with low doses. Hence, in the interest of safety and specificity of immune response, boosting doses of antigen from microspheres should be low.

Direct comparison of the intramuscular and intranasal routes of immunisation in BALB/c mice demonstrated that microencapsulated antigen by the intranasal route could enhance serum antibody responses to levels approaching those obtained with intramuscular administration(figures 5.8 and 5.9). It can be concluded that the PLA particles are an effective adjuvant formulation enhancing both the intensity and the length of duration of antibody responses to the incorporated antigen. Also the reports of the favorable efforts of co-incorporation of immunomodulators (Cox and Coulter 1999) (O'Hagan *et al.,* 1991) have been confirmed by our results. These microparticles show a high loading capacity and loading efficacy for the model antigen ovalbumin.

It may be concluded from this study that the incorporation of BSA into microspheres led to an increase in the immune response raised to the antigen in mice dosed either intranasally or intramuscularly, when compared to animals dosed with equivalent

amounts of free antigen. Administration of BSA loaded microspheres in the presence chitosan significantly enhanced immune responses after intramuscular administration. In addition, it was found that the administration of a boosting dose, given orally 16 weeks after the initial priming dose, led to a marked increase in observed serum anti-BSA IgG titres in specific treatment groups, suggesting the presence of an anamnestic response.

5.4.3. Effect of chitosan on nasal residence time of microspheres

The biodistribution data confirm that chitosan solutions are effective at protracting the nasopharangeal residence of intranasally applied microparticulates. The slower clearance of microparticles in the presence of chitosan can be explained, in part, by increased bioadhesion (as demonstrated in the Caco-2 cell model). Nasally administered bioadhesive materials are capable of slowing down the rate of mucocilliary clearance through interactions with the nasal mucosa (Soane *et al.,* 1999). The strong mucoadhesive behaviour of microspheres in the presence of chitosan can be attributed to electrostatic interactions between the positively charged amino groups in chitosan and negatively charged sialic acid residues that are present in mucins. In addition to charge effects, differences in molecular contact and flexibility may influence particle mucoadhesion. Similarly factors other than molecular interactions, for example physical hindrance related to inherent viscosity, can also affect mucoadhesion. In support of this, at the concentrations tested, poly-L-ornithine was not found to influence the transit times of NEN-TRAC microspheres through the upper respiratory and alimentary tracts *in vivo*. Both poly-L-ornithine and chitosan are polycations, although at the concentrations used in this experiment they have quite different viscosities (0.2 % w/w poly-L-ornithine has a viscosity not much different to saline solution). Hence although positively charged polymers have the potential to act as bioadhesieves (as demonstrated *in vitro*), due to electrostatic interaction with negatively charged sialic acid groups in mucin, the viscosity of the polycation vehicle is probably also a key factor influencing its capacity to retard microsphere clearance from the nasal passages.

Such findings suggest that intranasal administration of microencapsulated vaccines in a chitosan vehicle might result in improved immunological responses due to increased contact time with immunoresponsive elements in the nasopharangeal milieu.

CHAPTER: 6

6.0. The potential of absorption enhancers as nasal mucosal adjuvants

6.1. Introduction

The nasal mucosae is the first site of contact with inhaled pathogens, and is the initial line of physical and immunological defence of the mammalian organism. This defense is mediated through the sampling of proteins and antigens by the organized lymphoid tissue at the base of the nasal cavity, nasal-associated lymphoid tissue (NALT), and the prevention of pathogenic colonisation of the nasal mucosa is an effective method of halting infections. Herein lies the rationale for the development of mucosal vaccines (Almeida and Alpar 1996).

The effectiveness of the toxins CT and LT as adjuvants appears to be related to their ability to enhance the penetration of molecules through the mucosal membrane; i.e. they act as penetration enhancers. Absorption enhancers have been employed in attempts to increase the extent of peptide absorption from the nasal cavity. There has been some research on the function of absorption enhancers as mucosal adjuvants and in addition to CT and LT, *Zonula occludens* toxin (ZOT) from *Vibrio cholerae* and toxin A of *Clostridium difficile* (Marinaro *et al.*, 1999) have been shown to act as mucosal adjuvants. All of these agents are known to alter intestinal permeability. Another approach to absorption enhancement of proteins includes the co-administration of enzyme inhibitors (Morimoto *et al.*, 1995). The use of non-toxic absorption enhancers as effective mucosal vaccine adjuvants may be of considerable therapeutic benefit.

6.1.1. Mechanisms of action of penetration enhancers as mucosal adjuvants

Transport across the intestinal epithelium is often the rate-limiting step in the total process of absorption for orally administered hydrophilic macromolecular compounds. Passage of these compounds is most likely to occur extracellularly by passive diffusion through the paracellular pathway. However, tight junctions abolish passage of large molecules almost completely. Co-administration of an absorption enhancer is a potential way to increase the bioavailability of these compounds, and considerable effort has been directed toward identifying agents able to loosen tight junctions, thus increasing

paracellular permeability. Although the exact mechanisms of action are not fully identified, two general classes of absorption enhancers are distinguished: surfactants and calcium chelators (Artursson *et al.,* 1994; Hochman *et al.,* 1994). Surfactants (bile acids and salts, derivatives of fatty acids, Triton X-100 and so on) act by increasing the solubility of hydrophobic macromolecules in the aqueous boundary layer or by increasing the fluidity of the apical (and the basolateral) membrane. Calcium chelators (EGTA and EDTA) reduce the extracellular calcium concentration, which leads to disruption of cell-cell contacts. In general, the surfactants increase transcellular permeability, whereas the chelators increase paracellular permeability.

It has been proposed (Muranishi 1990) that penetration enhancers improve mucosal peptide absorption by: 1) Reducing the viscosity and/or elasticity of the mucuous layer, i.e. alteration in rheological properties of mucuous layer. 2) Facilitating transcellular transport by increasing the fluidity of the lipid bilayer of membranes through interaction with their lipid component or protein component. 3) Facilitating paracellular transport by altering the tight junctions either through chelating divalent cations like Ca+ present in the tight junctions, or through altering the macromolecules involved in the formation of intercellular junctions. 4) Overcoming the enzymatic barrier for peptide and protein. 5) Increasing the thermodynamic activity of peptide and protein drugs. Saponin has been demonstrated to have adjuvant effect after systemic administration by virtue of its ability to promote membrane interactions between antigens (Vogel 1995).

Schroder and Svenson (Schroder and Svenson 1999) found that mono-olein/oleic acid vesicles enhance the nasal immunogenicity of admixed DT in mice to the same level as injected alum adsorbed DT. It was shown that immunogenicity was linked to the length of the acyl chain of the lipids, where shorter acyl chains resulted in reduced titres and increased toxicity. Importantly, scanning electron microscopic analyses of nasal epithelial sections did not identify significant necrosis or alopecia in rats treated with the optimized mono-olein/oleic acid formulations. Work by Marinaro *et. al.* (Marinaro *et al.,* 1999) showed that the *Zonula occludens* toxin of *V. cholerae* could act as a mucosal adjuvant. ZOT disrupts the integrity of the epithelial tight junctions resulting in increased permeability to macromolecules.

The majority of immunological adjuvants, which have been shown to be effective orally, appear to exert an effect that could best be described as absorption enhancement.

Effective absorption enhancers include non-ionic surfactants, bile salts, fatty acids, salicylates and chelating agents.

For enhancing delivery of peptides and proteins, a reduction in the enzyme activity of the mucosal barrier may also be desired in addition to the enhancement. Nevertheless, the safety profile of absorption enhancers is of primary importance for human use. Most of the absorption enhancers are detergents, and it is their detergent properties, which are the source of their absorption enhancing capability (Aungst 2000). Detergents such as bile salts are soluble amphiphiles. Amphiphilic compounds are those which possess both polar and non-polar groups. Most amphiphilic compounds are surfactants, i.e., they exhibit significant surface activity (Muranishi 1990). Surfactants, which increase cellular permeability generally, do so by disturbing the cell's plasma membrane. Surfactant monomers are capable of partitioning into the plasma membrane, where they can form polar defects in the lipid bilayer. The use of chemical absorption enhancers increases nasal, pulmonary and gastrointestinal absorption of proteins and peptides.

6.1.2. Bile salts

Bile salts are synthesised in the liver from cholesterol and enter the duodenal lumen in the form of mixed micelles with lecithin and cholesterol. In the intestinal lumen, bile salts serve as detergents, which aid the solubilization of dietary fats. In the GIT, bile salts are generally found in mixed micelles with lecithin, cholesterol, monoglycerides, and fatty acids. The common bile salts in man are the glycine- and taurine-conjugates of the dihydroxy-bile salts deoxycholate (DOC) and chenodeoxycholate (CDOC), and of the trihydroxy-bile salt cholate.

Bile salts were used as early as 1932 to enhance the absorption of peptides and insulin and other drugs across the nasal, rectal and vaginal membranes (Sayani and Chien 1996). These enteric substrates are the most widely used surfactants for nasal absorption optimisation. Commonly used bile salts are sodium cholate, sodium deoxycholate, sodium glycocholate, and sodium taurocholate. Possible mechanisms by which these compounds exert their effects include increasing permeability of the membrane structure (they have surfactant properties akin to the saponins), and inhibition of proteolytic enzymes as previously discussed. Bile salts have a high capacity for solubilization of phospholipid, suggesting that extraction of phospholipid may disrupt

the intestinal brush-border membrane. Because these agents are endogenous residents of the gastrointestinal lumen, they are generally thought to be non-toxic. However, the ability to permeabilize and dissolve membranes through their surfactant action imply the potential for local irritation and toxicity. The use at sub-toxic levels should preclude this as a limiting factor in the further evaluation of bile salts as mucosal adjuvants.

6.1.3. Cyclodextrins

Cyclodextrins (CD's) are cyclic oligosaccharides of glucopyranose units with a lipophilic cavity in the center. They are produced by the action of a group of enzymes called cyclodextrin glycosyltransferases on starch. The natural product consists of a mixture of the various cyclodextrins, mainly α-cyclodextrin, β -cyclodextrin and γ-cyclodextrin, which consists of six, seven, and eight glucopyranose units, respectively. In aqueous solution and the solid state, the inner hydrophobic cavity of the cyclodextrin molecule provides accommodation for appropriate guest molecules and the formation of dynamic inclusion complexes leading to the modification of some physical and chemical properties, which characterize the drug. This ability to form inclusion complexes with many drugs by taking up whole drug molecules, or some part of it, into the cavity makes it a useful agent for altering many of the physicochemical properties of the drugs. The advantages in the drug formulation include improving the solubility of poorly water-soluble drugs and their consequent bioavalibility. Cyclodextrins are also useful to eliminate the undesirable properties of drugs, like irritation or unpleasant odour and taste. Through complexation, cyclodextrins may also stabilize and protect unstable compounds from degradation (Irie and Uekama 1999).

Cyclodextrins have a wide variety of uses in the food, cosmetic, and biotechnology industries. Recently, Hovgaard and Brondsted (Hovgaard and Brondsted 1995) have shown that cyclodextrin formulations enhance the transport across the intestinal epithelium. This is though to occur without affecting their barrier function, a property that makes cyclodextrins ideal penetration enhancers for intranasal drug delivery. Cyclodextrins can also act as solubilizers for lipophilic water-insoluble drugs, making it possible to formulate such drugs in aqueous nasal spray formulations. Furthermore, these molecules can stabilise drugs, which are chemically unstable in aqueous solutions,

and decrease drug irritation after nasal application (Marttin *et al.,* 1999). CDs have been used as absorption enhancers for calcitonin, glucagons, insulin, and recombinant human granulocyte colony stimulating factor. The dimethylated β form has been shown to improve the nasal absorptions of insulin in rats (Shao *et al.,* 1992).

A disruption of the paracellular barrier in the nasal mucosa by cyclodextrins could lead to increased accessibility of co-administered antigen to lymphoid tissue. In support of this idea, it has been shown that the ability of cholera toxin to increase mucosal permeability may play a role in its adjuvanticity (Goto *et al.,* 2000).

6.1.4. Polyaminoacids

Natsume *et al.* (Natsume *et al.,* 1999) screened several cationic compounds as potential nasal absorption enhancers for increasing the nasal absorption of FITC labeled dextran. They reported that poly arginine enhanced the nasal delivery of high molecular weight substances without severe damage to the nasal mucosal membrane. It was reported that the enhancing effect by poly-arginine was dependent on its molecular weight; it was proposed that a higher molecular weight would have a higher affinity for receptors, ion channels or glycoproteins on the nasal epithelium. Thus, the electrical property, molecular size and higher-ordered structure together with other physicochemical properties may give a different absorption mechanism, which allows further enhancement, without severe damage to the nasal mucosa. However, further clarification of the mechanism of absorption is required.

Poly-arginine resulted in a markedly higher bioavailability of dextran without undesirable effects to rat erythrocytes and isolated rabbit nasal mucosa, a property seemingly unique to this polyaminoacid. The authors emphasized that poly-arginine is a promising candidate for penetration enhancement of drugs, having a suitable balance between enhancing activity and safety for nasal peptide and protein delivery.

6.1.5. Vitamin E TPGS

Vitamin E TPGS (TPGS, tocopheryl polyethylene glycol 1000 succinate see figure 6.1 and table 6.1 for structure and chemical, physical properties respectively), a water-soluble derivative of a natural source of vitamin E, has been utilized for numerous

applications in pharmaceutical dosage forms. Its chemical structure contains both a lipophilic and a hydrophilic moiety, making it similar to a conventional surface-active agent.

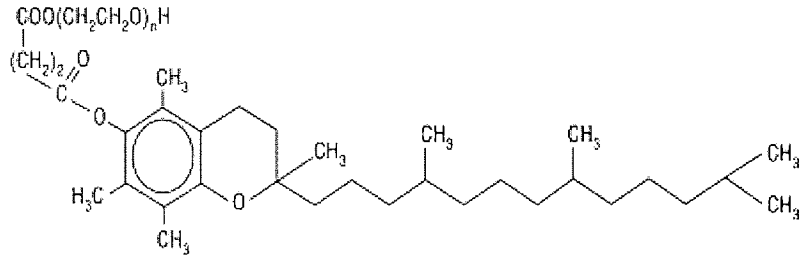


Figure 6.1: Chemical structure of the principal component of Vitamin E TPGS.

Its unique properties suggest these uses for TPGS: emulsifier, solubilizer, absorption enhancer, and water-soluble source of vitamin E.

Molecular weight	approx. 1513
Physical form	Waxy solid
Color (solid)	Pale yellow
Vitamin E content	mg/g min. 260 as d-α-tocopherol
Potency IU/g	387
Acid value	Max. 1.5
Specific gravity @ 45°C	Approx. 1.06
Melting point	Approx. 38°C (100°F)
HLB (hydrophile/lipophile balance)	~13

Table 6.1: Typical Properties of Vitamin E TPGS 1000.

Vitamin E TPGS is miscible with water and forms solutions with water at concentrations up to approximately 20 w/v beyond which the liquid crystalline phases may form. TPGS has a dual nature, with part of the molecule exhibiting hydrophilicity and the other lipophilicity. The exact portion of the molecule comprising the hydrophilic polar head or the lipophilic alkyl tail may not be elucidated from the

molecular structure. A generally accepted view is that the polyethylene glycol portion serves as the polar head while the tocopherol succinate portion serves as the lipophilic tail. This amphiphilic characteristic leads to its self-association in water when the concentration exceeds a threshold known as the critical micelle concentration (CMC).

It has a relatively high crystallinity, high degradation temperature, and good thermal stability. TPGS forms liquid crystalline phases in water. Micelles are formed at 0.02 % w/t. When the TPGS concentration is above 20 % w/v, higher-viscosity liquid crystalline phases start to form. With increasing TPGS concentrations in water, more complex liquid crystalline phases evolve, e.g., from isotropic globular micellar, to isotropic cylindrical micellar and hexagonal, mixed hexagonal and reversed hexagonal, reversed globular micellar, and to the lamellar phase.

TPGS is also believed to be a bioavailability enhancer when co-administered with some lipophilic drugs, including cyclosporine. TPGS is known as a surface-active agent or as an emulsifier for use in complex formulations that include a number of other excipients that function as solvent, binder, and filler.

In recent years, vitamin E TPGS, or d-.alpha.-tocopheryl polyethylene glycol 1000 succinate, has been used as one of many excipients in complex immediate release drug formulations, usually emulsions or micro-emulsions, wherein the active drug compound is poorly soluble in water.

Sokol *et al* (Sokol *et al.,* 1991), describes the oral co-administration of vitamin E TPGS with cyclosporine to improve the absorption of the drug by pediatric liver transplant patients. Because TPGS was known to be well absorbed in the absence of detectible bile flow, Sokol *et al.* postulated that TPGS co-administration with cyclosporine might enhance cyclosporine absorption in paediatric transplant patients if poor mixing of bile with cyclosporine or residual cholestasis were causing poor absorption. Similarly, Chang *et al.* (Chang *et al.,* 1996) discloses the concomitant administration of separate cyclosporine and Vitamin E TPGS dosages in an effort to enhance cyclosporine absorption, and decrease cyclosporine counter transport back into the intestine by P-glycoprotein.

Yu *et al.*(Yu *et al.,* 1999) observed in their studies that HIV protease inhibitor transport through the Caco-2 cell monolayers increased in the presence of vitamin E TPGS. Overall, vitamin E TPGS enhanced the absorption flux of HIV protease inhibitor by

increasing its solubility and permeability. The active efflux system was inhibited by vitamin E TPGS.

An important consideration for its use as a mucosal adjuvant is the safety of vitamin E TPGS, in comparison with alternative absorption enhancers. Vitamin E TPGS has been used as a vitamin E supplement for treatment of vitamin E deficiency in children. The acute oral LD_{50} of vitamin E TPGS is over 7000mg/kg for adult rats. In clinical studies, vitamin E TPGS is used at 2256 mg twice daily. Thus the concentrations in our studies were very low and should not cause any tissue damage. Although its mechanism of action has not been accurately defined, vitamin E TPGS is surface active at low concentrations (0.02% w/v) and the mechanism of absorption enhancement is likely to be similar to that of alternative surfactants. The enhancement of cyclosporin bioavailability is suspected to be due to enhanced solubility, improved permeability, and reduced intestinal metabolism. It is perhaps may, in part, be due to inhibition of P glycoprotein in the intestine.

6.1.6. Acylcarnitines

Acylcarnitines are endogenous amino acid-like compounds which play a role in the mitochondrial transport system of cells by carrying fatty acids across the mitochondrial membrane. L-carnitine has been extensively studied as an acyl acceptor in the mitochondrial acyltransferase system involved in fatty acid utilisation and apparently serves as an intramembrane carrier molecule to transport fatty acids to the mitochondrial interior. L-carnitine and their accompanying L-carnitine acyltransfersases are most highly concentrated in active muscle tissue.

It has been suggested that acylcarnitines enhance paracellular permeability in a calcium-independent way. The absorption-enhancing properties of these fatty acid derivatives of L-carnitine have been studied extensively (Fix *et al.*, 1986). The C16 conjugate palmitoyl-D, L-carnitine chloride (PCC) was found to be very effective in increasing transepithelial transport of poorly absorbed drugs *in vivo* in rats and dogs (Fix *et al.*, 1986; Sutton *et al.*, 1992) and *in vitro* in intestinal segments and Caco-2 cells (Hochman *et al.*, 1994).

Histological and ultrastructural examination of sections of rat jejunum and colon exposed to (PCC) showed slight alterations in the microvillus border but an intact cytoplasmic integrity and uncompromised junctional complexes. To elucidate the mechanism by which PCC exerts its action, *in vitro* studies were performed using Caco-2 cells (Hochman *et al.,* 1994). These studies have shown an immediate drop in transepithelial electrical resistance (TEER) and an increase in mannitol transport after apical exposure to submillimolar concentrations of PCC. The drop in TEER proved to be transient and returned to approximately control values in 10 to 12 h. No visible damage to the epithelium and no alterations in the filamentous actin were seen, but the appearance of tight junctions in freeze-fracture replicas seemed to be altered; that is, junctional strands showed a beaded appearance, several discontinuities and diminished cross-bridging. These observations indicate that PCC is able to increase permeability reversibly without causing major morphological alterations in the intestinal epithelium. On the other hand, other studies have shown that PCC causes damage to erythrocytes and the vaginal epithelium at concentrations likely to be used to achieve absorption-enhancing effects. In addition, LeCluyse *et al* 1993 reported that after PCC treatment, lucifer yellow transport could be enhanced without observed exfoliation of cells; however, an enhancement factor of 18 for lucifer yellow transport was accompanied by extensive cell exfoliation from rat colonic mucosa. The exact mechanism by which PCC exerts its permeability-enhancing action has still not been elucidated. Moreover, the sequence of events (transport enhancement, membrane damage and tight junction disruption) is unknown.

It is the medium and long chain fatty acid esters of carnitines that have shown most desired characteristics of ideal *in vivo* absorption enhancers. Fix *et al* 1986 have demonstrated that acylcarnitines enhance the absorption of cefoxitin and a cyclic hexapeptide analog of somatostatin, when dosed rectally or dudoenally in the rat. It was observed that palmitoylcarnitine was generally the most effective absorption enhancer, but lauroyl, myristoyl and steroylcarnitine were also effective. These acylcarnitines were above their CMC's in these experiments, which might suggest that they enhance absorption *via* detergent activity.

Kagatani, *et al* 1996 investigated the effect of different acylcarnitines on the nasal absorption of salmon calcitonin (sCT). Nasal absorption of sCT was significantly enhanced by carnitines with acyl groups of 12 or more carbon atoms. Enhancement of by lauroylcarnitine chloride was observed at its critical micelle concentration. Micelle formation was thus considered to be an important factor in the mechanism of enhancement of acylcarnitines on the nasal absorption of sCT.

The effect of several acylcarnitines with differing molecular weights was also investigated by Chao, *et al.,* 1998. The results compared well with the findings of fluorescence polarisation experiments, where brush border membranes vesicles isolated from rat small intestine were treated with long chain acylcarnitines (LeCluyse *et al.,* 1991). Both studies illustrate a relationship between the potency and chain length of the homologous series of the acylcarnitines. The 14 carbon acylcarnitines MCC was found to be the most potent at altering the microviscosity of the membrane bilayer. This was followed by rank ordering of the other long chain acyl carnitines, which closely paralled the order of the C values in the present study. The safety of PCC in intestinal mucosa has been demonstrated with reversible enhancement of absorption and no induction of morphological changes.

The aim of this chapter was to determine potential of a series of absorption enhancers as mucosal adjuvants. The following compounds were tested; vitamin E TPGS, sodium deoxycholic acid, dimethyl β cyclodextrin, polyornithine, acylcarnitine. Tetanus (TT) and diphtheria (DT) toxoids and F1 and V antigens of plague were used as clinically relevant antigens. These absorption enhancers were administered *via* the nasal route.

6.2. Materials and methods

6.2.1. Materials

Diphtheria and tetanus toxoids were supplied as a gift by the Serum Institute of India, Pune, India. Vitamin E TPGS was a gift from Eastman Co.Kingsport, TN, US. Acylcarnitine, dimethylcyclodextrin, polyornithine, sodium deoxycholic acid and all

other chemicals and reagents, unless otherwise stated, were obtained from Sigma Chemical Co., Poole, Dorset, UK.

6.2.2. Preparation of vitamin E TPGS Solution

The entire contents of a container of *Eastman* vitamin E TPGS NF was melted with constant stirring to ensure homogeneity. The molten TPGS was slowly poured, with vigorous stirring, into near-boiling water, up to a maximum of 20% w/v. The TPGS absorbed a portion of the water immediately. Forming high-viscosity gel particles; these particles dissolved with stirring. The mixture was gently stirred at room temperature for approximately 2 hours to ensure complete dissolution of the gel particles, and a clear fluid solution resulted from a mixture containing up to 20% TPGS by weight.

6.2.3. Immunisation schedule for nasal delivery of diphtheria and tetanus toxoids

The absorption enhancers used and their concentrations are tabled below.

Absorption Enhancer	**Concentrations used (% w/v)**
Sodium Deoxycholic Acid	1 and 0.5%
Vitamin E TPGS	20, 10, 5 and 0.5%
Dimethyl-β cyclodextrin	5 and 2.5%
Polyornithine (high molecular weight)	1 and 0.5%

Table 6.2: The various absorption enhancing agents used in this study and the concentrations used.

Groups of 5 BALB/c mice were dosed intranasally with 5Lf DT or TT as indicated, at day 1 and boosted with 2.5Lf on day 49 with or without the various absorption enhancers at the concentrations indicated in table 6.2 in 20 μl of PBS . Blood samples were collected at regular intervals for analysis by ELISA as described in the methods section.

6.2.4. Immunisation schedule for nasal delivery of *Yersinia pestis* V and F1 subunits

The absorption enhancers used and their concentrations are tabled below.

Absorption Enhancer	Concentrations used (% w/v)
Sodium Deoxycholic Acid	0.25%
Dimethyl-β cyclodextrin	2.5%
Polyornithine (high, medium and low molecular weight)	0.2%

Table 6.3: The various absorption enhancing agents used in this study and the relative concentrations.

Groups of 5 BALB/c mice were given a single dose intranasally comprising 1μg of F1 and 3μg of V antigen at day 1 with or without the various absorption enhancers at the concentrations indicated in table 6.3 in 20 μl of PBS Blood samples were collected at regular intervals for analysis by ELISA as described in the methods section.

6.2.5. Immunisation schedule for nasally delivered tetanus toxoid with various acylcarnitines

Groups of 5 BALB/c mice were dosed 20 μl intranasally with 1 μg of TT with or without the following acylcarnitine derivatives; DL-Octanoyl carnitine chloride (OCC), DL-Lauryl carnitine chloride (LCC), palmitoyl-DL- carnitine chloride (PCC), DL-Hexanoyl carnitine chloride (HCH), L-carnitine chloride (CH) and mistroyl-DL-carnitine chloride (MCC) in order to assess their potential to act as mucosal adjuvants. All derivatives were used at 0.5%w/v in PBS. Animals were boosted with the same formulations on day 35. Blood samples were collected at regular intervals for analysis by ELISA as described in the methods section.

6.3. Results

6.3.1. Vitamin E TPGS as an absorption enhancer and mucosal adjuvant

Groups of 5 mice were dosed on day 1 and day 49 with 5 Lf and 2.5 Lf TT with and without vitamin E TPGS. The following concentrations of vitamin E TPGS were used: 0.5%w/v, 5%w/v, 10%w/v and 20%w/v. Preparation of the vitamin E TPGS was carried out as outlined in the methods and materials section of this chapter(6.2.2).

6.3.1.1. Systemic immune responses to nasally delivered tetanus toxoid with vitamin E TPGS and the effect of concentration of vitamin E TPGS.

Blood was collected from the tail vain on day 21 and analysed for anti TT IgG. Free toxoid without the addition of vitamin E TPGS resulted in negligible or no toxoid-specific antibody response. Whereas, on day 21, TT administered in the presence of vitamin E TPGS produced significantly enhanced systemic immune responses directed against the antigen (Figure 6.2). This clearly shows that vitamin E TPGS acts as a mucosal adjuvant for nasally delivered tetanus toxoid. On day 21 the observed immune response generated by TT in the presence of vitamin E TPGS was shown to be dependent upon the concentration of vitamin E TPGS (Figure 6.2). Among the formulations used, the higher concentration of vitamin E TPGS resulted in higher antigen specific immune responses, as determined by serum IgG titres, to co-delivered tetanus toxoid. Even though 0.5%w/v vitamin E TPGS potentiated a lower immune response to TT; it is still significantly increased in comparison to the free toxoid. This clearly shows that vitamin E TPGS was able to enhance the systemic immune response to TT even after single administration. In order to elucidate the effect of vitamin E TPGS following boosting, all mice were dosed on day 49, blood was collected seven days after on day 56 and serum was analysed for toxoid specific IgG. Compared to the primary immune response, all formulations significantly elevated the serum anti TT titres after boosting. However, even following the booster dose the mice receiving free tetanus toxoid showed little improvement in the observed antigen- specific serum IgG titres. This is in agreement with the literature where, without mucosal adjuvant, TT after mucosal delivery produced negligible or no response. It has been shown that tetanus

toxoid, after nasal vaccination with cholera toxin or LT was able to facilitate elevated immune responses. Due to the toxic nature of these adjuvants, however, their use in humans may not be possible. There is a considerable interest in the development of safer, less toxic mucosal adjuvants.

In addition to the magnitude of the observed systemic humoral immune response, the duration of the response was shown to be maintained for in excess of six months (Figure 6.2).

With respect to the primary immune response, the optimal concentration range for achieving the highest serum titres was above 5% w/v and showed an increase between 5 and 20% w/v.

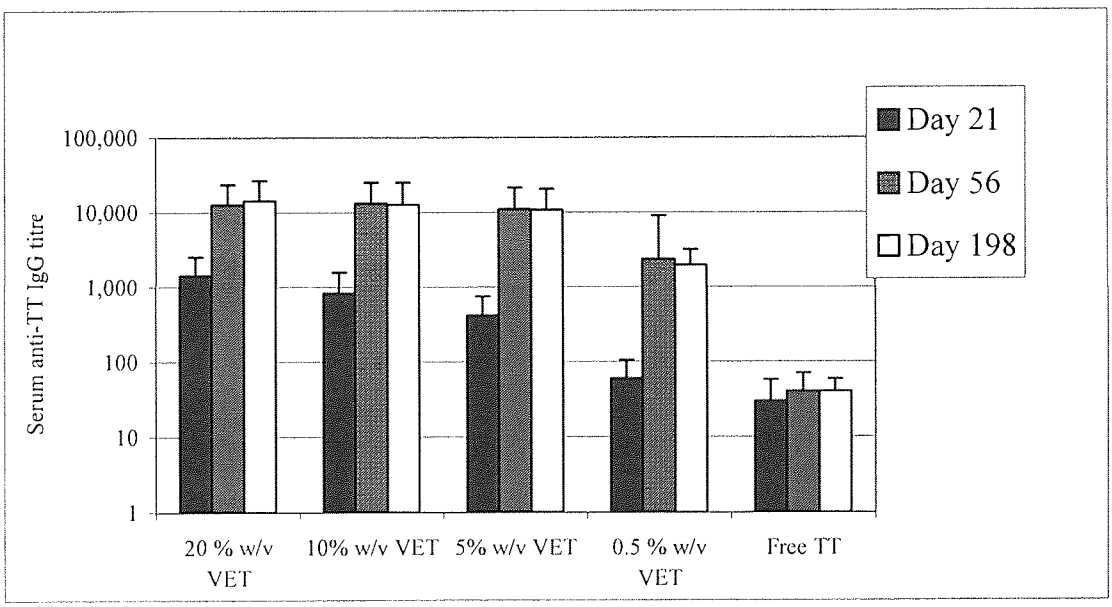


Figure 6.2: Anti-tetanus toxoid (TT) specific IgG end-point titres following intranasal administration of 5 LfTT on day 1 and 2.5 Lf TT on day 49. Toxoid was administered in the presence and absence of different concentrations of Vitamin E TPGS (VET) (n=5).

6.3.1.2. Systemic immune response to nasally delivered diphtheria toxoid with and without vitamin E TPGS

In order to confirm that the mucosal adjuvant action of vitamin E TPGS could be applied to alternative antigens we tested systemic immune responses using diphtheria toxoid delivered nasally. Groups of 5 mice were immunised with and without vitamin E

TPGS and we evaluated primary, secondary immune responses. Vitamin E TPGS enhanced significantly the serum immune response before and after the booster dose. The observed adjuvant effect and differences according to the concentration of vitamin E TPGS was shown to follow the same trend as that observed for tetanus toxoid. The primary and secondary systemic immune responses to nasally delivered diphtheria toxoid with and without vitamin E TPGS is shown in Figure 6.3.

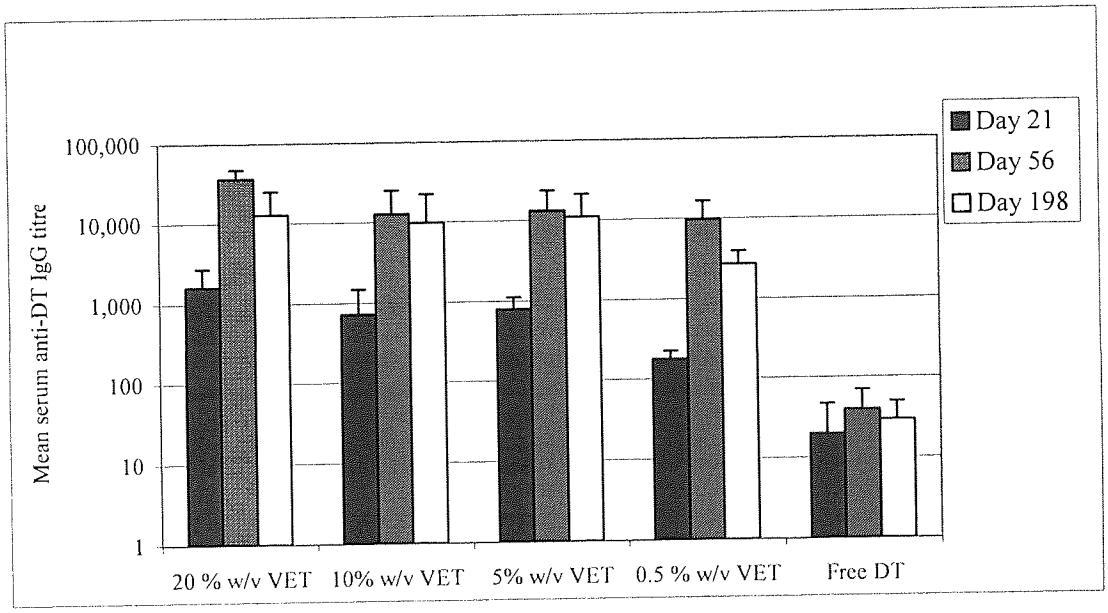


Figure 6.3: Anti-diphtheria toxoid (DT) specific IgG end-point titres following intranasal administration of 5 Lf DT on day 1 and 2.5 Lf DT on day 49. Toxoid was administered in the presence and absence of different concentrations of Vitamin E TPGS (VET) (n=5).

6.3.2. Cyclodextrin as an absorption enhancer and novel mucosal adjuvant delivered *via* the nasal route

Following confirmation that aborption enhancers can act as mucosal adjuvants, we tested wheather cyclodextrins are also able to act as mucsoal adjuvants. Groups of 5 mice were dosed on day 1 and day 49 with 5 Lf and 2.5 Lf TT with and without cyclodextrin. We tested two concentrations; 5%w/v and 2.5%w/v.

6.3.2.1. Systemic immune responses to nasally delivered tetanus toxoid with and without cyclodextrin

Blood was collected from the tail vain on day 21 and analysed for anti TT IgG. The primary serum immune response showed that the dimethyl β-cyclodextrin significantly enhanced the antigen specific antibody response (more than 25 times that observed following immunisation with free antigen). Free toxoid without the addition of the cyclodextrin thus produced markedly reduced toxoid specific antibody responses. There did not appear to be any difference in the immune response induced by using the two different concentrations of the enhancer (2.5%w/v and 5%w/v) following a single administration (Figure 6.4). Animals were boosted on day 49 and serum samples were taken 7 days after, on day 56. Following boosting there did appear to be increased discrimination between the groups with 5%w/v cyclodextrin facilitating higher antibody titres as compared to the 2.5% concentration although the difference was not significant (Figure 6.4). It is notable that the secondary response was increased by almost two orders of magnitude and was seen to be in excess of 150 times greater than the response generated by the free antigen. The immune response was long lived and TT specific antibody titres were still high but reduced in comparison to day 56 at day 198.

6.3.2.2. Systemic immune responses to nasally delivered diphtheria toxoid with and without cyclodextrin

As with vitamin E TPGS, we again wanted to confirm that the mucosal adjuvant action of cyclodextrin could be applied to alternative antigens. In order to do this we tested systemic immune responses using diphtheria toxoid delivered nasally in the presence of dimethyl β-cyclodextrin. Following the same immunization regime, the cyclodextrin again showed the same trend for the diphtheria toxoid as that observed for TT. The primary immune response was markedly enhanced in comparison to the free antigen (Figure 6.4). Boosting again engendered significant increases in antigen specific antibody titres and a similar discrimination was shown between the groups. The immune response was shown to be long lived but again the magnitude of the response was reduced.

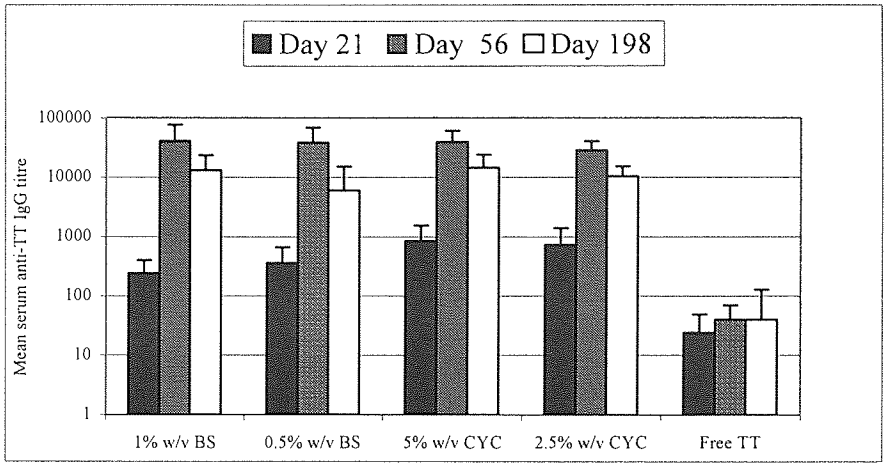


Figure 6.4: Anti-tetanus toxoid (TT) specific IgG end-point titres following intranasal administration of 5 Lf TT on day 1 and 2.5 Lf TT on day 49. Toxoid was administered in the presence and absence of different concentrations of dimethyl-β-cyclodextrin (CYC) or glyco deoxycholic acid (BS) (n=5).

6.3.2.3. Systemic immune responses to nasally delivered *Yersinia pestis* subunit F1 and V antigens with and without cyclodextrin

Groups of 5 mice were dosed on day 1 with a single administration of 1μg of the V antigen and 5μg of F1 in combination, with and without cyclodextrin.

The cyclodextrin was added at 2.5%w/v. Blood was taken at days 14 and 51 for analysis of V and F1 specific antibody titres. For both V and F1, the observed systemic immune response was increased in comparison to the free antigen at the two time points tested (figure 6.5). The results here show that the response is maintained up to day 51 following a single intranasal dose. Moreover, this is shown for both of the subunit antigens used in combination.

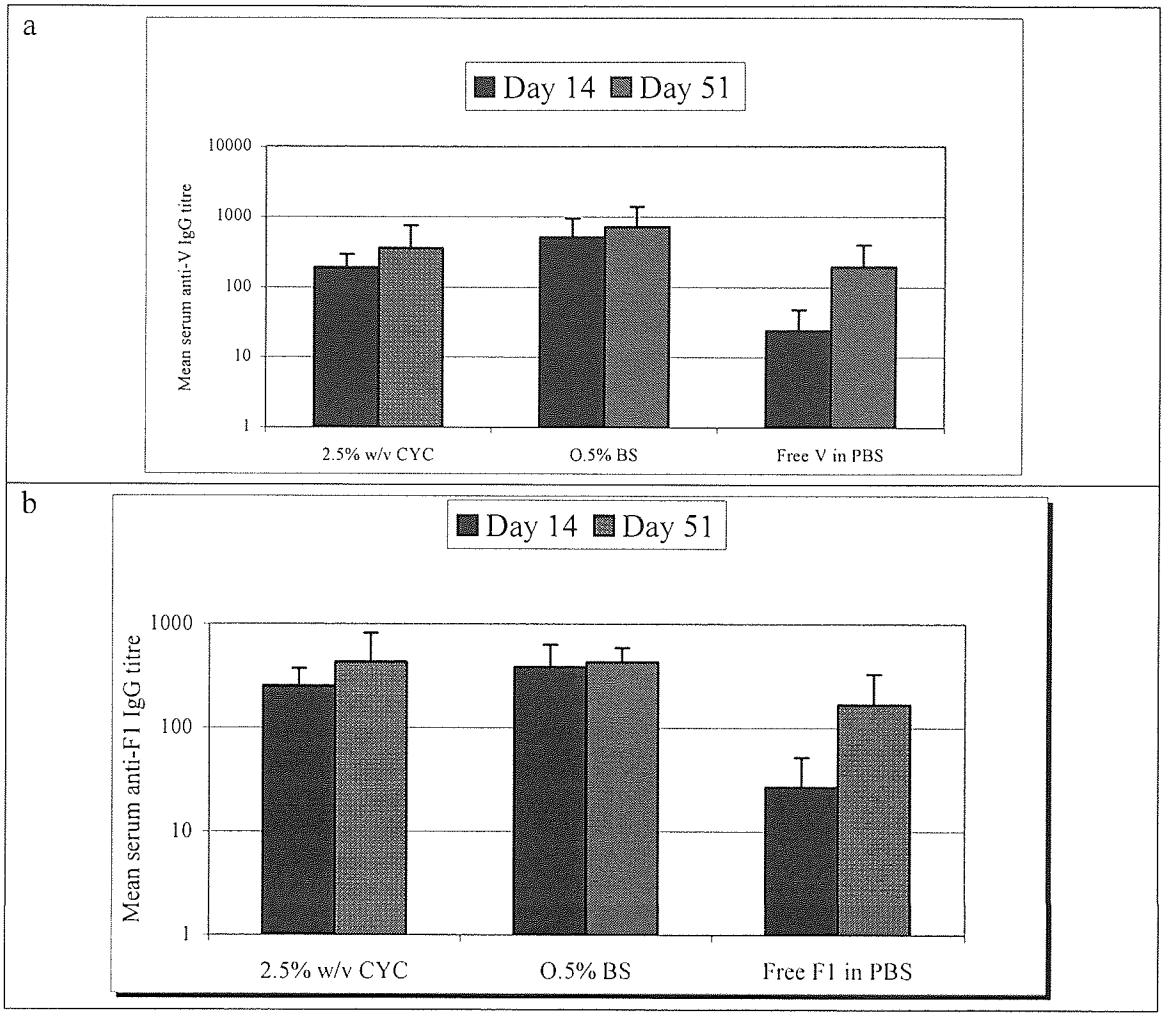


Figure 6.5 (a, b): Systemic immune responses to nasally delivered *Yersinia pestis* subunit F1 and V antigens with and without cyclodextrinGroups of 5 mice were dosed on day 1 with a single administration of 1μg of the V antigen and 5μg of F1 in combination, with and without cyclodextrin.

6.3.3. Bile salts as absorption enhancers and mucosal adjuvants.

In order to assess whether absorption enhancement of antigens through mucosal surfaces can enhance the immune response to mucosally delivered vaccines we tested wheather bile salts can improve the immune response to nasally delivered antigens. Sodium deoxycholic acid is known to increase the abosorption of insulin when given *via* the nasal route (Tengamnuay *et al.*, 2000). Two concentrations of sodium deoxycholic acid were investigated using tetanus and diphtheria toxoids and *Yersinia pestis* V and F1 subunit antigens.

Groups of 5 mice were immunised on day1 with 5 lf TT or DT with and without sodium deoxycholate at two concentrations; 1%w/v and 0.5%w/v. Mice were boosted on day 49 and blood was collected on day 21 and day 56. Serum was analysed for the antigen specific IgG response. In the case of the Yersinia antigens, groups of 5 mice were dosed on day 1 with a single administration of 1μg of the V antigen and 5μg of F1 in combination, with and without sodium deoxycholate at 0.25%w/v. Blood was taken at days 14 and 51 for analysis of V and F1 specific antibody titres.

6.3.3.1. Systemic immune responses to nasally delivered tetanus toxoid with and without sodium deoxycholate

The group of mice, which received TT with sodium deoxycholate, showed increased serum immune responses in comparison to the group of mice, which received TT alone. Mice, which received the free toxoid, exhibited negligible or no detectable response, as determined by antigen specific antibody titres. At both concentrations, deoxycholic acid significantly enhanced the immune response to nasally delivered tetanus toxoid (Figure 6.4).

After boosting in the presence of deoxycholic acid all mice showed significantly elevated serum immune responses in comparison to the primary immune response. There is not much difference between the lower and higher concentration of bile salts in the enhancement of the immune response to the nasally delivered tetanus toxoid (see also Figure 6.4). The immune response was long lived and TT specific antibody titres were still high but reduced at day 198 in comparison to day 56.

6.3.3.2. Systemic immune responses to nasally delivered diphtheria toxoid with and without sodium deoxycholate

In this case also, dexoycholic acid enhanced significantly the observed immune response to nasally delivered diphtheria toxoid. The primary response was again increased relative to the free antigen, boosting significantly enhanced antigen specific antibody titres and DT specific antibody titres were still high but reduced at termination of the experiment on day 198 in comparison to day 56 (figure 6.6). The mechanism of

action of bile salts as mucosal adjuvants may be due to the fact that bile salts are known to act on the lipid membranes of the nasal mucosa and enhance the absorption of proteins. The same principle may be applied to the absorption of antigens.

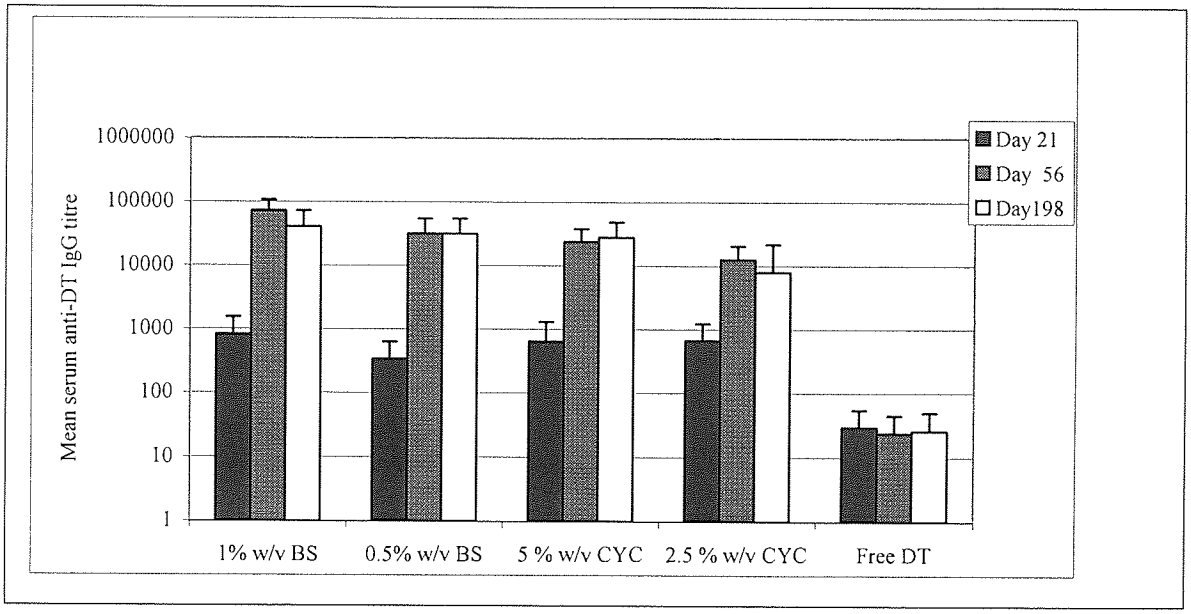


Figure 6.6: Anti-diphtheria toxoid (DT) specific IgG end-point titres following intranasal administration of 5 LF DT on day 1 and 2.5 LF DT on day 49. Toxoid was administered in the presence and absence of different concentrations of dimethyl-β-cyclodextrin (CYC) or glyco deoxycholic acid (BS) (n=5).

6.3.3.3. Systemic immune responses to nasally delivered *Yersinia pestis* V and F1 antigens with and without sodium deoxycholate

This aborption enhancer was also tested with the *Yersinia pestis* V and F1 subunit vaccine antigens. Dexoycholic acid enhanced significantly the immune response to nasally delivered subunit vaccines as determined by systemic antigen specific antibody titres even though the concentration was much below that previously used for diphtheria and tetanus toxoids. As with cyclodextrin, the deoxycholate was able to facilitate enhanced antibody titres to both the V and F1 subunit antigens independently when used in combination (figure 6.5) Again, the response was still markedly (716 vs 196) enhanced at day 51 from a single dose.

6.3.4. Poly-L-ornithine as an mucosal adjuvant

It was hypothesised that the charged nature of polyornithine may serve to enhance the absorption of antigens. In order to test this theory we used two concentration of poly-L-ornithine. For tetanus and diphtheria toxoids, groups of 5 mice were immunised on day 1 and day 49 and blood was collected and analysed for antigen specific IgG responses. High molecular weight poly-L-ornithine was tested at 1%w/v and 0.5% w/v.

6.3.4.1. Systemic immune responses to nasally delivered tetanus toxoid with and without polyornithine

The group of mice which received TT with polyornithine showed markedly increased (mean serum antibody titre 5600 vs 24) serum immune responses in comparison to the group of mice which received TT alone. Mice, which received the free toxoid, exhibited negligible or no detectable response, as determined by antigen specific antibody titres. The magnitude of the primary response conferred by the use of polyornithine was far greater than that observed for any of the other enhancers. Such a potent antigen specific primary response may have application where a swift and strong antibody response is required. For example in the development of post-exposure vaccination or single dose mucosally administered subunit vaccines. At both concentrations, polyornithine significantly enhanced the immune response to nasally delivered tetanus toxoid (Figure 6.7). After boosting with polyornithine formulated TT, all mice showed significantly elevated serum immune responses in comparison to the primary immune response (72500 vs 820). The difference between the lower and higher concentration of polyornithine was not significant. Again, the enhancement of the immune response to the nasally delivered tetanus toxoid subsequent to boosting was higher than that observed for any of the other enhancers (see also figure 6.4). The immune response was long lived and TT specific antibody titres were reduced at day 198 in comparison to day 56 but still as high as any of the other enhancers used in these studies.

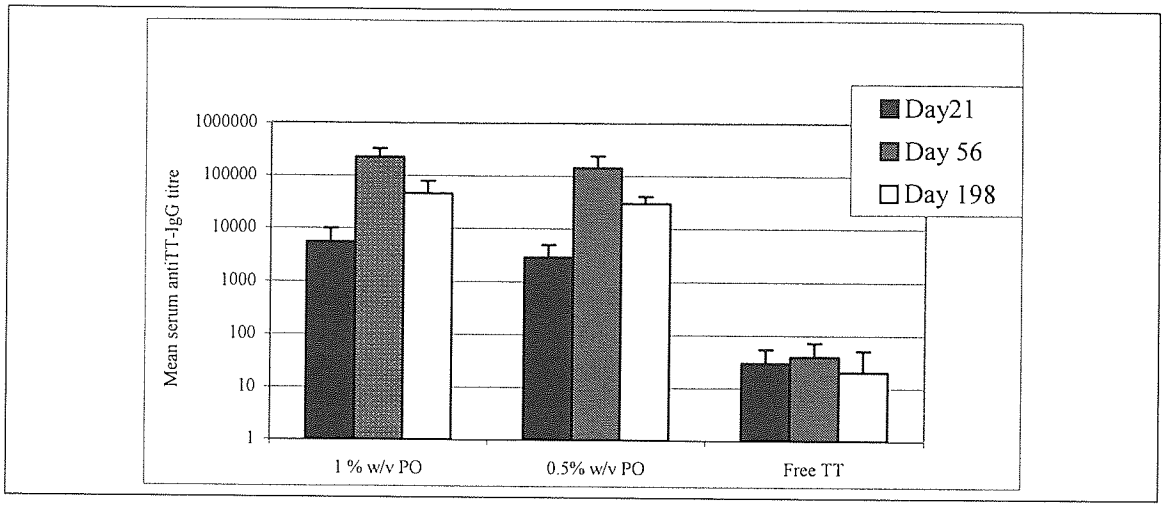


Figure 6.7: Anti-tetanus toxoid (TT) specific IgG end-point titres following intranasal administration of 5 LF TT on day 1 and 2.5 LF TT on day 49. Toxoid was administered in the presence and absence of different concentrations of poly-L-ornithine (PO) (n=5).

6.3.4.1.2. Response to mock challenge

In order to elucidate the memory response conferred by the polyornithine, mice were given 5 Lf free TT subcutaneously in 200µl PBS.

Antigen specific antibody responses were assessed 21 days post-mock challenge. At both of the concentrations of polyornithine used, the response to mock challenge was markedly increased (figure 6.8). In fact, the response in the group receiving the free antigen was also markedly increased and all titres were in excess of the measured range (titres of ~410 000 for the polyornithine formulations and ~16 000 for the free TT). This demonstrates that even a low antigen specific antibody response may give subsequent high titres following significant challenge with antigen. However, the real potential for protection may only be elucidated with challenge mediated by the disease causing agent, in this case the tetanus toxin.

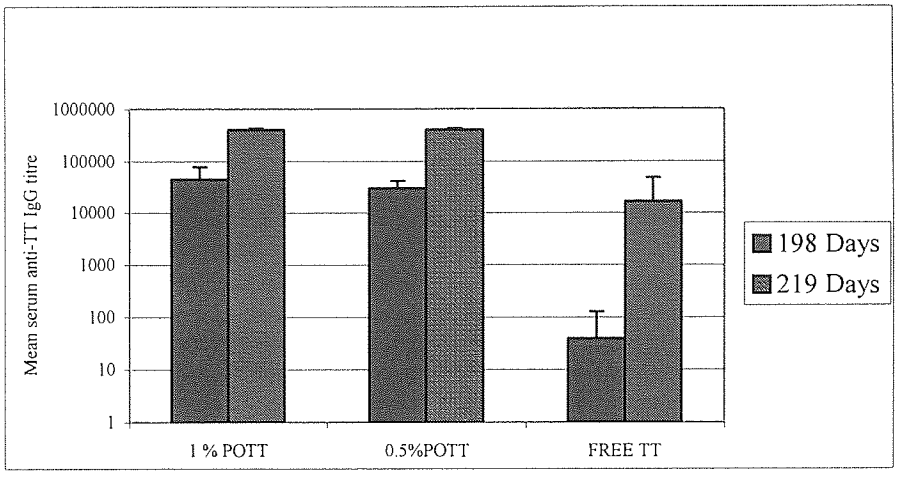


Figure 6.8: Systemic immune response to nasally delivered tetanus toxoid with or with out polyornithine before and after mock challenge subcutaneously with 5Lf of free TT.

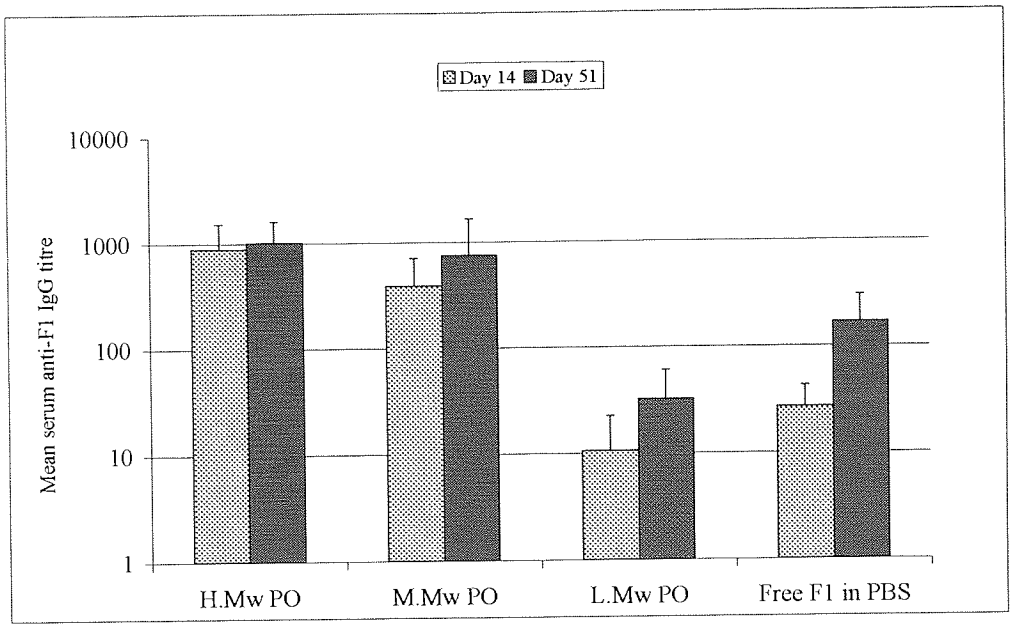
6.3.4.2. Effect of polyornithine on immune responses to nasally delivered V and F1 antigens and the effect of enhancer molecular weight

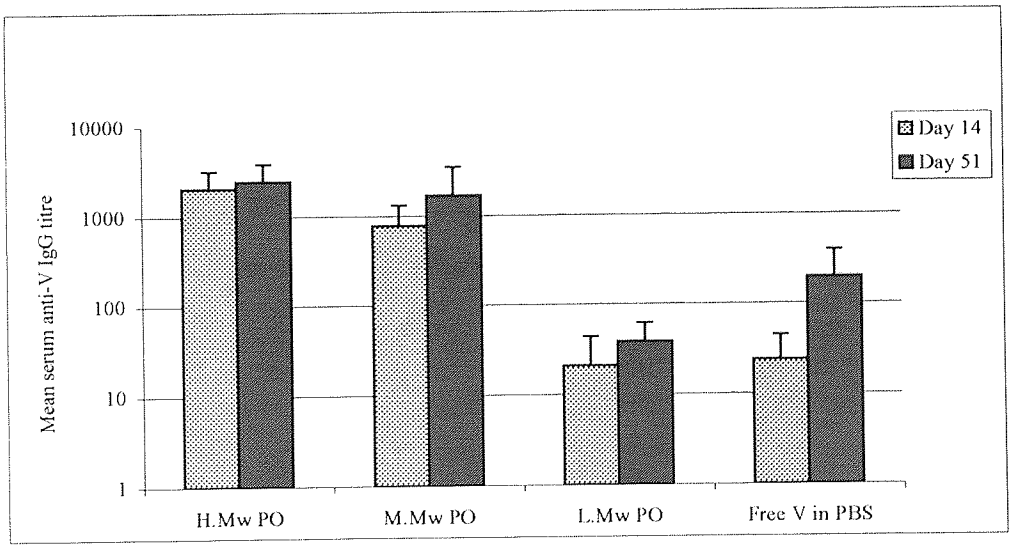
In the case of polyarginine, the molecular mass has been shown to be important for the absorption enhancing properties of this cationic agent. We hypothesised that similarly, there may be a difference observable when using a lower molecular weight polyornithine. In addition, we wanted to evaluate the potential for the use of this enhancer with another antigen. We addressed these questions by using the *Yersinia pestis* antigens V and F1 in combination with the polyornithine enhancer. Low, medium and high molecular weight polyornithine were tested at 0.2%w/v.

In the case of these antigens, groups of 5 mice were dosed on day 1 with a single administration of 1μg of the V antigen and 5μg of F1 in combination, with and without polyornithine. Blood was taken at days 14 and 51 for analysis of V and F1 specific antibody titres.

In the case of the F1 antigen, the low molecular weight at 0.2%w/v failed to enhance antigen specific antibody titres. In contrast, the medium and high molecular weight (mean serum titres 27 vs. 870) polyornithine facilitated markedly increased the observed immune responses on days 14 and 51. The low molecular weight polyornithine was also ineffective in the induction of immune responses to the V antigen. Again, the medium and high molecular weight agents engendered noticeably increased antibody titres. We thought that the higher molecular weight polyornithine may have increased the

absorption of antigen through the mucosal surface. The adjuvant activity observed with the polyornithine was shown to correlate well with molecular weight, increasing immunopotentiation with increased molecular weight.





b

Figure 6.9: Effect of molecular weight of polyornithine on immune responses to nasally delivered a) F1 and b) V antigens.
H.Mw PO: High molecular weight polyornithine, M.Mw PO: Medium molecular weight polyornithine, L.Mw PO: low molecular weight polyornithine.

6.3.5. Acylcarnitine as an mucosal adjuvant

It has been demonstrated that the powerful mucosal adjuvant *zona occludens* toxin (ZOT) has the ability to act on tight junctions (Marinaro *et a.;* 1999) Acylcarnitines are known for their properties as paracellular absorption enhancers and act on tight junctions. We tested various acylcarnitine derivatives; DL-octanoyl carnitine chloride (OCC), DL-lauryl carnitine chloride (LCC), palmitoyl-DL- carnitine chloride (PCC), DL-hexanoyl carnitine chloride (HCH), L-carnitine chloride (CH) and mistroyl-DL-carnitine chloride (MCC) in order to assess their potential to act as mucosal adjuvants.
All derivatives were used at 0.5%w/v in PBS.
Tetanus toxoid was used in this study. Groups of 5 mice were immunised on day 1 and day 40 with 10μg TT intranasally and blood was collected and analysed for antigen specific IgG responses on days 7 and 47(figure 6.10).

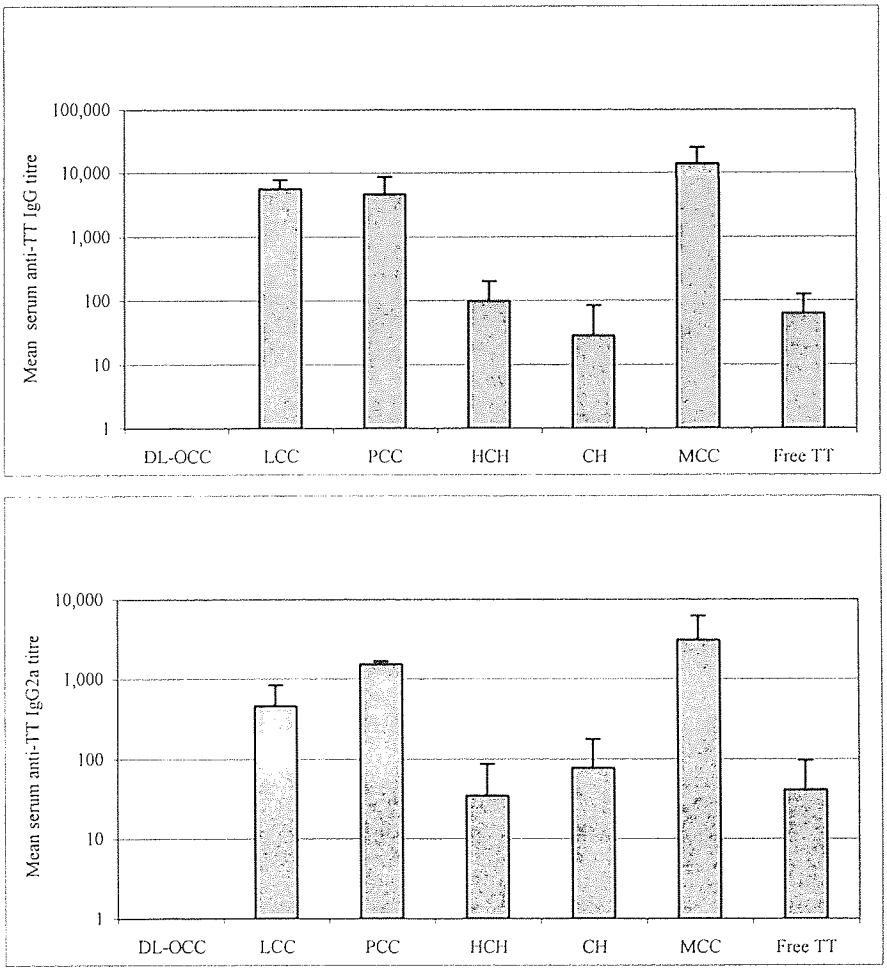


Figure. 6.10: Serum immune response to nasally delivered TT with and without various acylcarnitines on day 47.

6.3.5.2. Primary and secondary systemic immune responses

Intranasal immunisation of BALB/c mice with tetanus toxoid and coadministed with acylcarnitine resulted in increased antigen specific serum IgG titres but not following a single dose and boosting was necessary. An assay of TT specific IgG after the booster dose clearly showed that the enhanced immune response was dependent upon the acylcarnitine derivative used. Among these, the acylcarnitines which enhanced antigen-specific immune responses were MCC, LCC and PCC and the order in which they enhanced the immune response was MCC>PCC>LCC (Figure 6.10). In order to determine if the response elicited by acylcarnitine was more characteristic of Th1 or Th2 type responses, we additionally examined the production of antigen sepecific IgG2a. Titres of the IgG2a subclass were shown to be comparable to total IgG titres (Figure 6.10). In our hands this is characteristic of a mixed Th1/Th2 response.

6.3.5.3. Analysis of IgA in faecal samples

Faecal samples, 10 pellets from each group, were collected and dissolved in 0.5ml PBS containing 0.1 % w/v sodium azide. Samples were centrifuged and the supernatants were collected and analysed immediately by ELISA for antigen specific IgA. Titres were taken by comparison of O.D. values at 405nm.

The results are shown in figure 6.11. Titres correlated well with the serum total IgG; MCC>PCC>LCC. These results confirmed the induction of immune responses at mucosl surfaces.

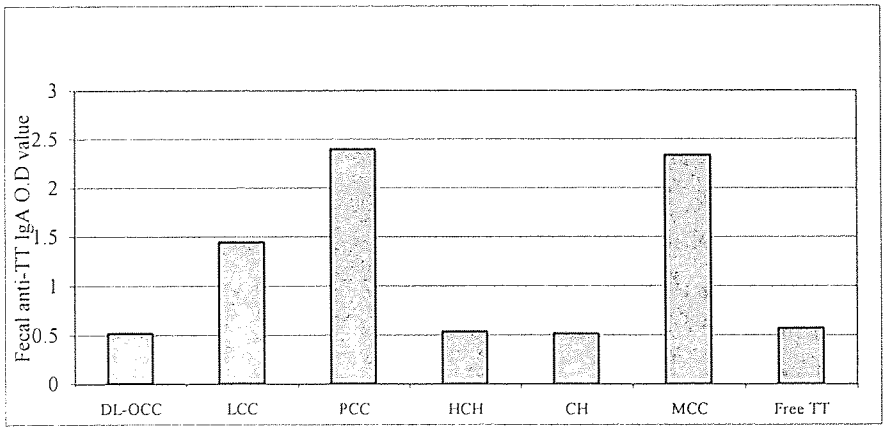


Figure 6.11: Mucosal (Faecal) immune responses to nasally delivered TT in the presence and absence of acylcarnitine on day 47.

6.4 General discussion

Mucosal vaccines generally require the use of adjuvants. Bacterial toxins such as cholera toxin are commonly used as mucosal adjuvants in animal models. However, toxicity prevents their use in humans.

We have evaluated the strength and nature of systemic and mucosal immune responses induced in mice after administration of tetanus and diphtheria toxoids and V and F1 with different absorption enhancers. Many of these have been shown to have absorption enhancing properties for macromolecules through mucosal membranes and we have shown that in addition, they offer significant potential as mucosal adjuvants.

6.4.1. Bile salts: mucosal adjuvant

In the case of bile salts, they have been shown to enhance immune responses to intramuscularly administered particulate antigen (Rafati *et al.,* 1997). Additionally they were also shown to augment the immune response to antigen adsorbed to alum *via* the intra muscular route.

Bile salts are the most widely used agents for enhancing the nasal absorption of macromolecules. The proposed mechanisms of action include increasing the permeability of the membrane structure and the inhibition of proteinases, solubilisation of the drug in the aqueous vehicle (although this last point is not thought to be important here as the antigens used are water soluble). It has been hypothesised that intercellular transport may be important; it is thought that these surface active adjuvants may temporarily convert hydrophobic contacts between junctional proteins into hydrophilic pore type pathways.

From these studies it is clear that absorption enhancers can be used as mucosal adjuvants. Before using these molecules as mucosal adjuvants, the safety of these molecules should be further assessed. It has been shown that bile salts below 0.3% w/v are safe to use for repeated administration (Morimoto *et al.,* 1998): we have shown that the adjuvant effect of the bile salts is evident at these safe concentrations. However, the toxic nature of these enhancers may not be that problematic because the frequency and quantity of these absorption enhancers required for the purposes of vaccination are very low (low quantity and infrequent administration).

6.4.2. VitaminE TPGS: mucosal adjuvant

From these studies it is clear that the amphiphilic molecule vitamin E TPGS can function as a safe mucosal adjuvant. Vitamin E TPGS which is the water-soluble form of vitamin E increases the absorption of vitamin E through mucosal surfaces. The antioxidant properties of Vitamin E could explain or be responsible for its adjuvant effect. The mechanism of action of vitamin E TPGS as a mucosal adjuvant is unknown and this work is the first exploration of its use in this role. The proposed mechanism of vitamin E TPGS as a mucosal adjuvant is due to its amphiphilic nature and also due to its surface-active properties. Vitamin E TPGS not only enhances the absorption of vitamin E but it has also been used to increase cyclosporin absorption after oral administration. Vitamin E TPGS forms micelles at very low concentrations and at higher concentrations vitamin E TPGS forms a liquid crystal structure, which is highly ordered. The mechanism proposed for enhancing cyclosporin absorption is micelle formation. Vitamin E TPGS can be considered as a bioenhancer and the enhanced absorption of antigens through mucosal surfaces is thought to be by the surface-active action of the modified vitamin E. The enhancement of immune responses may not only be due to the enhancement of antigens across mucosal surfaces but may also be due to the innate nature of vitamin E which is a known immunostimulant protecting rapidly proliferating cells of the immune system from oxidation damage (Tengerdy and Lacetera 1991). The mechanism action of this molecule needs to be explored more thourouhgly because compared to known mucosal adjuvants vitamin ETPGS has been shown to be an extremely safe molecule.

6.4.3. Cyclodextrins: mucosal adjuvant

Cyclodextrins have been used as absorption enhancers for many polypeptides, such as calcitonin, glucagons, insulin and human GMCSF (Irie and Uekama 1999). Surprisingly, the effect of their nasal administration was found to be comparable to parenteral administration of calcitonin alone. Dimethyl β-cyclodextrin as well as randomly methylated β-cyclodextrin are thought to be the most effective cyclodextrin derivatives for increasing the bioavailability of intranasally administered protein and peptide drugs (Marttin *et al.*, 1999). Active concentrations may have a lower effective

range of 2 to 5%w/v. Methylated β-cyclodextrins were found to be less toxic than sodium glycocholate, sodium taurodihydrofusidate, laureth-9 and L-α-phosphatidylcholine. Systemic toxicity after intranasal administration was not expected as low doses of cyclodextrins are administered and only very small amounts are absorbed. Additionally, cyclodextrins are less toxic than many other absorption enhancers, including some used in these studies (for example sodium deoxycholate (Behl *et al.,* 1998). The proposed mechanism of action of β-cyclodextrins may be explained by their interaction with epithelial membranes and their ability to open tight junctions (Marttin *et al.,* 1999). Our findings are in agreement with this hypothesis.

In the case of polyornithine, this positively charged polyaminoacid has been investigated extensively as an alternative to polylysine for transfection studies due to the reduced toxicity of this agent (Nead and McCance 1995) A key event in an immune response is how efficiently antigen presenting cell pickup and process antigens and present processed antigens to T lymphocytes. It has been shown that polyarginine, a similar polyaminoacid, also positively charged, was able to enhance uptake of FITC labelled dextran without any observed toxicity (Natsume *et al.,* 1999). Nakanishi *et al.* (Nakanishi *et al.,* 1999) demonstrated that positively charged multilamellar vesicles were taken up efficiently by macrophages, while negatively charged and neutral MLVs were hardly picked up. Consistent with this positively charged MLVs containing soluble OVA functioned as a more potent inducer of antigen specific cytotoxic T lymphocyte responses and antibody production than negatively and neutral MLV's (Nakanishi *et al.,* 1999). In addition, positively charged liposomal avridine has been shown to function as an efficient adjuvant to influenza antigens (Bergmann and Waldman 1988) and the cationisation of BSA has been reported to engender higher immune responses in comparison to free BSA when administered intramuscularly (Domen *et al.,* 1987; Muckerheide *et al.,* 1987). The increased uptake of cationised proteins by antigen presenting cells was thought to be due to the cationic nature of these agents (Apple *et al.,* 1988). The most obvious explanation for these data is the fact that cationized proteins tend to adhere better to the slightly anionic cell membrane. Fatunmbi *et al* (Fatunmbi *et al.,* 1992) showed that positively charged adjuvants are better adjutants than neutral or negative oil-emulsion adjuvants. We propose that the positive charge of polyornithine enhances the mucoadhesive and absorption properties of nasally delivered

antigens. There is the possibility that the molecule exhibits increased resistance to degradation and also that cationized antigen may be recognised more efficiently by APC and /or helper T cells because of its altered structure. Certainly, increased ability to interact with APC would play a key role in the activation of helper T cells. The formulation of TT, DT, F1 and V antigens along with polyornithine, resulting in a remarkably enhanced immune response after nasal delivery, may offer significant practical application in the development of mucosal vaccines. Further testing of these enhancers with other antigens and in vaccine/challenge experiments is needed to evaluate the potential of these compounds for use as adjuvants in human vaccines.
The mucosal administration of cholera toxin has been to shown to enhance Th2 type responses for coadminstered antigens resulting in antigen specific IgG1 responses.
The use of aceylcarnitine as an intranasal adjuvant showed that the engendered response was a mixed Th1/Th2 type response.

6.4.4. Acylcarnitines: mucosal adjuvant

In the present study, we have demonstrated that when co-administered intranasally with a soluble protein antigen (TT), acylcarnitine elicits a strong antigen-specific serum response. The pattern of antigen-specific IgG subclass antibodies elicited by acylcarnitines indicated a mixed Th1/Th2 response. However, a study of the cytokine profile of T-helper cells induced by acylcarnitne administration is necessary for a conclusive determination. The antigen-specific mucosal IgA response correlated well with the serum antibody titres. We did not detect significant adjuvant activity when some acylcarnitine derivatives were co-administered with TT. This suggests that the type of acylcarnitite may have a strong role in enhancing immune response to co-delivered antigen. Cholera toxin specifically binds to the monosialoganglioside GM1 present on all nucleated cells. Acylcarnitines act extracellularly apparently without the involvement of a receptor. Work by Marinaro *et al.* has shown that the zonula occludens toxin (ZOT) of *Vibrio cholerae* has potential as a mucosal adjuvant. They hypothesised that this toxin acts as an adjuvant due to its ability to specifically disrupt the paracellular barrier of epithelial cells. A disruption of the paracellular barrier in the nasal mucosa by acylcarnitines could lead to increased accessibility of co-administered antigen to lymphoid tissue. They demonstrated that co-administration of ZOT with an antigen

(OVA) resulted in both a systemic and mucosal antibody response consistent with a Th2-type response. Others have also shown that ZOT disrupts the integrity of the epithelial tight junctions resulting in increased permeability to macromolecules (Marinaro *et al.,* 1999). This is similar to the action of acylcarnitines on epithelial barriers (Fix *et al.,* 1986), however, the mechanism of the action of ZOT may be at least in part intracellular, involving a protein kinase C dependent polymerization of actin. Our study complements the ZOT work by showing that another absorption enhancer that disrupts the paracellular barrier can also function as an adjuvant when administered mucosally. An examination of the current data suggests that a common theme may be emerging with regards to absorption enhancers that function as adjuvants when administered mucosally. CT/LT, toxin A, ZOT, and absorption enhancers are all thought to disrupt the paracellular barrier resulting in increased mucosal permeability. Each has now been shown to act as an adjuvant for mucosally introduced antigens. Given that these toxins act through different molecular mechanisms, it seems likely that changes in paracellular permeability play an important role in adjuvanticity.

CHAPTER: 7

7.0. DNA vaccines

7.1. Introduction

One of the most exciting developments in vaccine technology in recent years has been the discovery of DNA vaccines, with which the antigen is synthesized *in vivo* after direct introduction of its encoding sequences into the host cell. This unique method of immunisation arose from the discovery that injection of pure plasmid DNA ('naked' DNA) into muscles of mice resulted in long-term reporter gene expression in transfected muscle fibers (Wolff *et al.*, 1990).

In 1992, Tang *et al.* (Tang *et al.*, 1992) found that plasmids coated onto gold beads resulted in foreign gene expression and the induction of an antibody response to the foreign gene product in mice. They used genetic immunisation as a means of generating a humoral immune response to a gene product by injecting plasmid DNA encoding the foreign gene into a host. Subsequently, a number of other investigators employed plasmid DNA immunisation to induce humoral and cell mediated immune responses to influenza A virus (Ulmer *et al.*, 1993) human immunodeficiency virus (HIV) (Wang *et al.*, 1994) hepatitis B virus surface antigen (Davis 1996) collectively demonstrated the potential for DNA immunisation as a vaccine strategy against viral pathogens. The potential of DNA immunisation as a method for cancer immunotherapy has also been demonstrated (Ciernik *et al.*, 1996) thus, it was clear that immunisation with DNA could generate antigen-specific immune responses to a specified gene product, and could therefore potentially serve as an immunotherapeutic agent.

The majority of DNA vaccines have been administered parenterally (e.g., by intramuscular (i.m.), intradermal (i.d.) or subcutaneous (s.c.) routes). Although these routes are able to induce potent systemic immune responses, they generally do not induce mucosal immunity. Mucosal immunity can prevent pathogen entry, whereas systemic immunity can only deal with pathogens once in the body (McGhee *et al.*,

1992). Furthermore, mucosal immunization at one site (e.g., lung) with antigen-based approaches has been shown to induce immunity at distant mucosal sites (e.g., vagina and nasal) (Mestecky *et al.,* 1997). In recent years there has been a considerable increase in reports on mucosal immunization using DNA vaccines. Such strategies may lead to the development of human vaccines, which are able to protect at the site of entry for mucosal pathogens, and may thereby better prevent many infectious diseases.

The adsorption of DNA onto gold particles and delivery through the use of a gene gun is a widespread an effective strategy for the delivery of DNA *in vivo* (Donnelly *et al.,* 1997). This method bombards the skin with plasmid DNA adsorbed to gold particles. The gold particles directly penetrate the skin due to the force of delivery, thereby increasing the rate of transfection without having to rely on the uptake of DNA by the host cell itself. At least 100-fold less plasmid DNA may be required for the induction of protective immune responses when administered in this manner when compared with inoculation of plasmid DNA in saline using conventional parenteral administration.

Although significant progress has been achieved in delivering DNA vaccines in murine models, the immunogenicity of DNA vaccines remains relatively low in large animals and non-human primates (Scheerlinck 2001). This low immunogenicity, which in most cases is measured by antibody (Ab) levels, is not always indicative of protective immunity. Indeed, DNA vaccines are particularly effective at priming immune responses and the induced memory is often sufficient to protect animals. Several approaches have been used to influence not only the magnitude but also the type of immune response induced by DNA vaccines.

Improving the delivery of plasmids to specific cells, such as APCs, or improving the intracellular trafficking of encoded proteins to relevant processing pathways such as the endosomale or lysosome (Ji *et al.,* 1999) could provide another opportunity for the improvement of DNA vaccines. Strategies that enhance delivery of plasmid DNA such as complexing or entrapment of plasmid DNA with cationic liposomes (Gregoriadis *et al.,* 1997) have been shown to be effective.

7.1.1. Nanoparticles as carriers for DNA vaccines

The use of nanoparticles as vehicles for drug and gene delivery has been an active area of research and development for over a decade (Chowdary and Srinivasa Rao 1997). Nanoparticle formulations have produced a wide spectrum of rigid polymer structures, which are suitable for encapsulation, delivery and controlled release of both low molecular mass drugs and biopolymer pharmaceuticals. Nanoparticles developed to carry oligonucleotides commonly comprise polylactide, poly (lactide-co-glycolide), and poly (alkylcyanoacrylate) (Zimmer 1999). These nanoparticles can be additionally modified by quaternary alkyl groups or DEAE-dextran (Zobel *et al.,* 1997) to introduce positive charges to enhance DNA binding. Polymers suitable for the preparation of nanoparticles include poly (alkylcyanoacrylates), poly (methylidene malonate), and polyesters such as poly (lactic acid), poly (glycolic acid), poly (caprolactone) and their copolymers.

Several groups are using particulate systems to elicit mucosal immune responses, for example, to increase resistance to microbial infection by this route (Almeida *et al.,* 1993). Nanoparticles were first developed in the mid-1970s (Speiser 1984). Later on, their application for the design of drug delivery systems was made available by the use of biodegradable polymers that were considered to be highly suitable for human applications.

Intranasally administered DNA encoding influenza haemagglutinin glycoproteins demonstrated the protection of mice against lethal influenza challenge (McCluskie and Davis 1999). A number of studies have reported, with varying degrees of success, that naked DNA delivered to mucosal surfaces is able to induce antigen-specific immune responses. In most cases, immune responses could be enhanced by the formulation of DNA with liposomes, microspheres or with powerful mucosal adjuvants such as CT or monophosphoryl lipid A. Despite the initial early success, progress has been considerably slower with mucosal than parenteral delivery of DNA vaccines. Delivery of genes to the mucosal surfaces, such as the genitourinary, gastrointestinal, and respiratory tracts, may be hampered by poor plasmid uptake across the epithelium. Barriers include secretion of enzymes (biochemical), mucus and ciliated surfaces

(physical) or mechanical clearance such as coughing. In order to increase plasmid uptake and limit degradation, various methods of formulating DNA for mucosal delivery have been utilized. These include biodegradable microspheres, such as those composed of poly (DL-lactide-co-glycolide), which have been used to encapsulate and deliver antigens, toxoids, or attenuated virus by systemic, oral, or intranasal routes, resulting in antigen-specific systemic and mucosal immune responses (O'Hagan *et al.,* 1995; Eyles *et al.,* 2000). Oral delivery of encapsulated DNA to the PP was first achieved using DNA encoding galactosidase in microspheres composed of polyanhydride copolymers of fumaric and sebacic acid [poly (FA: SA)] (Mathiowitz *et al.,* 1997). Subsequently poly (DL-lactide-co-glycolide)-encapsulated plasmid DNA has been shown to facilitate antigen-specific systemic and mucosal antibody responses after oral administration (Jones *et al.,* 1997) and, in the case of a rotavirus DNA vaccine, to protect against subsequent live challenge (Chen *et al.,* 1998).

Earlier work in our laboratory showed, the use of cationic particles to sucessfully adsorb and deliver DNA, inducing immune responses following mucosal administration. (Alpar 1996; Alpar *et al.,* 1997). In these studies non-biodegradable particles were used. More recently we have focused on the use of positively charged nanoparticles for the adsorption of plasmid DNA to deliver DNA vaccines. To achieve this goal, the concept of positively charged nanoparticles was explored. In the earlier chapters the potential of chitosan as adjuvant was explored alone or as a component of vaccine formulations. PLA microspheres were prepared using chitosan as a stabiliser and this resulted in positively charged microparticles.

Polyalkylcyanoacrylate nanoparticles have been used previously in drug delivery systems. Recently the potential of these as carriers for oligonucleotides has also been explored. To adsorb oligonucleotides to these particles the cationic surfactant CTAB has been used to achieve a positive surface charge.

In another study polyalkylcyaoacrylate nanoparticles were prepared in the presence of the positively charged DEAE-Dextran. In this study we explored chitosan as a stabilizer to achieve positively charged polyalkylcyanoacrylate nanoparticles facilitating the adsorption of DNA to these particles.

The polymer we have chosen to prepare microparticles is an ethylcyanoacrylate monomer, which is a biodegradable and biocompatible polymer (Couvreur 1988), and previously has been used for a range of biomedical purposes, including the preparation of controlled release drug delivery systems. Although cyanoacrylate nanoparticles have been used to deliver drugs and more recently, oligonucleotides, these particles have not previously been used as carriers for DNA in the context of vaccination.

Previous studies using microparticles have described the entrapment of DNA inside the carrier. Therefore, during microparticle preparation, the DNA was exposed to a range of conditions that have the potential to cause denaturation and degradation, including high shear, an organic/aqueous interface, localized high temperature, and freeze-drying. Not surprisingly, a recent study has reported that DNA is significantly degraded during encapsulation in PLG microparticles (Walter *et al.,* 1999). In addition, once entrapped in microparticles, the rate of release of DNA is slow, limiting the amount of DNA available for transfection of target cells and induction of immune responses.

The strategy adopted by Alpar *et al* (Alpar 1996; Alpar *et al.,* 1997) of presenting DNA on the surface of microparticles effectively overcomes the problems of DNA degradation during microencapsulation and enhances the amount of DNA immediately available to APCs. In achieving this, microparticles were prepared that displayed a positive surface charge for DNA adsorption; similar strategies have been adopted by other recent studies through the inclusion of cationic surfactants in the preparation process (Singh *et al.,* 2000). In general, however, these surfactants have some toxicity. We have therefore used biocompatible, biodegradable chitosan to alter the surface charge of PLA nanoparticles. We have also tested the use of chitosan to replace the stabilizer (dextran or pluronic F68) in the formulation of polyalkyl cyanoacrylate nanoparticles.

Chronic hepatitis B virus (HBV) infection is endemic in South East Asia. It is associated with serious consequences, including cirrhosis of the liver, hepatocellular carcinoma, polyarteritis nodosa, and membranous nephropathy. The basis for the prevention and treatment of chronic HBV infection are vaccination with recombinant hepatitis B surface antigen (HBsAg) and interferon-γ (IFN-γ) therapy. Although recombinant HBsAg vaccine is administered to newborn babies, a small but definite

proportion of the vaccinated individuals do not show an antibody response (Larsen *et al.*, 2000).

After preparation and characterization, cationic microparticles with adsorbed DNA were administered to BALB/c mice and the immune responses induced were compared with immunization with naked DNA.

7.1.2. Adjuvants for DNA vaccines

Recently, Ulmer and colleagues (Ulmer *et al.*, 1999) showed the potential of alum as an adjuvant for DNA vaccines following intramuscular administration. Isaka and coworkers (Isaka *et al.*, 1998) evaluated alum in order to deliver antigen *via* the nasal route and showed enhanced antibodies to alum adsorbed TT in the presence of CTB. The work by Ulmer *et al.* showed the use of conventional aluminium adjuvants were able to enhance antibody responses up to 100 fold in mice immunised parenterally with a plasmid encoding influenza haemagglutinin (HA) and guinea pigs immunised with a plasmid encoding HSV-2 glycoprotein D. There was also a 5-10 fold increase in transgene specific antibody titres in non-human primates receiving adjuvanted influenza HA DNA (Ulmer *et al.*, 1999). They noted that effective formulations had no detectable effect on the levels of antigen expression *in situ* and concluded that these adjuvants exerted their effects on antigen after expression. A similar study (Wang *et al.*, 2000) using plasmids encoding hepatitis B surface antigen also showed a 10-100 fold increase in antibody titres in mice when DNA was co-administered with calcium or aluminium phosphate. Both studies highlighted the requirement for unbound DNA in order to elucidate the adjuvant effect. One interesting observation was that, whilst aluminium adjuvanted vaccines have been shown to elicit essentially Th2 type immune responses (Cox and Coulter 1999), enhancement of Th1 associated IgG subclass titres was observed as well as increased production of Th1 type cytokines by antigen specific T cells following immunisation with aluminium adjuvanted plasmid DNA. Furthermore, it was possible to qualitatively alter an ongoing predominant Th2 response (elicited by priming with protein antigen) and markedly increase the production of antigen specific Th1 associated IgG subclass titres by boosting with the formulated DNA. Recognition of the contrasts between Th1 and Th2 type responses in disease states has alluded to the therapeutic possibilities of redirecting the Th1/Th2 balance in chronic diseases

(Coffman and Romagnani 1999). The potential of this facet of DNA vaccines may find application in allergy and autoimmunity in addition to therapeutic vaccines in pathogen mediated disease states. The fact that the adjuvant used is already approved for use with human vaccines and is effective in non-human primates is encouraging. Whether alum can act as a mucosal adjuvant for DNA vaccines was investigated using the co-administration of aluminium hydroxide and aluminium phosphate along with plasmid DNA.

As has been shown with several other polycations, there is electrostatic interaction between chitosan and DNA due to the positive charge of this agent. It has been shown that plain chitosan DNA nanospheres could transfect HEK 293, IB3, and HTE cell lines, though at lower levels than lipofectamine controls (Roy *et al.,* 1999). In recent years, the potential of chitosan as a polycationic gene carrier has been explored in several research groups (Leong *et al.,* 1998). Due to its good biocompatibility and toxicity profile, it has been widely used in pharmaceutical research and in industry as a carrier for drug delivery and as biomedical material for artificial skin and wound healing bandage applications. Chitosan has been shown to effectively bind DNA in saline or acetic acid solution and partially protect DNA from nuclease degradation.

The earliest work involving the effects of chitosan adsorption on negatively charged colloidal surfaces, however, took place in the field of lipid emulsions. A quite distinct approach was recently adopted in our laboratory for the formation of chitosan-coated microparticles. The formation of these three colloidal carriers was based on the well-known solvent displacement and solvent evaporation techniques. However, a key modification was introduced in these techniques: the incorporation of chitosan into the external aqueous phase in which the formation of the colloidal structures takes place. Using this approach we have produced chitosan-coated poly (lactide-co-glycolide) (PLA) nanoparticles. For PLA nanoparticles, chitosan molecules anchor to the surface due to entanglements between polymer chains, thus resulting in a core-coated structure.

Microorganisms contain surface-molecules such as LPS and a variety of soluble factors that function as adjuvants, alerting the immune system to 'danger' by inducing inflammation. The potency of genetic vaccines may be significantly enhanced by mimicking these signals with synthetic adjuvants such as QS21 (Sasaki *et al.,* 1998) or monophosphoryl lipid A (Lodmell *et al.,* 2000). More potent DNA vaccines may allow

a reduction in the quantity of DNA required for protective efficacy, which could be beneficial for vaccine cost and manufacturing.

An important aspect in optimising DNA delivery for the induction of immune responses is its route of administration. Over the last few years, intranasal administration of antigen has received considerable attention as a non-invasive route for vaccine delivery (Eyles *et al.*, 2000). Different vaccine formulations have been tested to improve their absorption *via* the nasal epithelium to protect protein from proteolytic degradation, and to vary the types of immune responses induced.

7.2. Materials and Methods

7.2.1. Materials

Hepatitis B surface recombinant protein and plasmid DNA encoding hepatitis B surface antigen were obtained from Aldevron (US), Chitosan chloride was from Pronova biopolymers (Norway), Aluminum phosphate (Adju-Phos®) and aluminum hydroxide (Alhydrogel®) and Quil-A, were purchased from Superfos (Denmark). Ethylcyanoacrylate monomer, DEAE-Dextran, Pluronic F-68 and all other reagents were obtained from Sigma (Poole, Dorset, UK).

7.2.2. Preparation of polyalkylcyanoacrylate nanoparticles

The preparation of polyethylcyanoacrylate nanoparticles was performed according to a previously published method of emulsion polymerisation. Polyethylcyanoacrylate nanoparticles were prepared by emulsion polymerisation of the ethylcyanoacrylate monomer in the presence of the cationic polysaccharide chitosan chloride. Two alternative polymerizing agents were used; pluronic F-68 (1% w/v) or chitosan hydrochloride (0.1% to 1% w/v plus pluronic F-68 (0.25% w/v)). 400 µl of ethylcyanoacrylate (ECA) monomer was slowly added to 25ml of filtered polymerisation medium composed of aqueous solution, acidified to pH 2 with hydrochloric acid, containing one of the above mentioned surfactants under mechanical stirring (1000 rpm). After the monomer polymerization was completed (~4 hours) the

colloidal milky suspensions were neutralized with 0.1M NaOH, washed two times with distilled water and freeze-dried.

7.2.3. Preparation of positively charged PLA nanoparticles

The positively charged nanoparticles were prepared by using a modified solvent evaporation process. Briefly, the microparticles were prepared by emulsifying 10 ml of a 5% (w/v) polymer(PLA 100kDa) solution in methylene chloride with 1.5ml of 0.25% chitosan (w/v) at high speed using a probe sonicator at high frequency. The primary emulsion was then added to 25 ml of 0.25% chitosan (w/v). This resulted in the formation of a water/oil/water emulsion that was stirred at 6,000 rpm for 12 hr at room temperature, allowing the methylene chloride to evaporate. The resulting nanoparticles were washed twice in distilled water by centrifugation at 20,000x g and freeze-dried.

7.2.4. Adsorption of plasmid DNA

After preparation, washing, and collection, DNA was adsorbed onto the nanoparticles by incubating 10 mg of cationic microparticles in a 10 mg/ml saline solution of DNA 30 minutes before immunisation. The aluminium concentration was 450 mg/ml and plasmid DNA was 100 μg/ml. After incubation, the nanoparticles were centrifuged at 21000 rpm for 10 minutes and supernatant taken for DNA quantification by spectrophotometric methods and agarose gel electrophoresis. The quantity of plasmid DNA adsorbed to the nanoparticles was determined indirectly by calculation of the difference between starting plasmid DNA concentration and final plasmid DNA in supernatants.

7.2.5. Electrophoretic analysis of plasmid DNA

Agarose gel electrophoresis was used to detect the size of DNA fragments present in a sample. The technique is based on the theory that when a voltage is applied negatively charged DNA will migrate towards the positive electrode. The larger the DNA fragment, the slower it will migrate through the gel. The conformation of the DNA can also be determined by this method since supercoiled, open circular and linear DNA of the same molecular weight will migrate at different rates.

A 1% agarose solution (approximately 50ml for a small gel) was prepared in 0.5x TBE solution and was heated until it became clear. The solution was allowed to cool until it was hand hot and 5µl of a 10mg/ml ethidium bromide solution was added. Ethidium bromide intercalates between the stacked base pairs in the DNA, which allows it to be detected using ultraviolet illumination. The gel solution was then poured into the gel-running rig, the comb was placed at the end furthest away from the positive electrode and the gel was allowed to set. The rig was filled with 0.5-x TBE solution (just enough to cover the gel) and the comb was removed. 10ml of each sample was added to 10ml of loading buffer and the entire sample was loaded onto the gel. The gel was run at a voltage of 80V for 90 minutes using a Bio-rad 300 power pack and was visualized using UV light using the UVP white/UV Transilluminator.

7.2.6. Quantification of plasmid DNA

The amount of DNA adsorbed to nanoparticles was assessed spectophtometrically in glass quartz cuvettes at 260 nm and 280 nm. For double stranded DNA, an OD260 of 1.0 corresponds to a nucleic acid concentration of approximately 50 mg /ml. The ratio between the OD readings at 260 nm and 280 nm gives an estimate of the purity of the plasmid DNA; pure plasmid gives a ratio of 1.8.

7.2.7. Nanoparticle Characterization

The size distribution of the nanoparticles was determined by using PCS particle size analyzer (Malvern Instruments, Malvern, U.K.) and the value was calculated by volume measurement, with loading determined as outlined above. The zeta potential of the microparticles, which is a measure of net surface charge, was measured using a Malvern Zeta master (Malvern Instruments, Malvern, U.K.).

7.2.8. Immunisation schedule for intranasal administration of plasmid DNA encoding Hepatitis B surface protein

Female BALB/c mice (25 g, 6-week-old) were lightly anaesthetized with an inhaled gaseous mixture of 3% (v/v) halothane (RMB Animal Health, UK) in oxygen (300 cm^3 min^{-1}) and nitrous oxide (100 cm^3 min^{-1}) for i.n. dosing procedures. 5 groups of mice (n

= 5) were dosed with 10μg plasmid DNA in the presence and absence of various adjuvants administered intranasally to mice using a micropipette. Each mouse received a 20μl volume (10μl per nostril), of particle suspension or plasmid DNA in solution. At day 1, group 1 received 10μg plasmid DNA in saline. Group 2 received 10 μg plasmid DNA adsorbed to 45μg of aluminium hydroxide. Group 3 received 10 μg plasmid DNA adsorbed to 45μg of aluminium phosphate. Group 4 received 10 μg plasmid DNA+10μg of cholera toxin B in saline. Group 5 received 10μg plasmid DNA in PBS + 7.5μg of Quil-A. On day 14, all groups received booster doses comprising the same formulations. All groups were boosted again on day 35 with 1 μg recombinant protein Hepatitis B adsorbed to 100μg of aluminium hydroxide intramuscularly in 50 μl of PBS. Blood samples were collected on day 28 and 42 for analysis by ELISA as described below.

7.2.9. Immunisation schedule for intramuscular administration of plasmid DNA adsorbed to positively charged nanoparticles

Six groups of female BALB/c mice were immunised intramuscularly with 50μl, two groups receiving 10μg of plasmid DNA adsorbed to 2 mg of either PLA or polyethylcyanoacrylate nanoparticles. A further two groups of mice received 1 μg of protein adsorbed to the above particles. A final two groups of mice received 10 μg of plasmid DNA and 1μg of protein antigen adsorbed to PLA or polyethylcyanoacrylate nanoparticles. Animals received a priming dose on day 1 and a booster dose on day 35. Blood samples were collected 1 week after the booster dose for analysis of antigen specific antibodies by ELISA.

7.2.10. Immunization schedule for subcutaneous administration of plasmid DNA and protein with saponin and chitosan alone or in combination

Six groups of female BALB/c mice were immunised subcutaneously with 200μl comprising 10 μg of plasmid DNA alone or in combination with 1 μg of antigen in the presence of Quil-A or Quil-A plus 0.05% chitosan chloride (w/v) as follows: Group 1 received 10 μg Quil-A and 1μg antigen. Group 2 received 10 μg Quil-A, 10 μg plasmid DNA and 1 μg antigen. Group 3 received 10μg Quil-A and 10 μg plasmid DNA; Group

4 received 10 μg Quil-A, 10μg plasmid DNA and 0.05% chitosan chloride (w/v); Group 5 received 10 μg Quil-A, 10μg plasmid DNA, 1 μg antigen and 0.05% chitosan chloride (w/v); Group 6 received 10μg Quil-A, 1μg antigen and 0.05% chitosan chloride (w/v). Animals received a priming dose on day 1 and a booster dose on day 35. Blood samples were collected 1 week after the booster dose for analysis of antigen specific antibody responses by ELISA.

7.2.11. Enzyme Linked Immunosorbent Assay (ELISA)

ELISA plates (96-well flat bottom, Dynatech) were coated with HBsAg (60μl /well, 3 μg/ml) dissolved in 0.05 M carbonate buffer (pH 9.6) and incubated at overnight at 4° C. The plates were washed three times in PBS-Tween 20 buffer (0.05% v/v, wash buffer) and blocked with 4% (w/v) BSA in wash buffer (50 μl /well) for 1 h at 37° C. After washing plates with washing buffer, serially diluted mouse sera (50 μl /well) was added, and incubated 1 h at 37° C. Following washing, peroxidase-conjugated goat anti-mouse IgG was added (1 in 2000 dilution in carbonate buffer, 50 μl /well). At the end of the incubation period, plates were washed and ABTS solution added. Plates were incubated at 37° C for 30 minutes. Colour development was determined spectrophotometrically at 405 nm. Results (means ± S.D.) were expressed as log10 of the reciprocal number of the final determined dilution, which gave a higher optical density at 405 nm than the highest measured optical density of negative serum.

7.3. Results

7.3.1. Nanoparticle characterization

To investigate the formation of positively charged nanoparticles (NP) stabilized with chitosan, positively charged poly (alkyl cyanoacrylate) (PACA) NP were prepared by emulsion polymerization in the presence of chitosan as a polymeric stabilizer at low pH(~2.5). The effect of the concentration of chitosan was evaluated in the formation the particles.

Polethylcyanoacrylate nanoparticles were traditionally prepared in a medium containing either pluronic F-68 or dextran. An attempt was made at preparing ethylcyanoacrylate

nanoparticles in the presence of chitosan. At higher than 0.2% w/v of chitosan, the monomer ethylcyanoacrylate failed to form nanoparticles. At that concentration, the combination gave a gel like preparation. Nanoparticles were formed at 0.1 % w/v chitosan concentration in the presence of 0.1 % w/v pluronic F-68. The nanoparticles were prepared with a mean volume diameter of about ~0.5 μm and showed a surface positive charge because of the inclusion of cationic polymer during the polymerization stage. This is in accordance with the formation of positively charged polyalkylcyanoacrylate nanoparticles in presence of positively charged DEAE-Dextran (Zobel *et al.,* 1997). Scanning electron microscopy of polyethylene cyanoacrylate nanoparticles shows a uniform size with a smooth surface (Figure 7.1). The particle size and net surface charge and loading efficiency of the plasmid DNA is shown in Table 7.1.

Formulation	Z-average dimeter nm	Zetapotential mV	Adsorption efficiency %
PLA nanoparticles	440 ± 190	28.3 ±1.6	100
PECN nanoparticles	370 ± 120	32.6 ±2.6	100

Table 7.1. Nanoparticles with adsorbed DNA: Particle size, net surface charge, loading efficiency, and DNA loading levels.

Positively charged PLA 100kDa nanoparticles are formed when PVA was replaced with chitosan in the preparation of PLA nanoparticles by the double emulsion method. The cationic polymer used in the current study was chitosan The surfactant or stabilizer most commonly used during PLG microparticle preparation, polyvinyl alcohol, imparts a negative surface charge on the microparticles, because of physical entrapment within the surface layer of the polymer (Rafati *et al.,* 1997). The positive charge on the surface of the cationic microparticles allowed the complete (100%) adsorption of plasmid DNA.

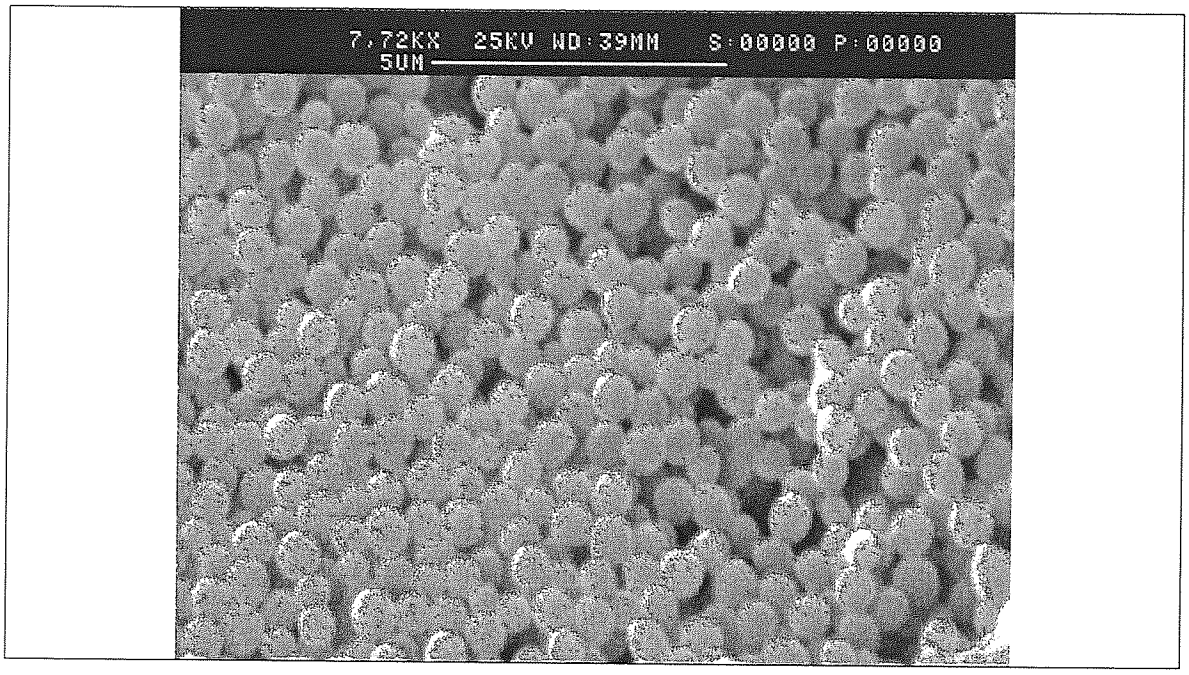


Figure 7.1: Scanning electron micrograph of polyethylcyanoacrylate nanoparticles prepared by emulsion polymerisation method in the presence of chitosan.

7.3.2. Electrophoretic analysis of plasmid DNA

The plasmid is tightly bound to the aluminium hydroxide gel as is observed by the fact that the plasmid does not move with the current in the gel (figure 7.2). The plasmid is not tightly bound to the aluminium phosphate as can be seen by the fact that the DNA moves through the gel under the influence of an electric current. This is due to the fact that aluminium hydroxide in saline exhibits a positive surface charge and the plasmid DNA is complexed with aluminium hydroxide due to charge interactions, whereas aluminium phosphate exhibits a negative surface charge and the plasmid is not complexed. This result is in accordance with the previous literature (Ulmer *et al.*, 1999).

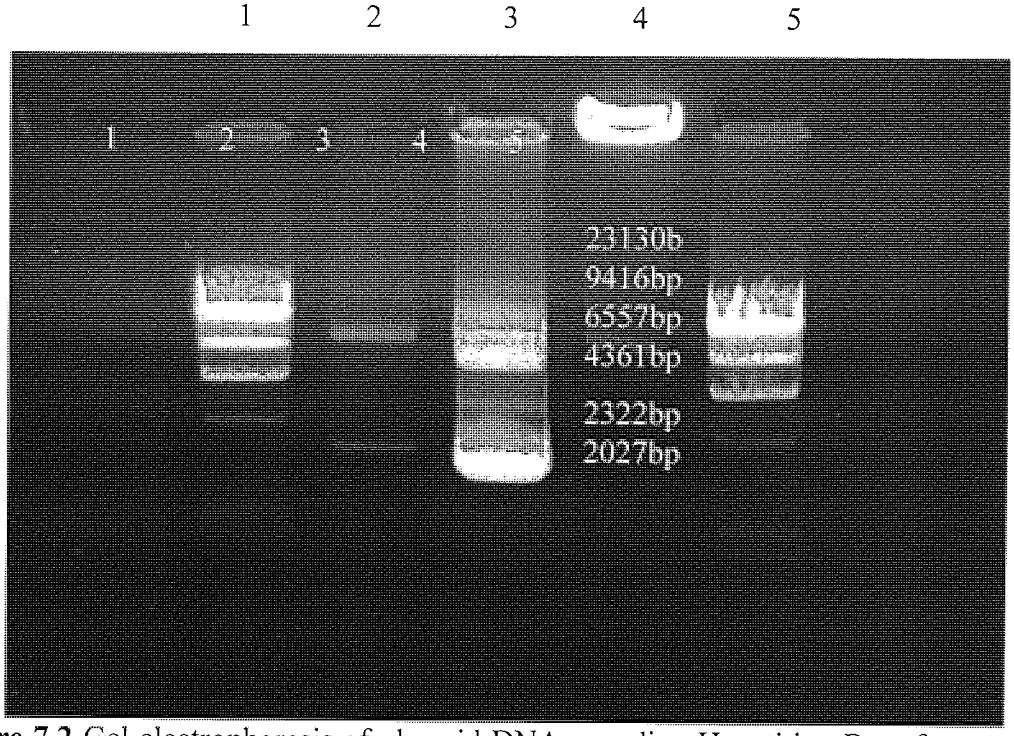


Figure 7.2 Gel electrophoresis of plasmid DNA encoding Hepatitis B surface protein. Plasmid DNA 100 μg/ml adsorbed to 45 μg of aluminium hydroxide or to 45 μg of aluminium phosphate. The samples were loaded as such on to the wells Lanes 1& 5: l-Hind DNA ladder Lane 2: Hepatitis B standard Lane 3: Hepatitis B & aluminium phosphate gel Lane 4: Hepatitis B & aluminium hydroxide gel.

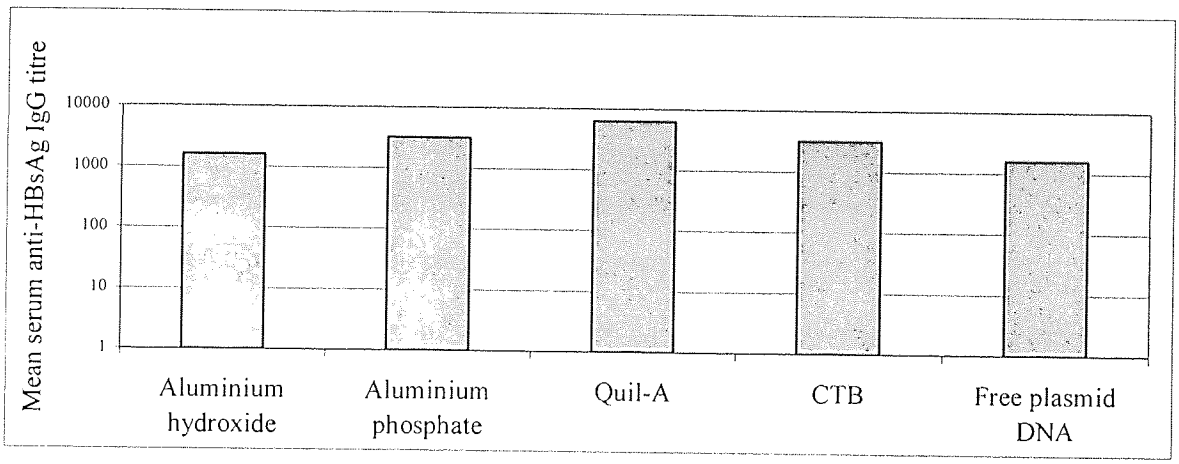
7.3.3. Serum immune response to nasally administered plasmid DNA encoding hepatitis B surface protein

To evaluate whether alum, CTB or Quil-A can be used as adjuvants for genetic immunisation, the humoral immune response elicited by 10 μg intranasal plasmid DNA, delivered as a free plasmid DNA or with adjuvants was evaluated. Sera was collected at various time points and examined by ELISA for specific anti-HBsAg antibodies. The *in vivo* results are shown in Figure 7.3.

None of the groups produced detectable serum transgene specific IgG, even after intranasal boosting with 10 μg plasmid DNA alone or in the presence of adjuvants. After boosting intramuscularly with protein antigen adsorbed to alum, all of the groups showed a resultant serum specific IgG response to the hepatitis B antigen. Compared to the free plasmid DNA group, the groups of mice, which received plasmid DNA nasally in the presence of adjuvants after intramuscular boosting, showed higher serum specific IgG responses. The group of mice which received plasmid, DNA adsorbed to

aluminium phosphate intranasally showed a higher immune response (3200) compared to aluminium hydroxide(1600) following boosting with recombinant antigen. The same trend was observed by Ulmer and colleagues (Ulmer *et al.,* 1999) after intramuscular administration of plasmid DNA in combination with various aluminium salts. Among the formulations the group dosed nasally with plasmid and Quil-A showed higher serum specific IgG responses following boosting.

These results indicated that even though all formulations initially failed to produce serum transgene specific IgG responses after nasal administration of the various formulations, following i.m. boosting with protein higher serum IgG was shown, which indicates that intranasal dosing with plasmid DNA has worked to prime the immune system. Further studies are needed to study cytokine profiles after mucosal administration to explain the mechanism of action of these adjuvants and explore the potential induction of other immunological responses (i.e. CTLs, T-cell proliferation and responses in challenge models).



Figugre 7.3: Serum immune response to nasally administered plasmid DNA encoding hepatitis B surface protein with various formulations. Female BALB/c mice (25 g, 6-week-old) were 5 groups of mice (n = 5) were dosed with 10μg plasmid DNA in the presence and absence of various adjuvants.

7.3.4. Serum immune response to intramuscularly administered plasmid DNA adsorbed to positively charged nanoparticles

To evaluate the positively charged nanoparticles as carriers for DNA vaccines, two types of nanoparticles were prepared. One with polyethylcyanoacrylate and another

with PLA 100kDa polymer. The results are shown in figure 7.4. After intramuscular immunisation the group of mice, which received plasmid DNA adsorbed to positively charged polyethylcyanoacrylate nanoparticles, showed serum IgG response whereas the plasmid DNA adsorbed to positively charged PLA failed to produce any detectable serum IgG response. The group of mice which received plasmid and antigen adsorbed to positively charged PLA nanoparticles showed higher antigen specific serum IgG levels compared to the group of mice which received antigen alone adsorbed to the nanoparticles. This study clearly shows that immunization with a combination of antigen and plasmid DNA adsorbed to nanoparticles produced higher serum specific IgG responses.

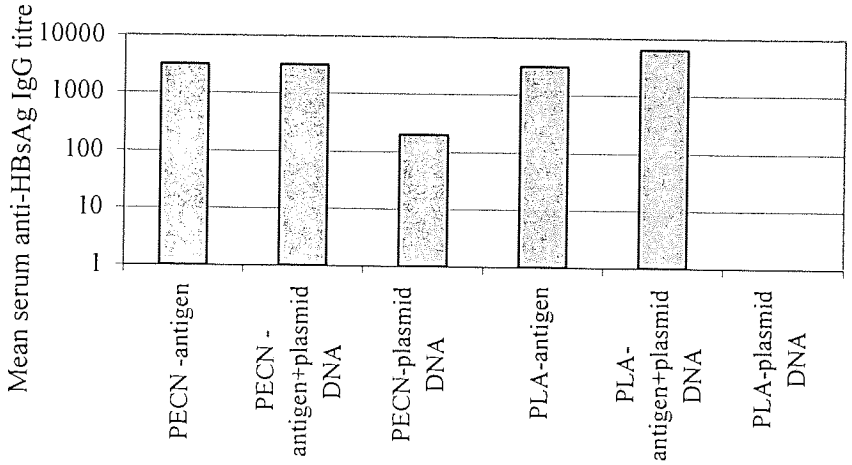


Figure 7.4. Serum immune response to intramuscularly administered plasmid DNA adsorbed to positively charged nanoparticles. Animals received a priming dose on day 1 and a booster dose on day 35. Blood samples were collected 1 week after the booster dose for analysis of antigen specific antibodies by ELISA.

7.3.5. Serum immune response to subcutaneously delivered plasmid DNA encoding hepatitis B surface protein

To evaluate the strategy of the addition of one or two adjuvants and combination of plasmid DNA and antigen groups of mice were immunised with various combinations. Plasmid DNA alone showed very low serum IgG responses compared to plasmid DNA plus protein antigen. The group of mice, which received plasmid and antigen in the presence of chitosan and Quil-A, showed higher serum IgG compared to the group,

which received Quil-A with plasmid and antigen. This is in accordance with previous reports where QS-21 induced higher immune responses to plasmid-encoded antigen (Sasaki *et al.*, 1998). Here it is shown that the combination of adjuvants resulted in a higher serum specific IgG response, even though these adjuvants alone failed to show any enhancement in the immune response to plasmid DNA after subcutaneous administration. These results are summarized in figure 7.5.

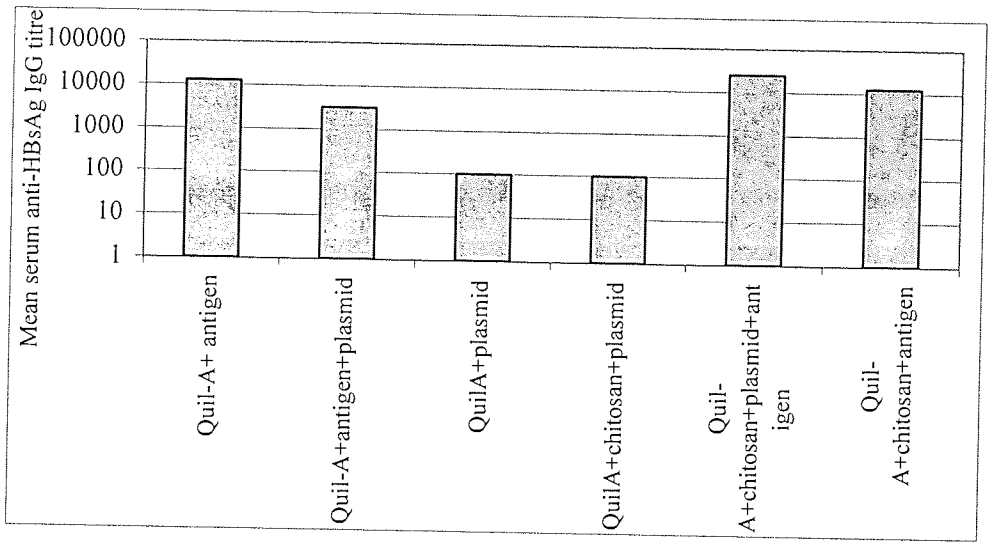


Figure7.5: Serum immune responses to subcutaneously delivered plasmid DNA encoding hepatitis B surface protein in combination with adjuvants Quil-A or Chitosan plus Quil-A.

7.4. Discussion and conclusions

The intranasal route of vaccination represents an attractive non-invasive route for vaccine delivery. In this study, the potential of alum as a mucosal adjuvant for DNA vaccine was examined. The co-administration of plasmid DNA with aluminium phosphate resulted in two fold higher serum IgG after protein boosting intramuscularly compared with plasmid DNA alone. This suggests that the presence of alum and other adjuvants in the DNA inoculum improved the priming. This is in accordance with the study done by Kanellos where they showed an enhanced immune response to lacZ after priming with plasmid DNA and boosting intraperitoneally with protein (Kanellos *et al.*, 2000).

Although it is not clear how alum increased the priming effect of DNA immunisation, it is interesting to note from the study by Ulmer and colleagues that after intramuscular

administration of hepatitis B plasmid, alum failed enhance the transfection of plasmid DNA (Ulmer *et al.,* 1999). Therefore, co-administration of alum might exert its adjuvant effect not by altering the *in vivo* transfection efficiency of the plasmid DNA, but by increasing antigen presentation of the DNA encoded protein, and/or by promoting antigen specific B and T cell priming within the nasal associated mucosal lymphoid tissue.

The use of mucosal adjuvants for intranasal DNA priming, followed by a protein booster might be an advantageous approach.

In the history of vaccine development since Jenner's innovation, a major goal has been enhancement of vaccine immunogenicity. An attractive approach to achieve this goal is the incorporation of immunological adjuvants into a vaccine formulation (Sasaki *et al.,* 1998).

In fact, a number of adjuvants have been explored and have showen respectable facilitatory effects on immune responses. This current study was designed to evaluate whether Quil-A, or Quil-A plus chitosan is able to enhance humoral immunity induced by DNA vaccination against hepatitis.

Sasaki and colleagues (Sasaki *et al.,* 1998) evaluated the immunomodulatory effect of the QS-21 adjuvant on the systemic and mucosal immunity induced by i.m and i.n DNA vaccination against HIV antigens and showed substantial facilitating effects on the antigen specific serum, mucosal antibody responses and cell-mediated immune responses. In their studies, antibody responses were only enhanced at a 25 μg dose of QS-21 whereas antigen specific cytolytic activity was enhanced by lower doses of QS-21. This may be one reason in the present studies why we did not get significantly higher serum IgG responses when mice were immunised with 10 μg of Quil-A and plasmid DNA.

Hepatitis B virus remains an important worldwide health problem with an estimated 250 million chronic carriers who face increased risk of development of liver cirrhosis and hepatocellular carcinoma. The development of effective protective and therapeutic vaccines for this pathogen are of prime importance, which is reflected, in the current implementation of DNA vaccine human trials for hepatitis B.

Cationic polyethylcyanoacrylate nanoparticles as carriers for DNA vaccines have not previously been explored for their potential in this field. Chitosan present during the polymerization process is strongly embedded into the polymer matrix resulting in a significant positive surface charge facilitating the adsorption of plasmid DNA. Chitosan has been shown to enhance transfection following oral delivery of plasmid DNA (Roy *et al.,* 1999) and this agent is known to be non-toxic and biodegradable (Jameela and Jayakrishnan 1995).

The DNA plus protein combined modality vaccination described in the present study combines the advantages offered by the DNA alone and potent antibody responses obtained using both DNA and protein. Further investigation is needed in order to assess the full potential of such combinations.

In the case of PACN adsorbed formulations, serum IgG responses were enhanced. This is in accordance with a previous report where cationic nanoparticles showed increased immune response to luciferase adsorbed to nanoparticles (Singh *et al.,* 2000). In contrast to their study, we have used biodegradable nanoparticles with a biodegradable positively charged polymer. This system has shown promising preliminary results, offering the significant advantage of biocompatibility and warrants further investigation for optimisation and evaluation.

The mechanism of the adjuvant effect achieved with cationic microparticles is not currently known, but we believe that efficient delivery of the adsorbed DNA to APCs is an important contributing factor. DNA co-administered with microparticles, but not adsorbed, does not induce a similar effect to adsorbed DNA. The fact that the presence of the cationic chitosan may activate macrophages may also make an additional contribution to the mechanism, because chitosan may contribute to the disruption of the endosome and the release of DNA into the cytoplasm. However, this hypothesis remains to be proven and further studies are necessary.

CHAPTER: 8

8. General conclusions and future work

In this thesis we have established the efficient mucosal delivery of vaccines using chitosan, a natural biopolymer obtained from crustacean shells. The results showed that nasal delivery of various antigens in the presence of chitosan exhibited markedly increased serum specific IgG responses in terms of both the magnitude and duration of responses achieved. We also investigated potential correlations of the observed immune responses generated by different chitosan derivatives with the characteristics of these derivatives. These results indicate that the degree of methylation is important for the trimethyl derivatives with a higher degree of methylation being advantageous and that in general, lower molecular weight or lower viscosity chitosan is more effective as a mucosal adjuvant. In addition, the use of chitosan was shown to enhance the action of other known adjuvants, such as CTB or Quil-A. Collectively, the data presented herein indicates that chitosan has excellent potential as a mucosal adjuvant.

The results in chapter three indicated that absorption enhancers can act as mucosal adjuvants. Among the absorption enhancers polyornithine gave higher serum immune responses to nasally delivered antigens. The molecular weight of polyornithine influenced the outcome of the immune response to nasally delivered antigens with higher molecular weight poly amino acids facilitating the best results. We have demonstrated for the first time that vitamin E TPGS can act as mucosal adjuvant. Dexoycholic acid was also tested for mucosal adjuvant properties and showed enhanced immune responses to nasally delivered antigens. Cyclodextrins were also identified as mucosal adjuvants. In addition the potential of acylcarnitine for the first time was shown in its ability to act as a mucosal adjuvant. Among the acylcarnitines tested, mistroyl-DL-carnitine chloride (MCC) showed the highest antigen specific serum antibody titres. Previously, none of these agents, common in their action as absorption enhancing agents, have been shown to have immunopotentiating activity for mucosal immunisation. These offer significant potential for the advancement and improvement

of vaccine strategies for mucosal application. The potential toxicity of some of these agents may be acceptable for vaccine delivery in that the concentrations required and frequency of administration is low. In any case, any toxicity is markedly reduced in comparison to CT, LT or LPS and some of the agents identified here, in particular vitamin E TPGS, are considered as safe and even beneficial.

The last decade has seen a massive increase in polymeric delivery systems for the purposes of vaccine administration. In an effort to enhance the efficacy of these delivery systems, we have successfully developed novel surface modified microspheres using chitosan as an emulsion stabiliser during preparation of PLA microspheres. We have extensively characterised this new particulate delivery system showing that the concentration of chitosan markedly alters the surface charge and formation of microspheres. In addition, we have found that immune responses could be substantially increased, effectively exploiting the immunopotentiating characteristics of both chitosan and PLA microspheres in the same delivery vehicle.

The effect of different release profiles has been the subject of much research. However, the relevance of such *in vitro* data in the generation of subsequent immune responses may be controversial. Our studies showed little correlation between *in vitro* release profiles and the observed immune responses, which may be expected from altered release profiles *in vivo*. Whilst the threshold for the generation of effective immune responses was undetermined from the formulations tested, it is likely that following administration, the degradation of biocompatible carriers is markedly influenced by the biological microenvironment such as uptake by phagocytic cells. In addition, activation of such cells along with localised cytokine production may provide the overriding stimulus in the generation of effective immune responses. However, further studies are required in order to assess these factors. In the same study, comparison of intranasal and intramuscular routes of administration showed that with these formulations, the nasal route could be as effective as intramuscular delivery, highlighting the potential of mucosal administration for these particulate delivery systems.

It was demonstrated that intramuscular co-administration of chitosan with polymer microspheres facilitated markedly enhanced immune responses in both magnitude and duration. We conclude that this combination shows potential for single dose administration of vaccines. However, following intranasal dosing, the addition of

chitosan was unable to enhance immune responses to antigen loaded polymer microspheres.

In another study, we have shown that the addition of chitosan to alum adsorbed TT was able to enhance immune responses, as has previously been shown for Quil-A. The most interesting facet of the chitosan was the ability of this formulation to induce TT specific IgG2a subclass production. This is indicative of the ability of chitosan to stimulate Th1 type responses and this aspect of the adjuvant activity of chitosan requires further elucidation.

Recent outbreaks of diphtheria in developed countries emphasise the importance of safe and effective mass vaccination strategies. Administration *via* mucosal routes may have significant advantage in efficient and effective implementation of vaccination programmes. This is ultimately dependent upon the discovery and elucidation of safe mucosal adjuvant formulations. In particular, chitosan or vitamin E TPGS show considerable promise in this context.

Using PLA microspheres, a smaller particle size was shown to facilitate higher serum IgG responses following nasal administration. A lower antigen loading was additionally identified as being preferential for the induction of immune responses with the smaller particle size. This may be due to the fact that the number of particles will be increased when antigen loading is low, which may in turn facilitate a more widespread uptake of particles.

Simple adsorption of antigen to lamellar particles was also shown to generate effective immune responses following intramuscular administration. This expands upon work already established previously in our laboratories.

Positively charged polyethylcyanoacrylate and PLA nanoparticles were designed and preliminary investigations into their potential as delivery vehicles for DNA vaccines undertaken. Successful preparation of particles with narrow size distribution and positive surface charge (imparted by the inclusion of chitosan) was achieved. Although the observed immune responses to encoded antigen were of low magnitude, this may reflect the low dose used, DNA binding characteristics, or the need for further analysis of the cellular immune response. DNA vaccines are often effective in the induction of Th1 type responses thus the evaluation of *in vitro* cell stimulation, CTL activity or challenge studies may be more informative. At least further investigation into cell

mediated immunity etc and optimisation is required. In the evaluation of antibody responses to DNA encoded antigen in the presence of alum administered intranasally, discrimination between the groups was only seen following intramuscular boosting with the corresponding protein. This study clearly shows that DNA vaccines in the presence of either alum or Quil-A may advantageously influence priming of the immune system by a mucosal route. Again, higher doses may be required in order to further enhance antibody responses which may be responsible for the inability to show the known adjuvant effect of Quil-A for plasmid encoded antigen. The potential for the combination of adjuvants, Quil-A and chitosan, to enhance antibody responses to plasmid encoded antigen co-administered with the corresponding protein antigen was shown and this is worthy of further investigation.

Ongoing and future work is focussed on mechanistic studies, differentiating between absorption enhancers; novel mucosal adjuvants identified here such as vitamin E TPGS, and other mucosal adjuvants. Currently we are evaluating absorption enhancers in combination with particulate vaccine delivery systems. More detailed and comprehensive analysis of immune responses *in vivo*, including investigation into CTL production, challenge studies and further characterisation of local and distal responses in the mucosal immune system for these and the other immunopotentiating formulations identified in these studies. This should be corroborated by studies in higher animals. We have shown that the magnitude of antigen dose may not correlate directly with the magnitude of the observed immune response. In the evaluation of different formulations, a lower dose may optimal for the purposes of comparison.In the case of polyethylcyanoacrylate and PLA nanoparticles for the delivery of DNA, transfection studies and cellular localisation studies and optimisation are planned.

The modification of PLA polymers by the grafting of chitosan is another approach for future studies offering potential for gene and vaccine delivery together with further evaluation of characteristics such as the alteration of the degradation of polymer microspheres by the inclusion of chitosan. The mode of association of antigen to adjuvant, or particulate delivery systems, will influence the manner in which antigen is presented to APC. The effects of such physicochemical characteristics can be evaluated *in vivo* and will further elucidate the mechanisms of action of particulate delivery

systems. This may be particularly relevant for the chitosan modified PLA formulations, where the localisation of the charged component may be of importance in targeting and uptake at mucosal surfaces. In addition, these biodegradable mucoadhesive delivery systems, surface modified in a single step process, may have application for other uses such as drug and gene delivery.

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