Characterisation of the immunological events induced by biodegradable polymer vaccines

Thesis

UNRESTRICTED

CHARACTERISATION OF THE IMMUNOLOGICAL EVENTS INDUCED BY BIODEGRADABLE POLYMER VACCINES

by

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ABSTRACT

This study was performed to unravel the adjuvant mechanisms of biodegradable microsphere vaccines based on poly (lactide-co-glycolide) (PLGA) polymer. Those microspheres have been extensively investigated as efficient vaccine adjuvant and delivery systems. However, their mode of action and their *in vivo* processing remain unclear. A better understanding of the adjuvant mechanisms would prove essential for the design of new generation vaccines required to be safer, given in less doses and stable at room temperature. This study focused on the sub-cutaneous administration of encapsulated protein models diphtheria and tetanus toxoids (DT and TT). The experimental work covered three aspects: (i) the uptake and transport of subcutaneously injected PLGA MS DT by macrophages and dendritic cells *in vivo* in mice; (ii) the influence of physico-chemical properties of the PLGA MS formulations on the toxoids immunogenicity in mice and guinea pigs; (iii) the immunogenicity of a multivalent encapsulated PLGS MS loaded with individual or multiple childhood vaccine antigens. This is probably the first study on the particle uptake and biodistribution of sub-cutaneously injected PLGA MS by antigen presenting cells in mice. Tracking of the fluorescent particles by microscopy confirmed previously postulated adjuvant mechanisms such as the depot effect at the injection site mimicking multiple injections; the efficient uptake of the particles by antigen presenting cells (APCs) and the active transport and relocalisation of the particles within lymphoid organs via APCs. These mechanisms were influenced mainly by the hydrophobic nature of the polymer and the size of the microspheres. When the formulation was optimized, a single dose of microencapsulated vaccine on its own (without addition of a potent adjuvant) protected better than two doses of the commercial alum-adsorbed vaccine. This study also demonstrates the feasibility of a multivalent paediatric encapsulated vaccine based on diphtheria, tetanus, pertussis and Hib antigens. The implementation of such a vaccine in the immunisation schedule would be a major achievement in the reduction of the number of injections given to the infants.

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ABBREVIATIONS AND DEFINITIONS

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
Ab	antibody
ADP	adenylase phosphate
Ag	antigen
Al(OOH)	aluminium hydroxide
$AlPO_4$	aluminium phosphate
aP	acellular pertussis
APC	allophycocyanin
APCs	antigen presenting cells
BSA	bovine serum albumin
°C	degree Celsius
CD	cluster of differentiation
Ci	curie (μCi=microCurie)
CMI	cell mediated immunity
CO	coacervation
cpm	counts per minute
CTL	cytotoxic T lymphocyte
CVI	children's vaccine initiative
DC	dendritic cell
DCM	dichloromethane
DH	Dunkin Hartley
DMF	dimethylformamide
DT	diphtheria toxoid
DTH	delayed type hypersensitivity
DTP	diphtheria-tetanus-pertussis
Dtx	diphtheria toxin
ED_{50}	effective dose 50%
EF	elongation factor
ELISA	enzyme linked immuno adsorbent assay
EM	emission maximal
EPI	expended program on immunisation
FCA	Freund's complete adjuvant
FCS	fetal calf serum
F-MS	fluorescent microspheres
FIA	Freund's incomplete adjuvant
FIM	fimbria
FITC	fluorescein isothiocyanate
FHA	filamentous haemaglutinin
g	gram
GSK	Glaxo SmithKline

h	hour(s)
H_2O_2	hydrogen peroxide
Hep B	hepatitis B
HBO-HAS	*Haemophilus influenzae* type b oligosaccharide conjugated tc human serum albumin
Hib	*Haemophilus influenzae* type b
HRP	horse radish peroxidase
i.d.	intra-dermal
IFN-γ	interferon gamma
Ig	immunoglobulin
i.m.	intra-muscular
IPV	inactivated polio virus
IL	interleukin
IS	injection site
IU	International Unit
KCl	potassium chloride
KDa	kilodalton
Lf	limit of flocculation
Ltd	limited
MALT	mucosal associated lymphoid tissues
MEM	minimum essential medium
MHC	major histocompatibility complex
µg	microgram
mins	minutes
mg	milligram
ml	millilitres
MLN	mesenteric lymph node
µm	micrometer
MMF	macrophagic myofasciitis
MMR	measles-mumps-rubella
MΦ	macrophage
M	molar
mM	millimolar
MRD	minimum reactive toxin dose
MS	microsphere
MTT	3-[4,5-Dimethylthiazol-2-γl]-2,5-diphenyl-tetrazolium bromide
Mw	molecular weight
nAb	neutralising antibody
NaCl	sodium chloride
NALT	nasal associated lymphoid tissues
NIBSC	National Institute for Biological Standards and Control
nm	nanometer

PBS	phosphate buffered saline
PBST	phosphate buffered saline tween
PC	peritoneal cavity
PerCP-CY5.5	peridinin chlorophyll protein coupled to cyanine dye 5.5
PfCS	*Plasmodium falciparum* synthetic peptide
Ph. Eur.	European Pharmacopoeia
PLA	poly (lactic acid)
PLGA	poly (lactic-co-glycolic) acid
PRN	pertactin
PRP	polyribosyl ribitol phosphate
PRP-TT	polyribosyl ribitol phosphate conjugated to tetanus toxoid
PS	polystyrene
PT	pertussis toxoid
s	second(s)
S	spleen
SD	spray drying
SHD	single human dose
SE	solvent evaporation
Sem	scanning electron microscopy
SEM	standard error of the mean
SM	surface marker
s.c.	sub-cutaneous
TCR	T cell receptor
TEM	transmission electron microscopy
Th-1 or-2	T helper cell type 1 or 2
TNF	tumour necrosis factor
TT	tetanus toxoid
Ttx	tetanus toxin
UK	United Kingdom
WHO	World Health Organisation
w/v	weight to volume

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Peyre M, Fleck R, Hockley D, Gander B, Sesardic D (2004). *In vivo* uptake of an experimental microencapsulated diphtheria vaccine following sub-cutaneous immunisation. *Vaccine*, **22**, 2430-2437

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Godefroy S, **Peyre M**, Garcia N, Muller S, Sesardic D, Partidos CD (2004). The effects of skin barrier disruption on the immune response to topically applied cross-reacting material (CRM_{197}) of diphtheria toxin. *Infection and Immunity* (In press)

Mawas F, **Peyre M**, Beignon A.S, Frost L, Del Giudice G, Rappuoli R, Muller S, Sesardic D, Partidos CD (2004). Successful induction of protective antibody responses against *Haemophilus influenzae* type b and diphtheria after transcutaneous immunisation with the glycoconjugate polyribosyl ribitol phosphate-cross-reacting $material_{197}$ vaccine. *The Journal of Infectious Diseases*, **190** (6), 1177-1182

AIM AND OUTLINE OF THE STUDY

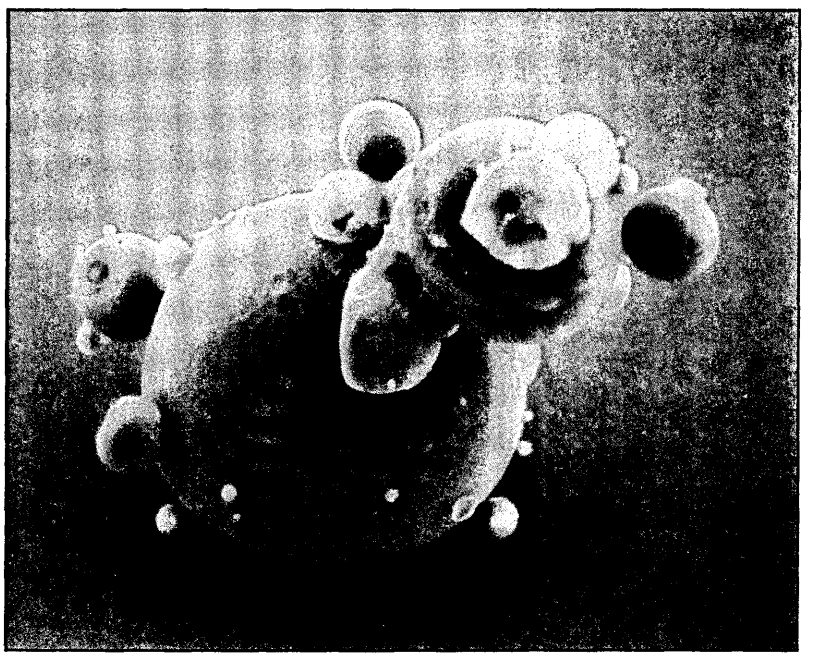
The main goal of this project was to enlighten the adjuvant mechanisms of PLGA MS vaccines. Until recently, the fate of injected PLGA MS was largely unknown (Jones et al., 1995) so that studies on particle uptake by phagocytic cells and retention times at the injection site were urgently needed. Only one localisation study has been performed on parenterally administered (intra-peritoneal and intra-dermal) PLGA MS (Newman et al., 2002) and no investigation on the trafficking of PLGA MS after intra-muscular (i.m) or sub-cutaneous (s.c) injection has yet been performed. Instead, the general interest increased towards the potential of PLGA MS for nasal or oral vaccines (Desai et al., 1996; McClean et al., 1998; Torche et al., 2000).

The first results chapter (3) describes the investigation of the trafficking of PLGA MS following a sub-cutaneous (s.c.) immunisation. As particle hydrophobicity and size had been described as key factors of phagocytosis (van Oss, 1978), these two parameters along with the type of reconstitution buffer for the MS and the volume of injection were studied here. Tracking techniques such as flow cytometry and immuno-labelling were used to quantify the amount of MS present in various tissues and organs and to identify the phenotype of cells involved in the MS uptake and transport. A range of microscopy techniques such as epifluorescence and confocal microscopy were also used to confirm the presence of the MS within the tissues and phagocytic cells.

The second results chapter (4) reports on the influence of formulation parameters on the type and extent of immune response induced. Polymer type, MS size, antigen content and administered dose have been investigated. The choice and effect of co-encapsulated stabiliser is not described in this work, as it has been thoroughly evaluated previously with similar formulations (Johansen et al., 1998b; Johansen et al., 1998a). Nonetheless, because of safety concerns regarding the use of bovine serum albumin as antigen stabiliser inside the MS, a new stabiliser was introduced and the efficacy of these new formulations was compared to the previous ones. Another issue was the co-administration of the conventional adjuvant aluminium hydroxide with the PLGA MS vaccines for further enhancement of the immune response. It was important to assess the role of alum in combination with PLGA MS in terms of the type and extent of immune response induced. In this chapter, the effect of different doses of admixed alum on both humoral and cellular anti-DT responses was examined. The impact of alum on the long term protection induced by PLGA MS vaccines was also determined.

The last results chapter (5) describes a highly relevant aspect of applying the PLGA MS technology to combined vaccines, i.e., the combination of multiple antigens within PLGA MS. New types of pediatric vaccines will be commercially viable only if multivalent formulations containing DT+TT+PT+Hib are feasible. One of the main issues with combination vaccines regards antigen-antigen interactions. The importance of combining multiple antigens within a single PLGA MS formulation versus mixing PLGA MS types loaded individually with single antigens (mixtures of monovalent PLGA MS) was assessed.

INTRODUCTION



CHAPTER 1: INTRODUCTION

Infectious diseases remain the highest cause of mortality worldwide and represent a particular threat in developing countries. Preventable diseases such as diphtheria and tetanus are not considered anymore as a threat in industrialised countries, although immunity against diphtheria has decreased due to reluctance of the population, especially amongst adults, to receive the necessary booster injections. Therefore, a resurgence of diphtheria has been observed over the last 20 years (Galazka et al., 1995; Maple et al., 1995). Moreover, such "old" preventable diseases continue to claim lives in remote parts of the world where vaccination coverage is not optimum (Figure 1.1). The following sections will put particular emphasis on diphtheria- and tetanus-related diseases and vaccines.

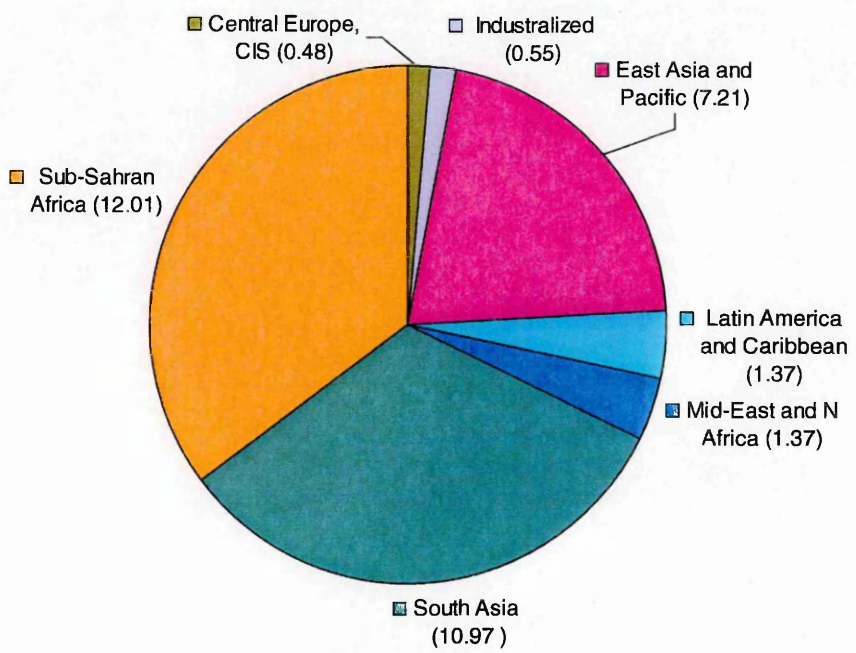


Figure 1.1. Worlwide distribution of children not immunised with DTP (33 millions). Source: WHO/UNICEF estimates 2003

1-1 Diphtheria and tetanus: diseases and vaccines

1-1-1. Diphtheria

Diphtheria is caused by the gram-positive *Corynebacterium diphtheriae*. In the acute phase of infection, the bacterium colonises pharynx, larynx and nose and produces a large greyish pseudomembrane (Figure 1.2). The large membranes consist of leukocytes, fibrin and bacteria and may choke the patient, while the bacterium secrets a toxin that enters the circulation and damages various organs. Although *C. diphtheriae* rarely infects the skin; it may serve as a microbial reservoir for later spreading of the organism. The initial symptoms of the disease are not specific: low to moderate fever, vomiting, cough and throat soreness. The severe development of the disease is caused by the effects of secreted diphtheria toxin on the myocardium, which can lead to heart failure and death (Aggerbeck, 1998; Pizza et al., 1999).

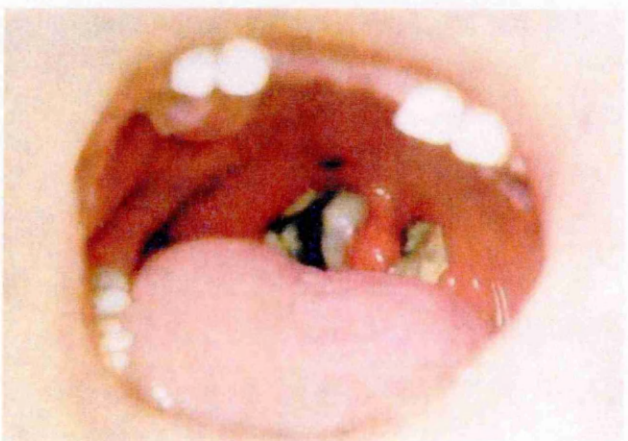


Figure 1.2. Pseudomembrane caused by *C. diphtheriae*. (with permission from C.V. Hunolstein (Eisenberg et al., 1996; von Hunolstein et al., 2003))

Observation made by Jonathan Dickenson, a local doctor, during the diphtheria epidemic that swept the northern American colonies in the years 1735-1740 (Eisenberg et al., 1996):

"The first assault was in a family about ten miles from me, which proved fatal to eight of the children in about a fortnight. Being called to visit the distressed family, I found upon my arrival, one of the children newly dead, which gave me the advantage of a dissection, and thereby a better acquaintance with the nature of the disease, than I could otherwise have had...

It frequently begins with a slight indisposition, much resembling an ordinary cold, with a litless habit, a slow and discernable fever, some soreness of the throat and tumefaction of the tonsils: and perhaps a running of the nose, the countenance pale, and the eye dull and heavy. The patient is not confined, nor any dangers apprehended for some days, till the fever gradually increases, the whole throat, and sometimes the roof of the mouth and nostrils are covered with a cankerous crust... When the lungs are thus affected, the patient is first afflicted with a dry hollow cough, which is quickly succeeded with an extraordinary hoarseness and total loss of voice, with the most distressing asthmatic symptoms and difficulty of breathing, under which the poor miserable creature struggles, until released by a prefect suffocation, or stoppage of the breath."

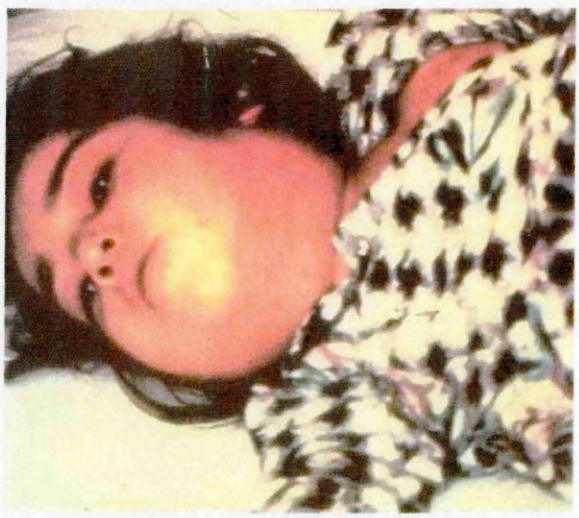


Figure 1.3. Child suffering from diphtheria, with a characterisitic swollen neck (1995, with permission from CDC, library source: PHIL, http://phil.cdc.gov)

Diphtheria toxin (Dtx) inhibits protein synthesis in the host cell. The secreted toxin (polypeptide of 535 amino acids) is easily cleaved into two subunits: (i) fragment B, which binds the toxin to cell surface receptors and facilitates the entry of the fragment A into the cytosol, and (ii) fragment A, which is an ADP-ribosylating enzyme that inhibits protein synthesis by inactivation of elongation factor 2 (EF-2) of eucaryotic cells (Pizza et al., 1999). A single fragment A in the cytosol is sufficient to kill the cell (Yamaizumi et al., 1978). The letal dose of Dtx in humans is ≤100 ng/kg body weight. Without specific treatment, fatality rates range between 12 to 86%, with the highest mortality rate being observed in children of less than 5 years of age (Aggerbeck, 1998).

The almost complete elimination of diphtheria in developed countries followed soon after the isolation of the toxin in 1888 (Roux and Yersin, 1888). In 1913, Emile von Behring (first Nobel Prize in physiology and medicine) reported on the immunisation of children against diphtheria by using a mixture of toxin and antitoxin, which had a most profound impact on public health (von Behring, 1913). This mixed toxin-antitoxin vaccine was replaced, after 1923, by a vaccine of a formaldehyde-treated Dtx, called diphtheria toxoid (DT) (Pizza et al., 1999).

Protein antigens administered alone are weak stimulators of the immune system, and the use of immunological adjuvants is required to enhance the immune response. In 1925, G. Ramon first demonstrated an increase in anti-diphtheria and anti-tetanus antitoxin levels by injecting the toxoids along with breadcrumbs, agar, tapioca, starch oil, lecithin or saponin to the vaccine (RAMON, 1955; RAMON, 1957; RAMON, 1959). Insoluble aluminium phosphates or hydroxides, commonly referred to as "alum", are nowadays the most widely used adjuvants

(Gupta and Siber, 1994; Gupta et al., 1995), and their use dates back to 1926, when Glenny discovered that a suspension of alum-precipitated DT was much more immunogenic than the soluble toxoid (Glenny, 1926). Although clinical efficacy trials have never been performed with DT, its effectiveness has been empirically confirmed by the disappearance of the disease in the countries with broad immunisation coverage. As Dtx is neutralised by specific antibodies of the IgG class, antibody levels were taken as a measure of efficacy (Aggerbeck, 1998; Sesardic et al., 1993; Simonsen, 1989; WHO, 1999a).

Diphtheria still causes epidemics in countries where the immunisation programme coverage is low (Mattos-Guaraldi et al., 2003); a resurgence of diphtheria has been observed in industrialised countries where the immunisation schedule was not optimal (Kelly and Efstratiou, 2003; Galazka and Robertson, 1996) and where the immunity against diphtheria decreased with age (Rappuoli et al., 1993). Between 1991 and 1998, more than 157,000 cases and 5,000 deaths were reported during the diphtheria epidemic in the former Soviet Union (Figure 1.4) (WHO, 1996; WHO, 1998; Galazka et al., 1995). At the beginning of the 21st century (2001) more than 10,000 children and adults, mostly from developing countries, are still affected by diphtheria with an annual death toll of more than 4000 children under the age of five years old (WHO, 2003).

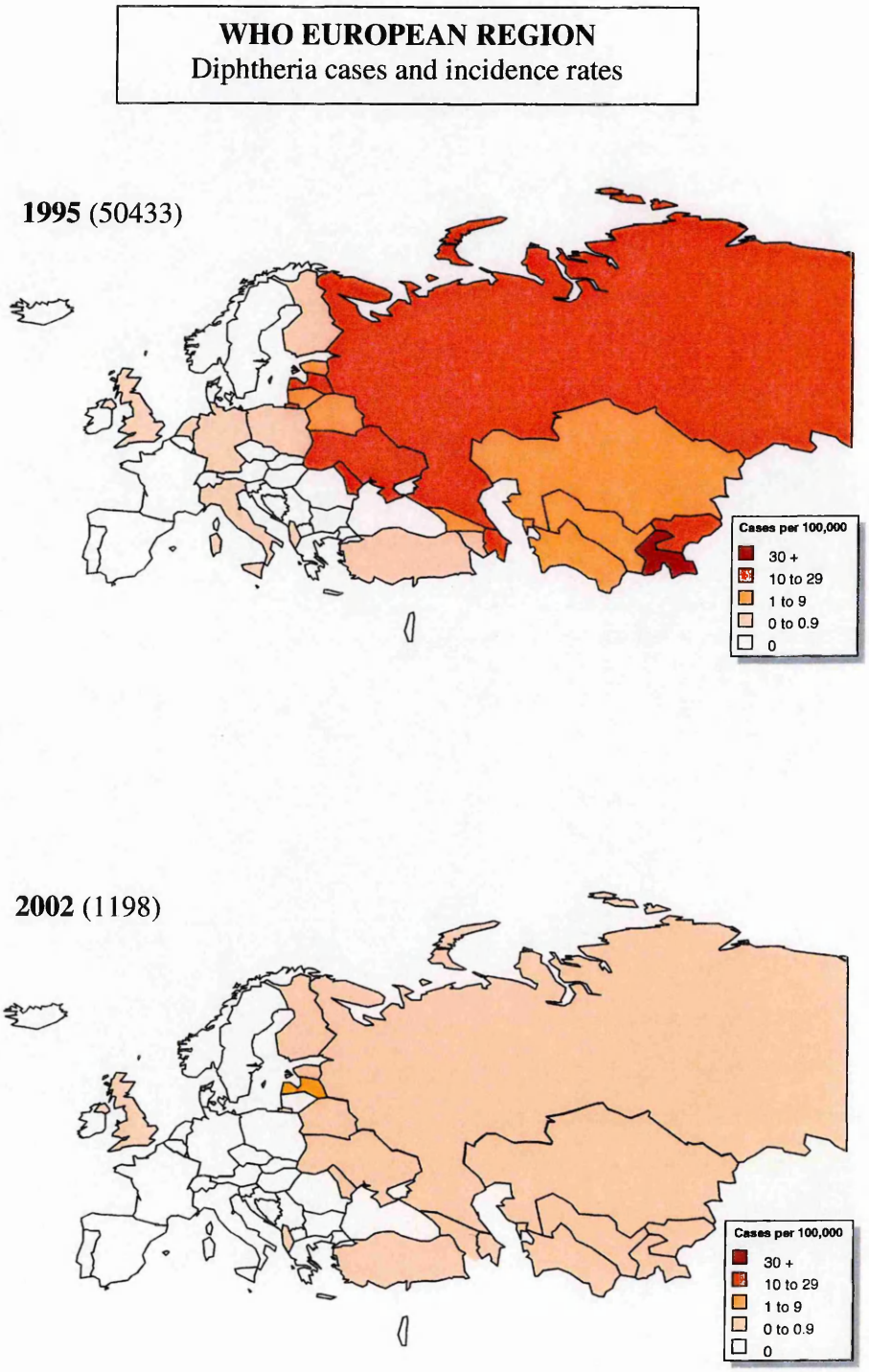


Figure 1.4. Diphtheria cases in European region at the peak (1995) and after (2002) the former Soviet Union diphtheria epidemic (1994-1996) (WHO data, with permission from C. von Hunolstein)

1-1-2. Tetanus

Tetanus is not a communicable disease, but is acquired through infection with the gram-positive spore-forming *Clostridium tetani* by environmental exposure. *C. tetani* is an ubiquitous organism commonly found in soils throughout the world. However, it is more widespread in southern countries of the northern hemisphere, and the incidence of tetanus is higher in warm countries than in northern areas. At the beginning of the disease, the clinical symptoms are unspecific, such as headache, neck and back pain, high fever and muscle stiffness (Herreros et al., 1999). The specific manifestations of the disease are more severe: risus sardonicus (sardonic smile) as a consequence of a lockjaw, opisthotonos (flexion of the arms, extension of the legs and contraction of the muscles of the back) (Figure 1.5), which can be caused by any visual or acoustic stimuli.

Figure 1.5. This neonate is displaying a bodily rigidity produced by *Clostridium tetani* exotoxin, called "neonatal tetanus" (1995, with permission from CDC, library source: PHIL, http://phil.cdc.gov)

These severe pathologies are caused by tetanus toxin (Ttx), a potent neurotoxin produced by *C. tetani*, which inhibits the exocytosis of neurotransmitter in the central nervous system and thereby causes muscle spasm (Herreros et al., 1999). Tetanus toxin is the second most potent toxin after botulinum toxin, with a lethal dose in humans below 2.5 ng/kg body weight (Popoff and Marvaud, 2004). The formaldehyde-detoxified Ttx is called tetanus toxoid (TT) which is widely used as a vaccine after adsorption onto insoluble aluminium salts.

Protection against tetanus relies upon toxin neutralising antibodies (WHO, 1999b), whose levels decrease throughout life so that booster doses are required to maintain protection in adults (Van Damme and Burgess, 2004). In developing countries, more than 300,000 infants die each year from neonatal tetanus acquired through infection at the umbilical stump of the newborn (WHO, 2003). Protection against neonatal tetanus is optimally achieved by immunising child-bearing mothers with at least two doses of TT-vaccine. Due to the rather low return frequency for receiving the booster doses, the development of a single administration TT-vaccine has a high priority for the World Health Organisation (WHO) (WHO, 1995)

1-2 Pediatric vaccines: limitations

In the first two years of life, a child will need 15 to 18 injections to acquire immunity against the main childhood threatening diseases. These injections include four doses of a combined diptheria-tetanus-pertussis (DTP) vaccine (Maple and al Wali, 2001) and one dose of a combined measles-mumps-rubella (MMR) vaccine (Miller et al., 2001). If these antigens were given separately, the number of injections would rise to more than 30. Combination vaccines have offered the benefit of including also antigens from *Haemophilus influenzae* type *b* (Hib), polio virus, hepatitis B virus (HBV) and varicella virus (VV) in the routine immunisation schedule (conjugated polysaccharide Hib vaccine and inactivated polio virus (IPV) vaccine are given in 3 to 4 doses, HBV in 3 doses and VV as a single dose) (Aristegui et al., 1998; Aristegui et al., 2003b; Kelly and Efstratiou, 2003).

Considering solely protection against diphtheria and tetanus, five doses of combined vaccine are offered before the age of ten and further booster doses after every ten years to maintain a life-time protection. Thus, any development of new vaccine delivery systems should address the need for fewer required injections and multivalent vaccines to facilitate current immunisation schedules. However, one needs to be aware that the immunogenicity of a particular antigen given individually may not be the same as when this antigen is included in a combination: the immunogenicity may be impaired or synergised. For some antigens, combination with more immunogenic antigens may be essential to raise protection (Vidor et al., 1999).

Eradication of diphtheria and tetanus causative organisms is impossible, as the environmental niche is unknown for the first one, and the latter is ubiquitously present. The only way to prevent the diseases is by worldwide vaccination, which might be more easily achievable by developing an optimal formulation, which is heat-stable and affords protection after a single or two administrations in a multivalent combination (WHO, 1995).

1-3 Improving immunisation coverage: search for improved vaccine formulations

The Expanded Program of Immunisation (EPI), initiated by the WHO in 1977, encompassed the provision of six basic childhood vaccines, including DT and TT, to developing countries (WHO, 1997). This effort of worldwide immunisation was followed by the Children's Vaccine Initiative (CVI) in 1990, which aimed at improving the current pediatric vaccines (WHO, 1995), i.e., developing formulations that are stable under ambient conditions and afford protection after only one or two injections. The CVI also promoted the development of new vaccines that could be given earlier in life or through easier routes of administration. To this aim, new types of adjuvants and delivery systems have been investigated over the past 15 years. The present study has therefore to be seen in this very context.

New adjuvants or delivery systems should ideally fulfil the following: (i) enhance long term immune response towards antigens upon nasal or other mucosal administration or single injection; (ii) elicit in an antigen-dependent manner an optimal immune response; (iii) remain stable at room temperature; (iv) be easy and cheap to produce; (v) be safe and biodegradable.

Realistically, no adjuvant or delivery system will probably ever meet all these criteria. Most adjuvants under current evaluation exhibit some adverse effects, mainly manifested as local reactions at the site of administration (Song and Katial, 2004; Aucouturier et al., 2001; Del Giudice et al., 2001; Degen et al., 2003). Adverse effects such as local tenderness, redness and swelling are usually mild, but sometimes more severe painful abscesses and nodules at the inoculum site are formed (Collier, 1980; Frost et al., 1985; Goto et al., 1997; Bergfors et al., 2003). The latter ones are often associated with poor degradability of the adjuvanted vaccine and persistence in tissues resulting in the formation of a reactive granuloma (White et al., 1955). Some inflammation might, however, be necessary to stimulate the chain of immunological reactions required to acquire a strong immune response and protection (Schijns, 2000; Sheikh et al., 1996). Adjuvants have generally been evaluated under balanced benefit/ risk considerations (Gupta et al., 1993; Kenney and Edelman, 2003; Dittmann, 2001). Most of the adverse effects observed are indirect consequences of the actual mechanism of action of the adjuvant itself. As the mode of action of adjuvants is intimately connected with the cells of the innate immune response, such as macrophages (MΦ) and dendritic cells (DCs), some of the main features of these two cell types will first be summarised in the following work.

1-3-1. Antigen presenting cells

Antigen presenting cells (APCs) such as macrophages and DCs play a critical role in the induction of the immune response, as they can ingest antigens, present them to T-cells and simultaneously release co-stimulatory molecules required for T-cell activation. They are the first cells to interact with antigens and adjuvants (Steinman, 1991; Unanue et al., 1969). An effective immune response is characterised by a good clonal expansion of antigen-specific T- and B-cells. To activate naïve T-cells, the antigen is presented to the T-cell receptor (TCR) by APCs on the so-called major histocompatibility complex (MHC) molecules of APCs. This first stimulus is known as signal 1 (Grakoui et al., 1999; Janeway, 1989; Schijns, 2001). Signal 1 alone is insufficient to activate T-cells and leads to tolerance and clonal deletion via apoptosis. Naïve T-cells require a second signal, the so-called signal 2, to become fully activated. Signal 2 appears upon the expression of co-stimulatory molecules on APCs, such as B7.1 and B7.2 in humans (CD86 in mice) and the production of IL-1, IL-12, TNF-α and IFN-γ. Adjuvants can induce both signals 1 and 2 to activate naïve T-cells to become specialised helper T-cells. The latter regulate the subsequent T-cell-dependent B- and/or T-cell responses (Schijns, 2002). The type of APC, the duration of stimulation via TCR, the temporal and spatial localisation of available antigen, and the type of adjuvant present during the initial contact all influence the differentiation of activated T-cells. An alternative molecular model suggests that adjuvants may induce or act like danger signals, causing distress or injured cells to release alarm signals that may bind to the same receptors to trigger APCs activation (Matzinger, 2002).

DCs (Fig 1.6A) originate from the bone marrow and migrate through the blood system to the tissues (mostly skin) (CD11b/c-positive cells) and through the lymphatic system to lymphoid organs (Steinman, 1991; Steinman et al., 2003). They are considered as the most competent APCs, as they present most efficiently antigens to naïve T-cells, thereby inducing specific T-cell-dependent immune responses (Macatonia et al., 1993; Macatonia et al., 1995). The induction of an immune response requires the migration of DCs from the site of first encounter to peripheral lymphoid organs such as draining lymph nodes. Adjuvants are assumed to promote DCs migration (Cumberbatch and Kimber, 1995) and thereby increase antigen presentation to naïve T-cells. DCs present in the T-cell-dependent areas of spleen and lymph nodes are termed interdigitating DCs and those in lymphoid follicles follicular DCs (FDCs). The latter are closely associated with B-lymphocytes (Szakal et al., 1989). Localisation of antigenic complexes on FDCs seems to be required to generate memory B-cells (Klaus et al., 1980). DCs can also produce IL-12, a dominant cytokine involved in the development of $CD4^+$ T-cells that produce IFN-γ (Macatonia et al., 1995). Production of IL-12 by DCs appears to be an early and crucial event in the cascade of cytokines formed in response to adjuvants. A subset of DCs are the Langerhans'cells, which, after migration from the bone marrow through the blood vessels to the skin, reside in the skin for approximately one week; Langerhans' cells are therefore mainly detected in the skin. Thereafter, the Langerhans' cells migrate further through afferent lymphatic ducts to the T-cell-dependent areas of lymph nodes, where they are finally termed interdigitating cells (Balfour et al., 1980).

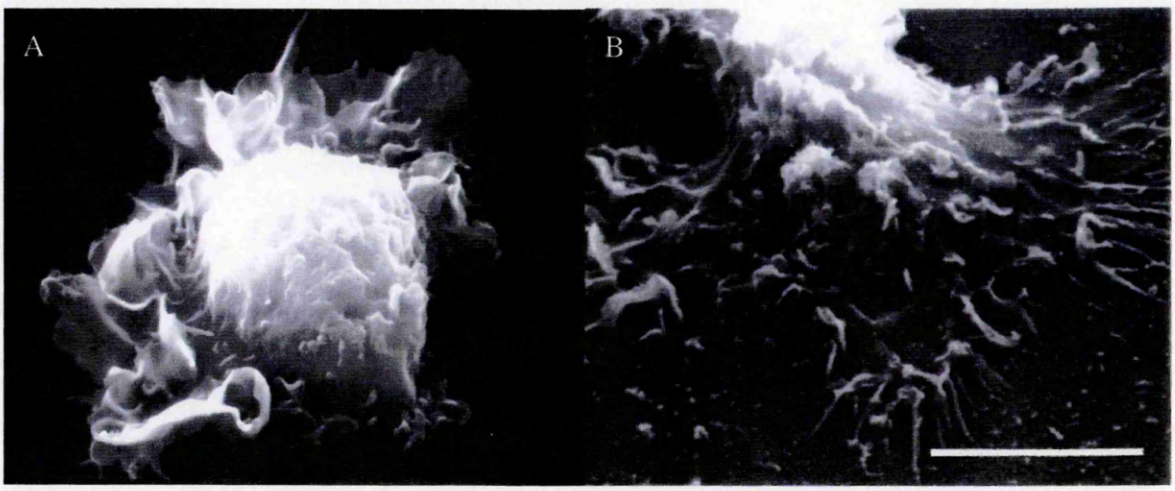


Figure 1.6. Scanning electron micrographs of a dendritic cell (A) and a macrophage (B). (Scale bar = 10μm). The dendritic cells were differentiated from Balb/c mice bone marrow in the presence of IL-4 and GM-CSF (Lutz et al., 1999). Macrophages were from the J774 cultured cell line (pictures from D.Hockley and R. Fleck, Cell Biology and Imaging division, NIBSC, UK).

Macrophages (Fig 1.6B) are considered as the main tissue-located phagocytic cells of the innate immune system. For decades, they have been associated exclusively with scavenger activities and non-specific clear up of intruding pathogens from the organism (Witmer-Pack et al., 1993). However, they have an essential role in the transportation and initiation of antigen specific immune responses (Kawai et al., 1987; Komatsubara et al., 1985). Macrophages originate from precursor cells in haemopoietic organs. Pro-monocytes from bone marrow mature and enter the circulation as monocytes, which then develop into tissue macrophages. Macrophages can recognise and ingest many types of extracellular bacteria, thereby destroying the pathogen and presenting pathogen-derived peptides to $CD4^+$ T-cells. An important function of activated T-cells is to act back on the macrophages thereby enhancing their capacity to kill ingested bacteria. Macrophages provide a first line of defence of the innate immune system as they can engulf and digest pathogens without requiring prior exposure (Allison, 1978). Upon phagocytosis, phagocytic cells induce secretion of chemical mediators (cytokines), which leads to inflammation (Langrish et al., 2004). Cytokines act directly on the local blood vessels and activate the dilation and increase of permeability of the blood vessels, leading to an increase in the local blood flow and leakage of fluid accounting for the heat, pain, redness and swelling of the inflammatory reaction.

1-3-2. Adjuvants as immunopotentiators and immunomodulators

Two main mechanisms of immunopotention by adjuvants have been described (Schijns, 2002; Kenney and Edelman, 2003; Alving, 2002; Audibert, 2003; Edelman, 2002; Marciani, 2003; O'Hagan and Valiante, 2003; Singh and Srivastava, 2003). In both these mechanisms, adjuvants act as "danger signals" to alert the immune system of the presence of an infection (Ridge et al., 1996) and to potentiate the response. More specifically, the first mode of action describes the retention of the antigen by the adjuvant at the injection site, thereby delaying the physical elimination of the antigen. This facilitates the uptake of antigen by APCs and thus, improves the transport of the antigen towards the lymphoid organs and/or increases the antigens' accessibility to the endogeneous pathways of antigen presentation. This first type of mechanism is referred to as the "depot effect". The second mode of action relates to the capacity of adjuvants to recruit and activate macrophages and DCs. Indeed, adjuvants can increase cellular infiltration, inflammation and trafficking to the injection site. They can also promote APCs activation by up-regulating MHC molecules and co-stimulatory signal expression, enhance antigen presentation and induce cytokine and chemokine release (Singh and O'Hagan, 1999).

Adjuvants can also modulate the immune response and promote the maturation of either Th1 or Th2 $CD4^+$ helper T-cells (Figure 1.7) (Yip et al., 1999). Adjuvants can further stimulate the production of IL-2, IFN- γ and TNF-β and thereby mediate a cell-mediated immune response (Th1); in this response, macrophages are activated to acquire microbiocidal properties, and cytotoxic T-lymphocytes (CTL) as well as higher levels of IgG2a (in the mouse model) are produced. Alternatively, adjuvants may increase the secretion of IL-4, IL-5 (stimulates B-cells in the mouse model only), IL-6 and IL-10, which are essential for a humoral response (Th2), where B-cells are activated to produce IgG1 (in the mouse model), IgA and IgE antibodies (Fig 1.7) (Mosmann et al., 1986; Romagnani, 1991; Romagnani, 2000).

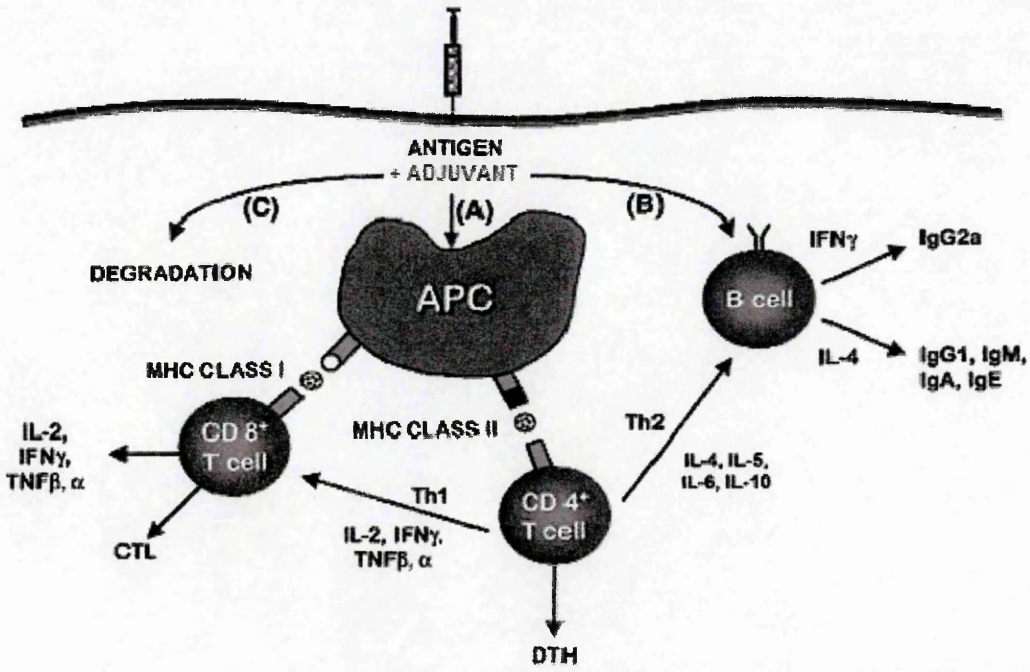


Figure 1.7 Antigen and adjuvant processing following injection. (A) Direct ingestion by APCs; (B) binding to the surface antibodies of B-cells; (C) chemical degradation. (Adapted from Singh et al, (Singh and O'Hagan, 1999))

1-4 Conventional aluminium hydroxide adjuvant: advantages and limitations

Until recently, aluminium-based compounds such as the insoluble aluminium phosphate ($AlPO_4$) and aluminium hydroxide [Al(OOH)] (alum), were the only adjuvants approved for human use (Kenney and Edelman, 2003). The mechanisms of action of alum consist of:

- formation of an antigen depot, which prolongs the interaction between antigen and APCs (Gupta et al., 1995);
- induction of an inflammatory response at the injection site, which attracts immunocompetent cells, and formation of a granuloma, which contains antibody-producing plasma cells (White et al., 1955);
- activation of complement (Ramanathan et al., 1979), which increases the inflammatory response and facilitates memory B-cell development (Klaus and Humphrey, 1977).

However, alum adjuvants have numerous limitations mainly in terms of efficacy, but also safety. The immune response induced in the presence of alum can not be modulated towards either a Th1- or Th2-cell maturation as a function of antigen. Alum preferentially stimulates a Th2-cell response with no or very poor stimulation of Th1-cell mediated immunity (Ramanathan et al., 1979; Lindblad, 2004; Brewer et al., 1999; Brewer et al., 1996). Therefore, alum favours production of IgG1 and IgE by stimulating APCs to release mainly IL-4 rather than IFN-γ (Allison and Byars, 1992). Protein antigens such as DT and TT require adsorption of the antigens to alum to enhance their immunogenicity (Gupta et al., 1993); conversely, adsorption of PRP-TT conjugate to alum alters the immunogenicity of PRP-TT (Claesson et al., 1988). Furthermore, alum does not show any adjuvant effect when used with

typhoid vaccine (Cvjetanovic, 1965) or influenza haemagglutinin antigen (Davenport et al., 1968).

Alum-based vaccines are considered relatively safe as they have already been used for over 70 years in billions of doses for human vaccination. Nonetheless, mild to severe adverse effects such as erythema (Collier, 1980; Goto et al., 1997), extensive swelling reaction (Rennels, 2003), subcutaneous nodules formation (Frost et al., 1985; Netterlid et al., 2004; Bergfors et al., 2003), and granulomatous inflammation at the injection site (White et al., 1955; Erdohazi and Newman, 1971) have been ascribed to alum, whereas its involvement in contact hypersensitivity (Clemmenson and Knudsen, 1979; Bajaj et al., 1997; Bohler-Sommeregger and Lindemayr, 1986) is controversial (Goto et al., 1997). In a more severe cases, the production of IgE can lead to serious allergic reactions (Relyveld et al., 1998) and recently, alum has even been associated with macrophagic myofasciitis (MMF) in patients with diffuse arthromyalgias and fatigue; MMF-lesions were ascribed to the infiltration of muscles by macrophages and lymphocytes, subsequent to intramuscular injection of aluminium hydroxide-containing vaccines (Gherardi et al., 2001). Rimaniol *et al.* showed that macrophages incubated *in vitro* with Al(OOH) contain large and persistent intracellular crystalline inclusions (Fig 1.8) (Rimaniol et al., 2004), which resembled observations in the context of MMF-lesions in patients (Gherardi et al., 2001). Another practical limitation of alum formulation is their inappropriateness for freezing and freeze-drying, as these processes cause the collapse of the alum gel structure, resulting in aggregation and precipitation of the formulation (Gupta et al., 1993). Finally, alum-based vaccine formulations are not sufficiently stable at room temperature and require refrigeration for storage (Galazka, 1989).

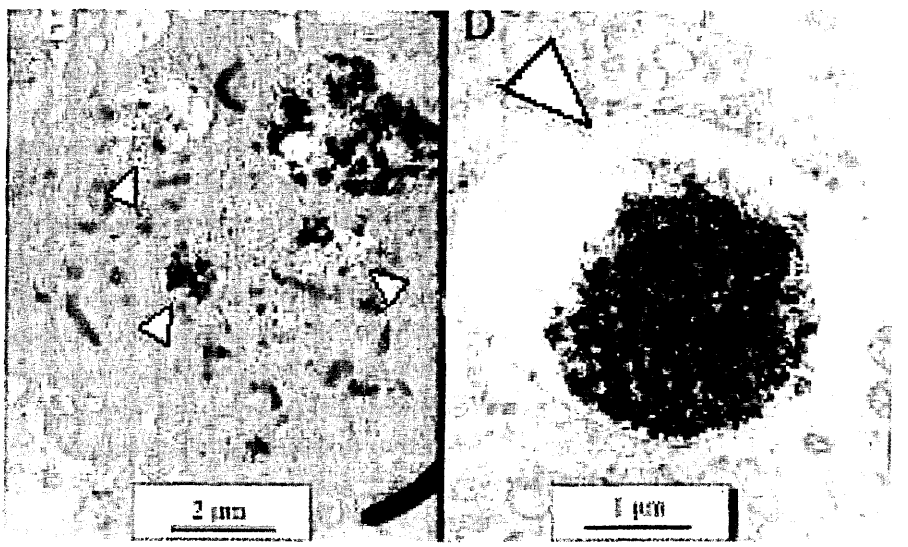


Figure 1.8. Electron micrographs of crystalline inclusions in AlOOH-loaded macrophages associated with macrophagic myofasciitis. (With permission from Rimaniol et al (Rimaniol et al., 2004))

Since aluminium compounds are presently the best known and most widely used adjuvants for human vaccines, they are often employed as golden standard for evaluating new adjuvant formulations, especially if the experimental adjuvant is intended to improve a current alum-adjuvanted vaccine. A commercially available aluminium hydroxide (Alhydrogel®, Superfos Biosector, Vedbaek, Denmark) was recommended as a standard for evaluating new adjuvant formulations (Stewart-Tull, 1989). Alhydrogel® is composed of $Al(OH)_3$ particles of fribrillar shape and a size range of 0.5-10 nm. It has a very large capacity to bind proteins and can adsorb 50-200 times its own mass.

Improving the safety and effectiveness of adjuvants and delivery systems remains an important challenge for vaccinologists and pharmaceutical formulations scientists (Plotkin, 2001; Gupta and Relyveld, 1991). Further development in novel adjuvants and delivery systems will likely be driven by a better understanding of the mechanism of action of currently available formulations, an area which requires much additional work as the mode of action of adjuvants is still poorly understood (Schijns, 2000; Singh and O'Hagan, 1999).

1-5 The potential of microspheres for antigen delivery

The drop-out rate for those receiving the doses of DTP vaccine required for full protection is significantly high in many parts of the world. In countries with good immunisation coverage for diphtheria, this disease has virtually been eradicated. The outbreak of severe diphtheria in Sweden from 1979-83 and, more recently, the re-emergence of epidemic diphtheria in the new independent states of the former Soviet Union has alerted the health authorities in developed countries. Bacteriological and serological surveys showed that all patients with neurological complications or those who died had sub-protective levels of antitoxin, i.e., < 0.01 IU/ml (WHO, 1996). Immunity in adults wanes low when general vaccination is applied only to children or when natural encounter with the microbial source is non-existent (WHO, 1999a). Many humanitarian organisations, such as the WHO, have promoted the development of new vaccines that can be used early in life, require fewer injections or can be administered through easier routes of administration (e.g., nasal, transdermal). One strategy was devoted to biodegradable microspheres (MS) made of poly(lactide-co-glycolide) (PLGA) varying in molecular mass and composition, i.e., 50:50 up to 100:0 of lactide:glycolide (Gupta et al., 1998c; Johansen et al., 2000a; Johansen et al., 2000c; Aguado, 1993; Hedley et al., 1998; Maloy et al., 1994; Men et al., 1997; Peyre et al., 2003; Walter et al., 2001). The rationale for investigating in some detail PLGA MS as a platform technology for antigen delivery was manifold. PLGA MS should warrant a high degree of vaccine safety, as this polymer class has been used for over 15 years in humans as suture material and as controlled release systems for peptide and protein drugs.

The biodegradability and antigen release properties of PLGA MS generally afford a controlled steady or pulsatile release of microencapsulated material. For antigens, these controlled release systems are expected to mimic the first one or two booster injections typically required to confer protection with alum-adsorbed vaccines (Gupta et al., 1998c). PLGA MS possess inherent adjuvant activity, which must be related to the fact that appropriately sized PLGA MS can be recognized and ingested readily by APCs (Prior et al., 2002; Thiele et al., 2002; Thiele et al., 2003; Lutsiak et al., 2002; Horisawa et al., 2002). Ingested PLGA MS release the antigen inside the cell for presentation on MHC molecules (Waeckerle-Men et al., 2004; Audran et al., 2003). Non-ingested MS will remain at the injection site and form a depot for prolonged antigen release. Phagocytosis of PLGA MS and depot formation at the injection site are considered to contribute primarily to the enhancement of the immune reponse (Djaldetti et al., 2002; Sinha and Trehan, 2003; Diwan et al., 2001). Previous studies have demonstrated the capacity of PLGA MS to act as efficient vaccine adjuvant; for example the immune response against DT was greatly enhanced and even afforded protection against direct challenge with the Dtx (Peyre et al., 2003). Other studies have demonstrated the adjuvant effect of PLGA MS for cell-mediated immunity (Maloy et al., 1994), including the induction of cytotoxic T cell response (Hedley et al., 1998; Men et al., 1997). Conversely, PLGA MS alone can probably not activate APCs in terms of increasing the synthesis of MHC molecules or enhancing cytokine release.

1-6 *In vitro* and *in vivo* uptake of MS vaccine by antigen presenting cells

In vitro, PLGA MS are efficiently phagocytosed by cultivated macrophages and DCs (Figure 1.9.). Ingested MS then degrade inside the APCs and release entrapped components (Walter et al., 2001; Tabata and Ikada, 1988b).

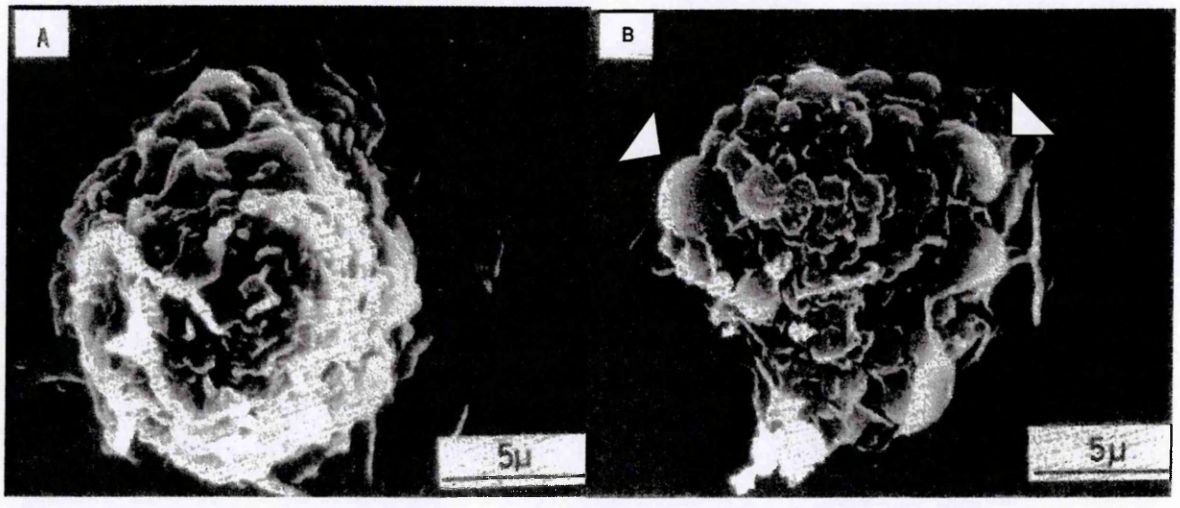


Figure. 1.9. Scanning electron micrographs of a macrophage before (A) and after (B) phagocytosis of PLGA MS (white arrows) (With permission from Tabata et al (Tabata and Ikada, 1988b).

In vivo, PLGA MS are also readily ingested by macrophages and DCs, although the kinetics and extent of *in vivo* phagocytosis depends on the immunisation route (Newman et al., 2002). Little is known about the *in vivo* fate of subcutaneously (s.c.) injected PLGA MS. Previous studies have monitored the antigen release from s.c.-injected PLGA MS without considering the fate of the particles themselves (Eyles et al., 1999; Gupta et al., 1996).

On the other hand, encapsulated fluorescent compounds have been used to follow spermin-alginate MS after esophageal intubation in mice (Lomotan et al., 1997) and PLGA MS after administration into the ileum (Damge et al., 1996) or nose (Eyles et al., 2001) of mice. The spermine-alginate MS were detected primarily in the gut-associated DCs. Nasally administered PLGA MS were found in nasal associated lymphoid tissues (NALT), lung, mesenteric lymph nodes (MLN), in spleen (S) and liver (Eyles et al., 2001). PLGA MS administered by oesophageal intubation were found in the intestinal epithelium and Peyers's patches (Damge et al., 1996; Eyles et al., 2001). In general, the encapsulation of protein antigens into PLGA MS significantly increases the immune response induced against the antigen, at a level comparable to that observed with the conventional adjuvant aluminium hydroxide (Johansen et al., 1999). The mechanisms involved in the adjuvant activity of PLGA and other polymeric MS are not yet well understood. This is not surprising considering that even the adjuvant mechanisms of action of alum lack full understanding (HogenEsch, 2002). A better knowledge of the phagocytic process of PLGA MS following s.c.-administration appears essential, as antigen presentation plays a determinant role in the induction of specific immune responses. An in-depth understanding of the adjuvant properties of particulate slow-release vaccines may provide further information that would be valuable for a better understanding of the mechanisms of adjuvantation.

1-7 Characteristics of MS vaccines: influence of formulation and process parameters

Over the past twenty years, many different types of MS formulations have been investigated, which varied in polymer type (PLGA with lactic/glycolic ratios varying between 50:50 and 100:0), polymer molecular weight (2kDa to 128kDa), and fabrication technique (solvent evaporation (SE), spray drying (SD), coacervation (CO)) (Tamber et al., 2005; Kissel et al., 1997a). All these variables influenced the MS characteristics such as particle morphology, release kinetics of encapsulated compounds, and the immunogenicity of the formulation. However, for the immunogenicity, no definite general trend or correlation with physical chemical or pharmaceutical particle characteristics is deducible from all the past studies.

Antigen integrity inside MS is probably critical for the immunogenicity of many antigens (Chang and Gupta, 1996; Johansen et al., 1999; Johansen et al., 1998b; Johansen et al., 1998a). Antigen stability during release and MS degradation is of particular concern as the generation of acidic polymer degradation products lowers the pH inside the MS (Lu and Park, 1995; Park et al., 1995). Exposure to low pH may induce chemical and/or physical alterations of the antigen. If specific epitope domains are affected, the antigenicity and immunogenicity of the antigen will be changed. Although evidence has accumulated that environmental stress, such as low pH or exposure to a W/O-interface (e.g., during antigen encapsulation), can lower substantially the ELISA-antigenicity of an antigen (Johansen et al., 1998b; Sasiak et al., 2000; Jiang et al., 2005; Jiang et al., 2005; Xing et al., 1996a), the effect of such alterations on the immunogenicity of microencapsulated antigens has not yet been demonstrated unambiguously. For illustration, microencapsulated TT underwent conformational changes and lost antigenicity upon incubation at 37 °C over 28 days, but this did not affect the

formation and persistance of protective antibody levels in mice (Sasiak et al., 2000). Similar discrepancies between *in vitro* and *in vivo* reactivity of microencapsulated TT was reported by others (Johansen et al., 2000a; Kersten et al., 1996).

An early motivation for using PLGA MS for DT and TT delivery lay in the hypothesis that one or two booster injections of conventional diphtheria-tetanus vaccines may be mimicked by a pulsatile antigen release from PLGA MS. Such release kinetics was indeed obtained by single PLGA type MS and mixtures of low and high molecular weight PLGA MS, as peptide and protein release generally depend on the polymer degradation rate (Men et al., 1995; Men et al., 1996; Partidos et al., 1994; Singh et al., 1998b; Cohen et al., 1991; O'Hagan et al., 1993a; Singh et al., 1997a; Thomasin et al., 1996). This hypothesis was supported by data showing that a single injection of DT in a single polymer type MS induced a lower antibody response than did three injections of the alum adsorbed DT, whereas a single injection of DT in a MS mixture yielded a similar response to the latter (Singh et al., 1998b). Similarly, (Men et al., 1995; Men et al., 1996) mixtures of two fast degrading small-sized MS types with a slowly degrading large-sized MS type produced a stronger T-cell response up to 45 weeks than the respective single MS types. However, the small-sized fast degrading polymer type MS yielded similar kinetics of Ab titer development as the MS mixture and the three injections of alum adsorbates; incidentally, no typical booster effect could be observed *in vivo* in the animals immunized with the MS formulations. In other studies, however, single polymer type MS induced similar immune responses as did mixtures of MS types (Johansen et al., 2000a).

Obviously, factors others than the antigen release kinetics must play an important role in the kinetics of immune response development; amongst these factors, the MS size, antigen content, MS hydrophobicity and administered dose should be mentioned.

The most commonly used size range of MS for antigen delivery lies between 0.3 and 100 μm (Thomasin et al., 1996; O'Hagan et al., 1993b). Small-sized (< 10 μm) MS tend to induce a stronger primary response than larger MS (O'Hagan et al., 1993a). Particles of below 10 μm are readily taken up by macrophages and DCs, whereas larger particles should be particularly useful for prolonged supply of antigen at the administration site. Under *in vivo* conditions, however, small-sized particles were shown to induce higher antibody titres only in the early response with the larger MS catching up after few months (Thomasin et al., 1996); further, the duration of the response did not appear to depend on the MS size.

Vaccine dose and antigen content were also of interest in past investigations. Variation of the administered dose of microencapsulated TT (0.2-20 Lf) (Kersten et al., 1996; Gupta et al., 1997) and DT (2.5-75 Lf) (Johansen et al., 1999; Singh et al., 1992) did not affect consistently the antibody response in mice and guinea pigs. However, one needs to be aware that the antigen dose could modulate the type of response induced as Th1 clones are more susceptible to apoptosis than Th2 clones at high dose of Ag (Constant and Bottomly, 1997). In a dose-escalating study, Gupta *et al.* determined that 1/10 of a single human dose (SHD) of microencapsulated antigen (equivalent to 5 Lf DT and 2 Lf TT) was adequate for eliciting an immune response similar to that observed with two injections of alum-adsorbed vaccines at equivalent dose (Gupta et al., 1998b).

Although particle hydrophobicity appeared to affect the extent of particle phagocytosis *in vitro* and *in vivo* (Tabata and Ikada, 1988b; Thiele et al., 2003; Constant and Bottomly, 1997; Jones et al., 2002; O'Hagan et al., 1993b; Gupta et al., 1998b; Tabata and Ikada, 1994), its effect on MS vaccine immunogenicity is less obvious from data in the literature. In some studies, the particle hydrophobicity was a key factor in the enhancement of the humoral response (Alpar and Almeida, 1994; Conway et al., 1997; Raghuvanshi et al., 2002). It was linked to an avid uptake of hydrophobic particles by MALT when administered intranasally, inducing a stronger and long lasting immune response than free Ag (Almeida A.J et al., 1993; Alpar and Almeida, 1994).

1-8 Co-administration of MS vaccine with alum

PLGA MS-based DT and TT vaccines have been proven to stimulate efficiently a primary response after a single injection and to boost the response upon a second injection. Some of the investigators speculated that a single injection of PLGA MS might be sufficient to replace two injections of alum-adsorbed vaccines (Desai et al., 2000; Gupta et al., 1998a; Men et al., 1995; O'Hagan et al., 1991; Singh et al., 1992; Singh et al., 1991; Singh et al., 1995; Singh et al., 1997c; Stivaktakis et al., 2004; Tobio et al., 1999). Other reports doubted that a single injection of PLGA MS vaccines alone is sufficient to induce and sustain protective levels of antibodies (Walker et al., 1997; Singh et al., 1998b; Johansen et al., 2000a). Thus, combinations of MS vaccines with other adjuvants such as alum were explored. Co-administration of microencapsulated TT and alum substantially enhanced the antibody response (Johansen et al., 2000a).

Part of the motivation behind developing new antigen delivery systems arose from the desire to replace aluminium compounds, which have until recently been the only adjuvants approved for human use. By helping the immune system in inducing a strong inflammatory response, alum sometimes also induces severe local reactions (Gupta et al., 1995). Reducing or replacing the dose of alum in vaccines might help to prevent such adverse effects. Moreover, alum is important for the early response, but not for long-term immunity (Singh et al., 1998b). Alum primes the immune system for antibody production better than microparticles alone (Singh et al., 1998b; Diwan et al., 1998; Shi et al., 2002). Further, alum drives the response towards a Th2 type, whereas PLGA MS appears to mediate a mixed Th1/Th2-reponse (Singh et al., 1998a; Jilek et al., 2004; Carcaboso et al., 2004; Conway et al., 2001). Thus, the potential usefulness of mixing alum and PLGA MS will be a function of the type of response required.

1-9 Multivalent PLGA MS vaccines

When developing new types of vaccine delivery systems, the feasibility of multivalent vaccines must also be considered. An improvement in the immunisation schedule would then lie in the administration of combined vaccine by a single or maximum two injections (WHO, 1995). There would indeed be little future for single injection monovalent vaccines of DT, TT, PT, and Hib (Aristegui et al., 2003b). In combination vaccines, the immunogenicity of particular components may be altered with respect to the individually administered antigen: interference with other antigens would result in impaired immunogenicity, whereas synergistic effects would enhance the immunogenicity. For some components, combination

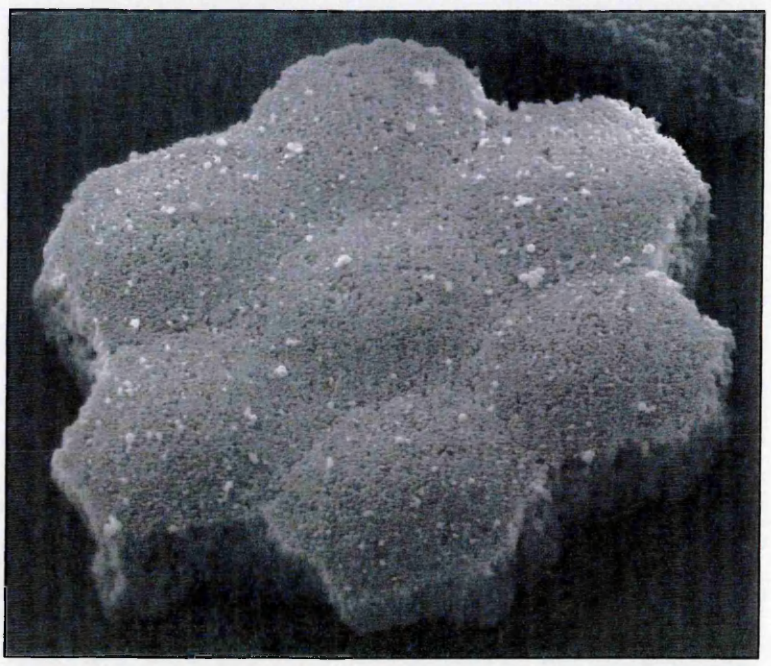
with other more immunogenic antigens may even be essential to raise protection (Vidor et al., 1999).

Developing a multivalent PLGA MS vaccine may indeed improve vaccination coverage by reducing the number of vaccination sessions required to generate immunity. Microspheres loaded with TT or DT have demonstrated strong immune stimulation with long lasting antibodies in mice and guinea pigs after a single inoculation (Johansen et al., 1999). Furthermore, co-immunisation of individually encapsulated DT and TT has elicited a strong neutralising antibody (nAb) response and a good protection efficacy against challenge with the corresponding toxins (Peyre et al., 2003). Conversely, microencapsulated Hib-conjugate antigen in monovalent MS induced higher anti-Hib Ab response in rats than a tetravalent MS vaccine containing the Hib antigen together with DT, TT and PT (F.Mawas personal communication NIBSC, unpublished data). However, it remains to be determined whether these findings are due to the lower content of the Hib-conjugate in the tetravalent PLGA MS as compared to the monovalent MS. Co-encapsulation of DT and TT has also proven efficient in inducing high total and neutralising Abs (Singh et al., 1998b; Singh et al., 1997b). However, the protection efficacy of the vaccine has not yet been confirmed. As with any multivalent vaccine formulations, it is important to compare the co-encapsulated antigens in a single PLGA MS type with the simple physical mixture of monovalent formulations.

1-10 Final remarks

The design of PLGA MS in terms of polymer type and microsphere size is critical. The MS properties have to be finely tuned so that a good balance is reached between efficient particle phagocytosis and immunogenicity on the one side, and well-controlled induction of inflammation or other adverse reactions on the other side. Probably one of the main limitations in the study of PLGA MS and other particulate antigen delivery systems is the incomplete understanding of the mechanism of action of adjuvant. Moreover, the feasibility of multivalency of PLGA MS vaccines needs to be proven before further progress in clinical trials can be achieved.

MATERIALS AND METHODS



CHAPTER 2: MATERIALS AND METHODS

2-1 Materials

2-1-1. Antigens and control vaccine

The antigens used in this study encompassed tetanus toxoid (TT) (1400 Lf/ml ; 2mg/ml Massachusetts Public Health Biologic Laboratory, Boston MA), diphtheria toxoid (DT) (6500 Lf/ml, 14.5 mg/ml ; Aventis Pasteur, Lyon, France), genetically attenuated pertussis toxin (PT) (Chiron Vaccines, Siena, Italy) and PRP antigen from *Haemophilus influenzae* type b (Hib) conjugated to tetanus toxoid (Berna Biotech, Berne, Switzerland) (PRP-TT). PfCS is a synthetic peptide representing the C-terminal region (amino acids 282-383) of the circumsporozoite protein of the *P.falciparum* strain NF54 (kindly provided by G. Corradin, University of Lausanne, Switzerland). The peptide was chemically synthetised by solid-phase Fmoc chemistry as described (Lopez et al, Eur. J. Immunol 31 (2001); Roggero et al, Parassitologia 41 (1999)).

Commercial alum-adsorbed DT-TT vaccine (50 Lf/ml of DT and 20 Lf/ml of TT) was provided by Aventis Pasteur, Lyon, France, and will henceforth be referred to as alum control. Commercial alum- adsorbed DT-TT-aP vaccine was provided by Aventis Connaught, Toronto, Canada. Hib-conjugate antigen (PRP-TT), in powder form, was provided by Aventis Pasteur, Lyon, France. The mixture of alum- adsorbed DT-TT-aP and Hib-conjugate powder will henceforth be referred to as DT-TT-aP-Hib-alum. For both the DT-TT and DT-TT-aP alum vaccines, the estimated lower fiducial limit of potency stated by the manufacturer was >30 IU/standard human dose (SHD) for DT and >40 IU/SHD for TT, which meets the Ph. Eur.

specifications (European Pharmacopoeia, 2005b; European Pharmacopoeia, 2005a). The alum-adjuvant was identical for the commercial control vaccines and the experimental test formulations (Alhydrogel®, 2% $Al(OH)_3$, Superfos, DK-Vedbaek), and will henceforth be referred to as alum.

2-1-2. Reference reagents (antitoxins, toxoids and toxins)

Guinea-pig anti-DT and anti-TT antitoxin standards, NIBSC code 98/572 with 3.0 and 3.5 IU/ampoule for DT and TT, respectively, calibrated *in vivo* against the corresponding international standards (equine anti-DT, code 97/762 and human anti-TT, code TE-3). Mouse anti-DT and anti-TT antitoxin NIBSC standard, made of pooled sera from mice immunised with a DT-TT-aP-alum vaccine (Biken, Japan). The guinea-pig antitoxin reference will henceforth be referred to as "98/572" and the mouse reference as "Biken".

The ELISA coating antigens were: DT (1650 Lf/ml, 7.4 mg/ml ; code BJGL 1004/F) and TT (2560 Lf/ml, 17.8 mg/ml ; code MWC S208/A/F-6) from Wellcome Foundation Limited (Kent, UK) ; PT from Glaxo Smithkline (lot PAC057, Belgium) ; HbO-HA from Wyeth Lederle (Pearl River, NY, USA).

The Dtx was from Aventis Pasteur (Lyon, France) (code FA016723, 400Lf/ml) and the Ttx from Wellcome Foundation Limited (Kent, UK) (code AWX 4664, containing between $1x10^6$ to $16x10^6$ mouse paralytic doses per vial).

2-1-3. Polymers and other reagents

The biodegradable polymers used for encapsulating the antigens were end-group capped 23 kDa poly(D,L-lactide) (PLA; Resomer® R203), end-group capped and uncapped 35 kDa poly(lactide-co-glycolide) 50:50 (PLGA50:50 and PLGA50:50H; Resomer® RG503 and RG503H), and end-group uncapped 14 kDa PLGA50:50H (Resomer® RG502H) all from Boehringer-Ingelheim, D-Ingelheim. Bovine serum albumin (5%) (Biochemica, for enzyme immuno assay, >98% Fluka, CH-Buchs), D(+)-trehalose dihydrate (5%) (Fluka, CH-Buchs), or succinylated gelatine (4%) (Physiogel®, Braun Medical, D-Melsungen) were co-encapsulated as antigen stabiliser (Table 2.1).

Table 2.1. Polymers used for microencapsulation of tetanus and diphtheria toxoids by spray-drying (SD) and coacervation (CO) methods.

Polymer[a]	Code	Mw (kDa)
PLGA 50:50	503	35
	503H[b]	35
	502H[b]	14
PLA	203	23
PLGA 50:50	503H[b]	35
	502H[b]	14
PLA	203	23

[a] *The polymer brand names were Resomer® RG503, 503H or 502H and Resomer® RG 203, from Boehringer Ingelheim;*

[b] *H-types: PLGA with open carboxylic end-groups*

Unless stated otherwise, all other reagents were purchased from Sigma, Poole, UK and were of analytical grade.

2-2 Microsphere preparation and antigen microencapsulation

Antigens were microencapsulated individually or in combinations into various PLA/PLGA types by spray-drying or coacervation, as described below. BSA (5%, w/w, relative to polymer mass), trehalose (5%), or succinylated gelatine (4%) were co-encapsulated as antigen stabilisers, as specified below. To produce fluorescent MS (F-MS), fluorescein and rhodamine were co-encapsulated by dissolving the dyes either in the aqueous antigen solution or organic polymer solution for further processing.

2-2-1. Spray-drying

Volumes of 2.0 - 2.5 ml of aqueous antigen solution (0.5 - 9 Lf/ml), with or without co-dissolved stabilizer (4-5%), were mixed with 20 ml of polymer solution in dichloromethane (DCM) (5% w/v). The obtained water-in-oil (W/O) dispersion was homogenised on ice by ultrasonication (2×10 s, 20 kHz, 50 W) and spray-dried with a Büchi Mini Spray-Dryer 191 (Flawil, Switzerland) at a rate of 2 ml/min and an inlet temperature of 38 – 39 °C (Figure 2.1). The collected MS were washed with aqueous poloxamer 188 (0.1%) (ICI, Middlesborough, UK), passed through a 50 µm mesh sieve, collected on a 0.8 µm cellulose acetate filter (Schleicher & Schuell, Dassel, Germany), washed with heptane, and dried at room temperature and 50 mbar for 12 h before storage at 4 °C until further use.

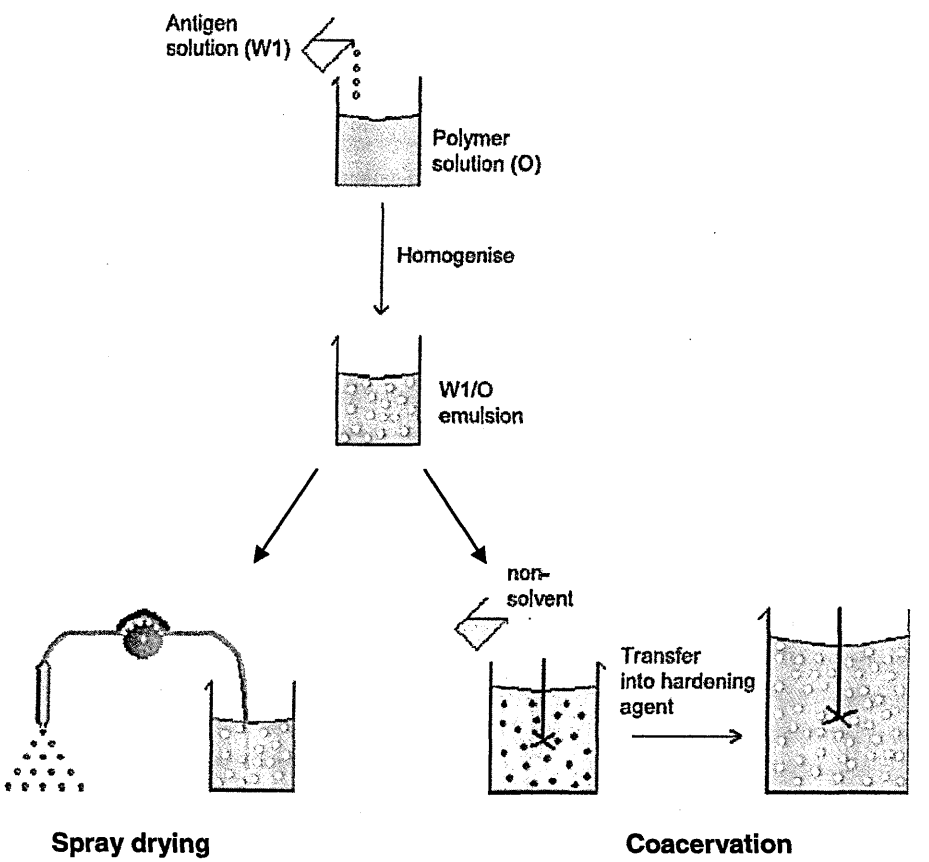


Figure 2.1. Conventional microencapsulation methods. An aqueous antigen solution is dispersed into an organic polymer solution by ultrasonication (W_1/O emulsion). The W_1/O emulsion is processed further by the specific methods to prepare antigen containing MS: Spray-drying or Coacervation. In the final stages before drying and storage, the MS are collected and washed to remove nonencapsulated antigen. Adapted with permission from Dr. H. Tamber (Tamber et al., 2005).

2-2-2. Coacervation technique

Volumes of 2.0 - 2.5 ml of aqueous antigen solution (1.6 - 2 Lf/ml), with or without co-dissolved stabilizer (4-5%), were mixed with 20 ml of polymer solution in dichloromethane (DCM) (5% w/v). The obtained water-in-oil (W/O) dispersion was homogenised on ice by ultrasonication (2×10 s, 20 kHz, 50 W). This dispersion was introduced into a jacketed-vessel (250 ml) equipped with baffles and an anchor stirrer. Coacervation was induced by introducing a pre-determined amount of silicone oil (DC-200, PDMS, viscosity grade of 1070 mPa s) at a rate of 4 g min^{-1} to obtain stable coacervate droplets according to established phase diagrams (Thomasin et al., 1998). Stirring was set at 1000 min^{-1} and the temperature maintained at 10-15 °C. The coacervation dispersion was slowly transferred into 1200 ml of hardening agent (octamethylcyclotetrasiloxane (Scheller AG, Zürich, Switzerland)) to solidify the microparticles (Figure 2.1). Stirring was continued for 30 min, after which the MS were collected on a sintered glass filter and washed with 100 ml of hexane. The MS were air-dried for 5 min and re-suspended three times in water containing 0.1 % poloxamer F 68. After washing, the powder was dried for 12 h under laminar air-flow followed by vacuum drying at 0.5 mbar.

2-3 Microsphere characterisation

2-3-1. Particle size measurement by laser light scattering

Particle size distributions were determined directly after drying of the MS. The particles were re-dispersed in 0.5% (w/w) polysorbat 20 solution and subsequently treated with ultrasound for 5s (6 mm probe, 20 kHz, 50 W; VibraCell VC50T, Sonics & Materials, Danbury, USA). MS size was determined by laser light scattering (Mastersizer X, Malvern, Worcestershire, UK, equipped with a 100 mm lens) using a Fraunhofer diffraction model for the analysis of the raw data. All size distributions are presented in the volume-weighted mode with the mean diameter being calculated from the volume-moment average of the size distribution.

2-3-2. Particle morphology and ultra-structure analysis using scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

The dry MS powder or MS reconstituted in alum or lecithine solutions (1%; w/v) were scattered on to 12 mm diameter double-sided adhesive carbon pads attached to SEM mounts. The samples were sputter-coated with a layer of gold/palladium (5 nm thick) before examination with a Philips SEM501B electron microscope operating at 15 or 30 kV.

For TEM, tissues samples were fixed in 2.5% gluteraldehyde (R1011, Agar, UK) in 0.1 M cacodylate buffer of pH 7.2 at room temperature for 30 min and then at 4 °C for at least 24 h. Fixed samples were embedded, as a pellet, in 1% low melting temperature agarose (A-4018, Sigma, UK), and the agarose blocks were briefly fixed in gluteraldehyde and washed in

buffer. Blocks were processed in a Lynx EM Tissue Processor (Leica, Germany) and treated with 1% osmium tetroxide (166010, Johnson Matthey) in cacodylate buffer for 2 h and with 0.5% uranyl acetate for a further 3 h. After dehydration in ethanol, blocks were embedded via propylene oxide in Spurr resin (S024, TAAB, UK). Sections were cut with a Reichert-Jung Ultracut E ultramicrotome, post-stained with uranyl acetate and lead citrate and examined with a Philips CM12 electron microscope operating at 80 kV.

2-3-3. Determination of antigen content in the MS by ELISA

The antigens were extracted from the MS using a filtration method: 20 mg of dried MS were dissolved in 8 ml of DCM, the non-dissolved antigens were separated from the dissolved polymer by filtration over a 0.2 µm- regenerated cellulose filter (RC, Schleicher & Schuell, Dassel, Germany). The filters were washed twice with additional 4 ml of DCM, and the toxoid was eluted into 2 ml of PBS by mixing and soaking for 2 hours.

The amounts of extracted DT, TT and PT were determined by capture enzyme-linked immunosorbent assay (ELISA) as described previously (Xing et al., 1996a). Briefly, 96 wells plates were coated overnight with an in–house anti-DT, -TT or-PT monoclonal antibodies and the test samples and reference toxoids (described under 2.1.) were incubated at 37 °C for 2 h. Anti-diphtheria or anti-tetanus IgG, raised in guinea-pigs, or standard sheep anti-pertussis toxoid serum (NIBSC code 97/572) were used to detect to the toxoids upon incubation at 37 °C for 2 h. A peroxidase conjugated anti-guinea-pig antibody (SIGMA, Poole, UK) and an alkaline phosphatase conjugated donkey anti-sheep IgG (The Binding Site, Birmingham, UK) were used as secondary antibodies. The substrate solutions containing 0.5 mg/ml of ABTS and

0.04% H_2O_2 (BDH, Poole, UK) in 0.05 M citric acid of pH 4 for DT and TT, and 1 mg/ml of p-nitrophenyl phosphate (Sigma) in 10% (w/v) diethanol-amine buffer of pH 9.6 for pertussis toxoid were added and left at room temperature for 30 – 40 min for colour development. The absorbance was read at 405 nm in a plate reader (Anthos Labtec Ins, model 2001). The extracted toxoids were compared to the reference solutions using a parallel line assay. The results were expressed in Lf content per milligram of MS. All samples were assayed in duplicate.

Encapsulated PRP and PfCS content were assessed by Dr. Pal Johansen, ETH, Zurich, Switzerland and the techniques are described below:

Encapsulated PRP content, after extraction from the MS, was assayed fluorimetrically using excitation and emission wavelengths of 280 and 320 nm, respectively. In the presence of the other antigens, ribosyl content of Hib was quantified with the orcinol reaction, as proposed by Bial (Ashwell et al., 1957). Briefly, 0.6 ml of 0.1% $FeCl_3$ in concentrated hydrochloric acid and 60 µl of 100 mg/ml orcinol in 95% ethanol were added to 0.6 ml of Hib solution. The samples were heated at 95 °C for 40 min and cooled to room temperature before assaying photometrically at 670 nm.

PfCS content was measured using a dot-blot technique. PfCS extracted from the MS was adsorbed on nitrocellulose and captured by antibodies from hyper-immune mice serum. Blots were visualised using a goat anti-mouse antibody conjugated to alkaline phosphatase and the substrate p-nitrophenyl phosphate. The membrane was scanned, and the PfCS concentrations analysed against known amounts of PfCS (r^2=0.985).

2-3-4. In vitro antigen release from MS

For each DT-MS or TT-MS formulation, 3 samples of 30 mg each were accurately weighed into 3.00 ml of PBS (67 mM, pH 7.4) containing 0.02% (w/v) sodium azide with 5% skimmed milk (Marvel, Premier Brands, Dublin, Ireland). The MS were dispersed by vortexing (Jencons) and ultrasonication in a bath for 10 seconds and incubated at 37 °C under gentle rotational movement (3 rpm). At predefined time points, the vials were centrifuged at 3700 rpm for 5 min, the supernatants were collected and replaced by fresh 5% (w/v) skimmed milk in PBS. The supernatants were assayed by capture ELISA as described previously for diphtheria and tetanus toxoid (Xing et al., 1996a). Briefly, flat-bottom 96-well plates were coated with anti-diphtheria (DT05) or anti-tetanus (TT010) monoclonal antibodies in 0.05 M carbonate buffer of pH 9.6 by overnight incubation at 4 °C. After 1 hour of blocking with 5% (w/v) skimmed milk in PBS at 37 °C, the plates were incubated at 37 °C for a further 2 h with serial dilutions of test samples and reference DT or TT (Welcome, Beckenham, UK) (detection range: 0.002-0.5 Lf/ml). Anti-diphtheria or anti-tetanus polyclonal antibodies, raised in guinea-pigs, were used to detect the captured toxoids at 37°C for 2 h. A peroxidase conjugated anti-guinea-pig antibody (SIGMA, Poole, UK) was used as secondary antibody. A substrate solution of pH 4, containing 0.5 mg/ml of ABTS and 0.04% H_2O_2 in 0.05 M citric acid (BDH, Poole, UK) was added and left at room temperature for 30 min for colour development. The absorbance was read at 405 nm in a plate reader (Anthos Labtec Ins, model 2001). The toxoid content in the samples was assessed against the reference using the parallel line assay and the final results expressed in Lf/mg of MS. All samples were assayed in duplicate.

2-4 General methods

2-4-1. Animals

Female, 6-8 weeks old mice strains BALB/c or NIH were purchased from Harlan (Oxon, UK) or Charles River (Kent, UK), female or male, 6-8 weeks old Dunkin Hartley guinea-pigs (250-300 g) were purchased from Harlan (Oxon, UK). Animals were kept and monitored by NIBSC scientific staff (Biological Services Division), received food and water *ad libitum*, and were allowed to settle at NIBSC for 1 week prior to immunisation.

All *in vivo* experiments were performed according to the UK Home Office regulations and were approved by the local ethics committee.

2-4-2. Immunisation procedures

Immediately before immunisation, individual or mixed MS formulations were dispersed in a lecithin solution (1-5% w/v of L-α-phosphatidylcholine in sterile water) or in a 0.02% alum (Alhydrogel®, 2% $Al(OH)_3$, Superfos, DK-Vedbaek) in sterile saline composed of 140 mM NaCl, 5 mM KCl and 1% glucose. To obtain homogeneous MS dispersions, the reconstituted MS were mixed by vortexing for at least 1 min and ultrasonicated in a bath (50-60Hz, Sonicor Instrument Corporation, Copiague, NY) for a maximum of 10 seconds.

The immunisation protocols are summarised in table 2.2 and described below:

Table 2.2.(1) Influence of formulation parameters (antigen type, polymer type, particle size) on PLGA MS immune response: groups of 5 mice (Balb/c) were immunised s.c. with 1 Lf DT (equivalent to 1/25 of SHD) in MS reconstituted in 0.5 ml of 1% lecithin in distilled water; 5 naive animals were used as control. Animals were terminally bled 4 weeks later by cardiac puncture under anaesthesia and their spleen collected. The serum was prepared from clotted blood and stored at –20 °C before analysis of the anti-DT antibodies by ELISA. The spleen cells were processed for lymphocyte proliferation and cytokine production assays.

Table 2.2.(2A & 2B) Influence of alum on PLGA MS immunogenicity: the effect of admixing alum to DT- and TT-loaded PLGA MS on the cellular responses was studied in mice and on the protective responses against tetanus toxin and diphtheria toxin challenges in mice and guinea –pigs, respectively. Groups of 5 mice or 8 guinea pigs were immunised s.c. with 2.8 mg of divalent DT-TT-MS containing 2.5 Lf DT and 1 Lf TT; the MS were freshly dispersed in 0.5 ml of differently concentrated alum solutions (equivalent to 0.35, 0.035 and 0.0035 mg Al^{3+}) or in 1% lecithin (in aqueous solution). One group of mice and one group of guinea pigs were also immunised with 2 injections of 1/20 of a SHD of DT-TT alum-adsorbed control vaccine in saline (equivalent to 0.06 mg Al^{3+}) at a 4 weeks interval. Mice and guinea-pigs were test bled at various time points between 2 and 44 weeks post-immunisation by tail bleeding and cardiac puncture under anaesthesia. The mice were challenged 3 days after the last bleed with tetanus toxin (as described under 2.4.6); some groups of mice were killed at the same time points, and their spleen cells collected for lymphocyte proliferation and cytokine production assays. The guinea pigs were challenged 5 days after the last bleed with diphtheria toxin (as described under 2.4.6).

Table 2.2. Immunisation protocols

Protocol title	Chapter	Animals (n=)	Vaccines	Antigen	Immunisation dose (SHD equivalent)	Reconstitution buffer	Time	Assays
(1) Influence of formulation parameters	4.3	Mice (5)	DT-MS	DT	1 Lf (1/25 SHD)	1% lecithin	4 weeks	Total Ab T-cell prolif.* Cytokines
(2A) Influence of Alum on PLGA MS immunogenicity	4.4	Mice (5)	DT-TT-MS or Alum control x2	DT TT	2.5 Lf 1 Lf (1/10 SHD)	Alum (0.35, 0.035, 0.0035mg Al^{3+}) Or 1% lecithine Alum (0.06mg Al^{3+})	2-24 weeks	Total Ab T-cell prolif.* Cytokines Protection Ttx
(2B)		Guinea-pigs (8)	DT-TT-MS or Alum control x2	DT TT	2.5 L f 1 Lf (1/10 SHD)	Alum (0.35, 0.035, 0.0035mg Al^{3+}) Or 1% lecithine Alum (0.06mg Al^{3+})	6-44 weeks	Total Ab Protection Dtx
(3) Immunogenicity of PLGA MS versus alum control	4.5	Mice (3)	DT-MS + TT-MS or Alum control x1	DT TT	2.5-0.4 Lf 1-0.16 Lf (1/10- 1/62.5 SHD)	1% lecithin Alum	2-6 weeks	Total Ab T-cell prolif.* Cytokines
(4) PLGA MS immunogenicity after single dose immunisation	4.6	Guinea-pigs (8)	DT-MS + TT-MS or Alum control x2	DT TT	6.25-0.5 Lf 2.5-0.2 Lf (1/4-1/50 SHD)	1% lecithin Alum	4-52 weeks	Total Ab nAb Protection Dtx

*T-cell proliferation assay

Protocol title	Chapter	Animals (n=)	Vaccines	Antigen	Immunisation dose (SHD equivalent)	Reconstitution buffer	Time	Assays
(5A)Immunogenicity of divalent PLGA MS[5]	5.3	Guinea-pigs (10)	DT-MS + TT-MS Or Alum control x1 Alum control x2	DT TT	2.5 Lf 1 Lf (1/10 SHD)	Alum (0.035 mg Al^{3+}) Alum (0.06mg Al^{3+})	2-16 weeks	Total Ab Subclasses Ab nAb
(5B)		Guinea-pigs (8)	DT-TT-MS or Alum control x1	DT TT	0.625-5 Lf 0.25-2 Lf (1/80-1/5 SHD)	Alum (0.07-0.009 mg Al^{3+}) Alum (0.12-0.015mg Al^{3+})	6 weeks	Protection Dtx
(5C)		Mice (8)	MS-DT MS-TT Alum control x1	DT TT	0.125-0.5 0.05-0.2 Lf (1/400-1/20 SHD) (1/300-1/100 SHD)	Alum (0.07-0.0017mg Al^{3+}) Alum (0.006-0.002mg Al^{3+})	6 weeks	Protection Ttx
(6A) Immunogenicity of tetravalent PLGA MS	5.4	Guinea-pigs (8-10)	DT-TT-PT-Hib-MS + PfCS-MS or DT-TT-aP-Hib alum control x2	DT TT PT Hib PfCS	2.5 Lf 1 Lf 2 µg 1 µg (1/10 SHD) 5 µg	Alum (0.035 mg Al^{3+})	2-16 weeks	Total Ab nAb Protection Dtx
(6B)		Mice (5)	DT-TT-PT-Hib-MS + PfCS-MS or PfCS-MS	DT TT PT Hib PfCS	2.5 Lf 1 Lf 2 µg 1 µg (1/10 SHD) 2 µg	5% lecithine	2-12 weeks	Total Ab

Table 2.2.(3) Immunogenicity of PLGA MS versus alum control: the kinetics and dose-dependency of the anti-DT and anti-TT immune responses were assessed with the most efficient DT-MS + TT-MS formulation (made of small-sized PLGA 50:50, RG503H) and compared with a commercial DT-TT alum adsorbed control vaccine, which is in use for infant vaccination. Groups of 3 mice were immunised s.c. with a single injection of mixtures of MS-DT + MS-TT at 1/10, 1/25 or 1/62.5 of SHD, or with a single injection of 1/10, 1/20 or 1/25 of SHD of the commercial DT-TT alum adsorbed control vaccine. The animals were terminally bled by cardiac puncture under anaesthesia at 2, 4 and 6 weeks post–immunisation, and serum was prepared from clotted blood and stored at –20 °C, the spleen were removed and processed for lymphocyte proliferation and cytokine production assays.

Table 2.2.(4) Immunogenicity after single dose immunisation: to investigate the controlled release properties of PLGA MS vaccines and their suitability for single injection, as opposed to multiple injections of alum-adsorbate vaccines, a one year protection study in guinea pigs was performed. Groups of 8 guinea pigs were immunised s.c. at 4 doses (1/4, 1/10, 1/20 or 1/50 of SHD) with mixtures of MS-DT + MS-TT injected once, or with a commercial DT-TT alum control vaccine administered, at equivalent total doses, twice at a 4 weeks interval. The animals were test bleed by cardiac puncture under anaesthesia at intervals of 4-52 weeks post-immunisation and challenged with diphtheria toxin (as described under section 2.4.6) 5 days after the last test bleed.

Table 2.2.(5A,5B,5C) Immunogenicity of divalent PLGA MS: groups of 10 guinea pigs were immunised s.c. with a single dose of 2.5 Lf DT and 1.0 Lf TT in MS dispersed in 0.5 ml of 0.02% alum (equivalent to 0.035 mg Al^{3+}) of different polymer type and microsphere sizes (Table 5.1). The commercial DT-TT alum-adsorbed vaccine was used as positive control. Prior to injection, the alum control vaccine was diluted in saline to yield 2.5 Lf/ml of DT, 1.0 Lf/ml of TT, and 0.06 mg Al^{3+} per dose. The control groups received either a single injection in 1.0 ml vehicle or two injections in 0.5 ml vehicle at a 4 weeks interval. Animals were bled by cardiac puncture under anaesthesia at intervals of 2 - 16 weeks post-immunisation, and serum was prepared from clotted blood and stored at -20°C. Protection assays against Dtx and Ttx were performed as described in section 2.4.6.

(6A) Immunogenicity of tetravalent PLGA MS: female guinea pigs were vaccinated once s.c. with the experimental MS vaccines (mixtures of monovalent or a tetravalent MS formulation) dispersed in 0.5 ml of alum (2.5 Lf DT, 1 Lf TT, 2 μg PT, 1 μg PRP, 5 μg PfCS and 0.035 mg Al^{3+} per dose). The amount of MS was calculated from the experimentally determined antigen loadings (Table 5.2). The commercial DT-TT-aP-Hib alum adsorbed vaccine was used as positive control, but given twice at a four week interval. Animals were bled by cardiac puncture under anaesthesia for serum preparation at 2 – 16 weeks post-vaccination.

(6B) In addition, BALB/c mice (12 weeks old) were injected once s.c. at the base of the tail with 2 μg PfCS contained in MS suspended in 200 μl of 5% aqueous lecithin without alum (work performed by G. Corradin group, Lausanne University, Switzerland). Other mice received the same amount of PfCS in MS mixed with the tetravalent DT-TT-aP-Hib-MS

formulation or received the tetravalent formulation alone. Mice were bled at 2 – 12 weeks for serum collection, and anti-PfCS antibody levels were measured using standard ELISA.

2-4-3. Measurement of lymphocyte proliferation and cytokine production

Spleen cells from immunised or naïve animals were resuspended at $2x10^6$ cells/ml in AIM-V medium (GIBCO, Invitrogen) supplemented with 5% fetal calf serum (FCS) and 2% antibiotics/antimycotic solution (10,000 units penicillin, 10mg streptomycin and 25µg amphotericin B per ml). Cells were cultured in triplicate at 37 °C and 5% CO_2 in 96- U well microtitre plates (Falcon, Becton Dickinson, UK) in the presence of DT or TT (0.01-100 µg/ml; 1:10 dilutions). Concanavalin A (10 µg/ml) and medium only were used as positive and negative controls, respectively. After 3 days of incubation, the cells were pulsed overnight with 0.25 µCi of ^{3}H-thymidine (Amersham, UK) and then harvested on glass fibre filters for liquid scintillation counting. Results were expressed as counts per minute (cpm).

For cytokine analysis, splenocytes were cultured as described above for 48 h for IL-6 and IFNγ or 72 h for IL-2, IL-4 and IL-10. The culture supernatants were removed and stored at -20 °C. Cytokine levels in the supernatant of stimulated cells were measured by standard sandwich ELISA, following the kit manufacturer's instruction (Pharmingen, BD Bioscience, Oxford, UK). Values were expressed in pg/ml using a mouse cytokine recombinant protein standard (Pharmingen, BD Bioscience, Oxford, UK).

2-4-4. Detection of serum antibodies by ELISA

Anti-DT and anti-TT antibodies were determined by ELISA. Briefly, Nunc Maxisorb 96-well ELISA plates were coated with 100 µl/well (0.5 Lf/ml) of DT or TT (Welcome, Beckenham, UK) in carbonate buffer of pH 9.6 and left at 4 °C overnight. The plates were then washed and blocked with 5% skimmed milk solution in 0.05% PBS-Tween (PBS-T) for 1 h. Serum samples were diluted across the plate, starting at no less than 1:10 dilution, and incubated at 37 °C for 2 h. Guinea-pig anti-DT and anti-TT (98/572) or in-house mouse anti-DT and anti-TT (Biken) standards were diluted 1/100 and used in each plate as references. Secondary anti-guinea-pig and anti-mouse IgG HRP conjugated Ab (SIGMA, Poole, UK) were used as secondary Ab. The results were expressed in IU/ml against the guinea-pig reference and in titers against the mouse reference. Subclasses of anti-TT and anti-DT IgG were determined on pooled samples using anti-guinea pig IgG1 and IgG2 conjugates (Nordic Immunological Labs) or anti-mouse IgG1 and IgG2a conjugates (Pharmingen, BD Bioscience, Oxford, UK). The substrate solution of pH 4, containing 0.5 mg/ml of ABTS and 0.04% H_2O_2 in 0.05M citric acid, was added and left at room temperature for 30 min for colour development. The absorbance was read at 405 nm in a plate reader (Anthos Labtec Ins, model 2001). The results were expressed in arbitrary titres. The geometric mean ± SEM of two sets of experiments was calculated.

Anti-pertussis toxoid and anti-Hib antibodies were determined by an in-house method after coating ELISA plates with 2 µg/ml of a pertussis toxoid (GSK, Belgium) or with 1 µg/ml of a *Haemophilus influenzae* type b oligosaccharide conjugated to human serum albumin (HBO-

HAS) (Wyeth Lederley, Pearl River, NY, USA). HRP-conjugated anti-guinea-pig Ab (SIGMA, Poole, UK) was used as secondary Ab. Substrate solution (0.5 mg/ml ABTS and 0.04% H_2O_2 in 0.05 M citric acid of pH 4.0) was added, and the plates left at room temperature for 30 min. The absorbance was read at 405 nm with an Anthos plate reader (Anthos Labtec Ins, model 2001). The results were expressed as mean optical density (OD) ± SEM from two sets of experiments. An in-house reference serum was used in each plate.

For detection of Ab against PfCS synthetic peptide (work performed by Dr.G.Corradin group, Lausanne University, Switzerland), microtitre 96-well plates were coated in a humid chamber at 4 °C overnight with 50 µl of 1 µg/ml PfCS in PBS of pH 7.0. Plates were washed with PBS-0.05% Tween 20 (PBST) and saturated with 5% of non-fat dry milk (PBSTM) at room temperature for 1 h. Then, duplicate serial dilutions of individual sera in PBSTM were added to the plates and incubated at room temperature for 1 h. The plates were then incubated with rabbit anti-guinea pig or rabbit anti-mouse polyvalent IgG1 (Sigma, Buchs, Switzerland) in PBSTM. Finally, HRP-conjugated anti-rabbit IgG (Bio-Rad Laboratories, Reinach, Switzerland) was added to the plates and incubated for 1 h before washing and adding the enzyme substrate phenylenediamine dihydrochloride. Absorption was read at 492 nm, and the titer of the antigen-specific antibody represents the last positive serum dilution. Endpoint titers were defined as the highest serum dilution that resulted in an absorbance value that was three standard deviations higher than that of negative control sera from pre-immune animals.

2-4-5. Anti-DT and anti-TT antibody toxin neutralisation capacity

Tetanus: Tetanus antitoxin neutralising capacity was determined in mice (strain NIH; 15-22 g, Harlan, UK) by using the onset of paralysis as the end point (Tierney et al, 2003). A series of dilutions of the species specific reference standards (98/572 for guinea-pig samples or Biken for mice samples) and of pooled sera from each immunisation group (5-7 dilutions in the range of 0.01-0.025 IU/ml, values predicted from ELISA data) was made in 0.25% (w/v) gelatine in PBS of pH 7.0 in the presence of a fixed concentration of tetanus toxin (50 x PD50). All mixtures were allowed to stand at room temperature for 30 min before 0.5 ml volumes were injected subcutaneously into groups of 4 mice. The mice were observed for 96 h for signs of paralysis. The protective capacity of the test samples was compared to the antitoxin reference standard using the Probit method of parallel line analysis. The protective concentration was expressed in international units (IU) per milliliter, with upper and lower limits calculated (Probit analysis).

Diphtheria: Neutralising antibodies specific for diphtheria toxin were measured both *in vivo* and *in vitro* by toxin neutralising assays as previously described (Mawas et al., 2004; Redhead et al., 1994) and adopted for validation of direct challenge potency method for diphtheria vaccines (Winsnes et al., 2002).

Diphtheria antitoxin neutralising capacity was determined *in vivo* in guinea pigs by the onset of dermonecrosis as the end point. A series of dilutions of the species specific reference standards (98/572 for guinea-pig samples or Biken for mice samples) and of pooled sera from each group (6 dilutions in the range of 0.00025-0.025 IU/ml, values predicted from ELISA

data) was made in saline in the presence of a fixed concentration of diphtheria toxin (0.025 Lf/ml equivalent to 12.5 x MRD). All mixtures were allowed to stand at room temperature for 30 min before 0.2 ml volumes were injected intradermally into 2 guinea pigs. Each shaved guinea pig was injected with increasing amounts of the six reference toxin dilutions, and reactions were observed for 48 hours. The protective capacity of test sample was expressed against the reference in IU per millilitre with upper and lower limits calculated (Probit analysis).

The *in vitro* Vero cell toxin neutralisation method relies on the inhibition of a cytotoxic dose of diphtheria toxin and the end point is taken as the highest serum dilution protecting the cells. Briefly, in 96-flat well culture plates, 50 µl of serial dilutions of each individual serum were mixed with 50 µl of diphtheria toxin at 0.25 mLf/ml (10 x MRD). Plates were incubated at 37 °C for 1 h . At the end of the incubation period, 50 µl of a Vero cell suspension containing $4x10^5$ cells/ml in minimum essential medium (MEM, supplemented with 5% fetal calf serum, 1.5% Hepes buffer, 1% glucose and 2% antibiotic/antimycotic solution) were added into each well, and plates were incubated at 37 °C for 7 days. At the end of the incubation period, 10 µl of MTT solution (0.5% w/v in PBS) were added to each well, and the plates sealed and incubated for a further 4 h. Viable cells (reduction of MTT to a blue product by the mitochondrial dehydrogenase) were extracted with an aqueous solution of 10% (w/v) sodium dodecylsulphate and 50% dimethylformamide. The plates were read at 570 nm in a plate reader (Anthos Labtec Ins, model 2001). The neutralising potency was calculated relative to the NIBSC guinea-pig (IU/ml) or mice reference standards (relative titres). The results were expressed as geometric mean ± SEM.

2-4-6. Protection studies against challenge with Dtx and Ttx

Diphtheria: Guinea pigs (female, strain DH, 250-300g) were immunised once sub-cutaneously with MS vaccines or with alum control vaccine given once or twice at equivalent doses (Table 2.1). At various time post-immunisation (Table 2.1), the animals were challenged intradermally with escalating amounts of diphtheria toxin (0.00025-0.256 Lf/ml equivalent to 8-128 MRD) and observed for 48 h for appearance of erythema (red mark larger than 5mm diameter, as described in the Ph. Eur., 2005, for intra-dermal challenge procedure). Results were expressed as percentage of animals showing protection at each of the toxin challenge. The mean ± SEM was calculated using data from three independent observers.

Tetanus: mice (female, strain NIH, 18-22g) were immunised once s.c.. with MS vaccines or alum control vaccine given once or twice at equivalent doses (Table 2.1). At various time post-immunisation (Table 2.1), animals were challenged s.c. with $50xPD_{50}$ of tetanus toxin, as described in the Ph. Eur. (2005) for paralytic challenge procedure. Results were expressed in percentage of animals protected at each of the vaccine dose.

2-5 Localisation studies

2-5-1. Immunisation and tissue collection

Groups of one to three mice were immunised s.c. with 1 mg of small-sized fluorescent DT-MS (F-MS), 5 mg of large-sized F-MS (both equivalent to 1 Lf DT, 1/25 SHD), or 1mg of synthetic fluorescent polystyrene beads (PS) (Fluospheres® of 0.46 μm diameter, Molecular Probes, Oregon, USA), all freshly reconstituted and homogenised in 1% lecithin in sterile

water or in 0.02% alum in sterile saline (1:100 dilution of Alhydrogel®, 0.035mg Al^{3+} per dose). Animals immunised with lecithin alone or naïve animals were used as controls. The volume of injected formulation was 0.5 ml.

The PS beads were used to standardise the detection technique, as the degradation of the PLGA MS was assumed to induce a substantial decrease in the fluorescence signal. The aim of this experiment was to investigate the feasibility of the detection techniques used for PS beads to follow the fate of PLGA-based F-MS. No conventional statistics could be applied as only one animal per group and per time point was used. However, differences were considered as meaningful when there was at least a 2.5-fold difference between groups.

Mice were killed at different time points from 10 min to 8 weeks post-immunisation; tissues from the injection site (IS), mesenteric lymph nodes (LN) and spleen (S) were removed, and macrophages (MΦ) were collected from the peritoneal cavity (PC) by simple flushing with medium (4 ml AIMV medium, Gibco, Invitrogen, Paisley, UK, supplemented with 5% fetal calf serum and 2% antibiotic-antimycotic solution). Tissues were homogenised by extruding through nylon filters (Falcon, BD Biosciences, Oxford, UK). The cells were washed twice followed by centrifugation at 1500 rpm for 5 min in 10 ml medium. In-between washes, the red blood cells were lysed by osmolytic shock using ice-cold water. Cell pellets were reconstituted in medium at a concentration of 1 x 10^6 cells/ml (the cells were diluted in trypan blue and the viable cells counted with a haemocytometer) and kept at 4°C until further use.

The type and percentage of fluorescent cells were determined by FACS, using specific CD markers for MΦ and DC , as described below. The presence of fluorescent particles (F-MS or PS beads) was assessed by FACS and epifluorescence microscopy (λ_{em}(fluorescein) = 514 nm;

(λ_{em}(rhodamine 123) = 529 nm); for the latter cells were fixed onto poly(lysine) coverslips. The data obtained by FACS were sufficient to confirm the presence of fluorescent particles in samples such as IS and PC. However, for the LN and S, a visual confirmation of presence or absence of fluorescent particles was necessary, because of the much lower number of internalised fluorescent particles.

2-5-2. Discrimination between macrophages and dendritic cells involved in MS uptake and MS uptake kinetics

Cells involved in MS uptake were analysed for their phenotype by flow cytometry. Single cell suspensions were isolated as described above and incubated with rat IgG (Caltag-MedSystems, Silverstone, UK) in the dark for 15 min to block the Fc-receptors and prevent unspecific staining. The cells were then incubated for 30 min with fluorophore-conjugated antibodies directed against surface proteins specific for Mϕ (PerCP-CY5.5-conjugated rat anti-mouse CD11b Ab) or DC (*APC*-conjugated hamster anti-mouse CD11c Ab) (Pharmingen, BD Biosciences, Oxford, UK). Control samples including unstained cells and cells incubated with antibody isotypes (PerCP-CY5.5-conjugated rat IgG1 and *APC*-conjugated hamster IgG) were also used. Cells were washed twice with FACS wash solution (PBS, 4% FCS, 0.01% sodium azide), fixed with formaldehyde (1% in FACS wash solution) and stored overnight in the dark at 4 °C prior to analysis using a Becton Dickinson fluorescence-activated cell sorter (FACSCALIBUR®).

2-5-3. Determination of macrophage activation

Groups of 3 mice were immunised subcutaneously twice with 1 mg of non-fluorescent DT-loaded MS (MS-DT) (1 Lf DT, equivalent to 1/25 SHD) in 0.5 ml injection vehicle at 2 weeks interval; one naïve animal was used as control. At 4h after the second immunisation, the animals were killed and the peritoneal Mϕ collected as described above. Cell samples were analysed by flow cytometry as described in section 2.5.2, but using different markers as follows. (i) for specific marking of Mϕ: *APC*-conjugated rat anti-mouse CD11b Ab (Caltag-MedSystems, Silverstone, UK), RPE-conjugated rat anti-mouse CD14 Ab (Pharmingen, BD Biosciences, Oxford, UK), and FITC-conjugated anti-mouse DC marker (Leinco Technologies, St. Louis, USA); (ii) for cell activation: FITC-conjugated rat anti-mouse CD80 and CD86 Ab (Pharmingen, BD Biosciences, Oxford, UK) and MHC class II Ab (Caltag-MedSystems, Silverstone, UK). Correspondingly, unspecific isotype antibodies were also used to account for unspecific binding.

2-5-4. In vitro MS uptake and qualitative observation of MS degradation

In vitro uptake and processing of F-MS was performed using a murine macrophage cell line (J774 cells, ECACC, CAMR, Salisbury, UK) and primary macrophage cells collected from the peritoneal cavity of naïve Balb/c mice, as described in section 2.5.1. The primary macrophages were allowed to settle at 37°C and 5% CO_2 for 2 h. Macrophages were gently washed with medium (4 ml AIMV medium (Gibco, Invitrogen, Paisley, UK) supplemented with 5% fetal calf serum and 2% antibiotic-antimycotic solution to remove dead or unattached cells. The adherent cells were collected in 10 ml medium with a cell scraper (Flacon BD

Labware, NJ, USA), centrifuged at 1500 rpm for 5 min, and the pellet reconstituted in 1 ml medium before counting. The cells were diluted 1:50 in trypan blue and counted under bright field microscopy using a haemocytometer (BDH, Poole, UK). Primary and J774 cells, both at a number of $1x10^6$, were settled at 37°C and 5% CO_2 for 2 and 48 h respectively in $25cm^3$ cell culture flasks (Flacon) or onto cover slips placed in 24-well microplates (Flacon). The cells were then incubated with 3 mg/ml of small-sized or large-sized DT-loaded F-MS for 10 min or 12 h respectively, gently washed twice with medium to remove free particles and incubated for another 2 h. The cells were collected from the flasks, centrifuged at 1500rpm for 5min, and the pellet fixed in fresh 2.4% glutaraldehyde solution for TEM analysis. The cells in the 24-well plates were washed, fixed with paraformaldehyde in PBS, and kept in the dark at 4 °C before observation by epifluorescence microscopy.

F-MS degradation was qualitatively observed *in vitro*. For this, the dry DT-loaded F-MS were reconstituted in PBS of pH 7.4 and incubated at 37 °C on a rotative shaker for up to 8 weeks. The pH was maintained at approximately 7.4 by exchanging the buffer solution every 3 days. F-MS samples were collected from 24 h to 8 weeks and analysed by SEM and epifluorescence microscopy.

2-5-5. In situ MS localisation and qualitative observation of MS degradation

Groups of two mice were immunised as described in section 2.5.1 with DT-loaded F-MS and killed after 4 h, 7 days, 2 or 4 weeks. Various tissue samples from one animal were extracted and embedded in a cryostat medium (OCT), cryo-fixed by floatation on liquid nitrogen and stored at –80 °C, before frozen sections of 10 µm thickness were cut with a microtome (Bright Cryostat®). Tissues from the other animal were disintegrated to obtain a single cell

suspension, which was analysed by fluorescent microscopy (epifluorescent and confocal microscopy, see below) and flow cytometry, as described above.

2-5-6. Qualitative observation of ex vivo particle degradation following uptake by phagocytic cells.

Groups of three mice were immunised as described above and killed after 4 h. Cells from the peritoneal cavity were collected by simple flushing, washed twice, and allowed to adhere to glass cover slips (10 mm in diameter) placed at the bottom of a 24-well plate. After 2 h incubation at 37 °C and 5% CO_2, the cells were gently washed twice with 1 ml medium (4 ml AIMV medium (Gibco, Invitrogen, Paisley, UK) supplemented with 5% fetal calf serum and 2% antibiotic-antimycotic solution) to remove unattached cells and placed back into the incubator for up to 2 weeks. Cover slips were removed from the plate at regular intervals (24 h, 48 h, 3 days, 7 days and 2 weeks), fixed in 4% paraformaldehyde solution and kept in the dark at 4 °C prior to staining and analysis by confocal microscopy.

2-5-7. Cell staining and laser scanning confocal microscopy (CSLM)

Cells from naïve and immunised mice were generally stained for the F-actin cytoskeleton with a phallacidin derivative (Molecular Probes, USA) and for cell nucleus with Hoechst 33342 (Molecular Probes, USA), diluted at 1:100 in dH_2O), both according to the manufacturer's protocol. Briefly, the cell membranes were first permeabilised by soaking the samples in 1% Tween® 20 in PBS for 30 min, followed by 30 min impregnation in 1% phallacidin in PBS. The samples were further rinsed 3 times with PBS, impregnated in 0.2% Hoechst 33342 stain in PBS for 10s, rinsed 3 times again with PBS and mounted onto a poly(lysine)-coated

miscrocope slide using a fluorescent mounting agent (Vectashield, Vector Laboratories, Burlingame, CA). Samples were observed and imaged with a laser-scanning microscope (Leica SP2 AOBS) mounted on an upright microscope (DM RE-7 Leica) and operated with the manufacturers software (Leica Confocal Software v 2.5, Leica). Mounted samples were scanned for areas of interest using conventional epi-fluorescent illumination with a blue diode laser (λ_{ex} = 405 nm), an argon-ion laser (λ_{ex} = 488 nm), and a helium-neon laser (λ_{ex} = 543 nm), in combination with FITC-type, TRITC-type and DAPI- type wide-band pass filter blocks at low magnifications (10x and 20x). Images were taken using an oil-immersion objective (HCX PL Apo λBL 63x), separated with spectral filtering (user optimised) for each fluorophore, and collected by sequential scanning for exciting individually each fluorophore. The same settings were kept for all samples (excitation, gain, pinhole size, distance between optical slices, and spectral filter settings). Images were analysed and exported using the Leica 3D software package.

2-6 Statistical analysis

Geometric means were calculated as the antilog of the arithmetic mean of the log transformed values. The graphs error bars represent the antilog of the SEM of the log transformed values.

Comparisons between polymer types and MS sizes were assessed by analysis of variance using PROC GLM in SAS. Where appropriate and prior to the assessment of any differences between polymer types or MS sizes, the effect of antigen content in the MS was included in the statistical evaluation (as a covariant in the ANOVA) to account for differences in loading in the formulation. In addition, Tukey's multiple comparison test (Hsu, 1996) was used to make comparisons between each pair of polymer types.

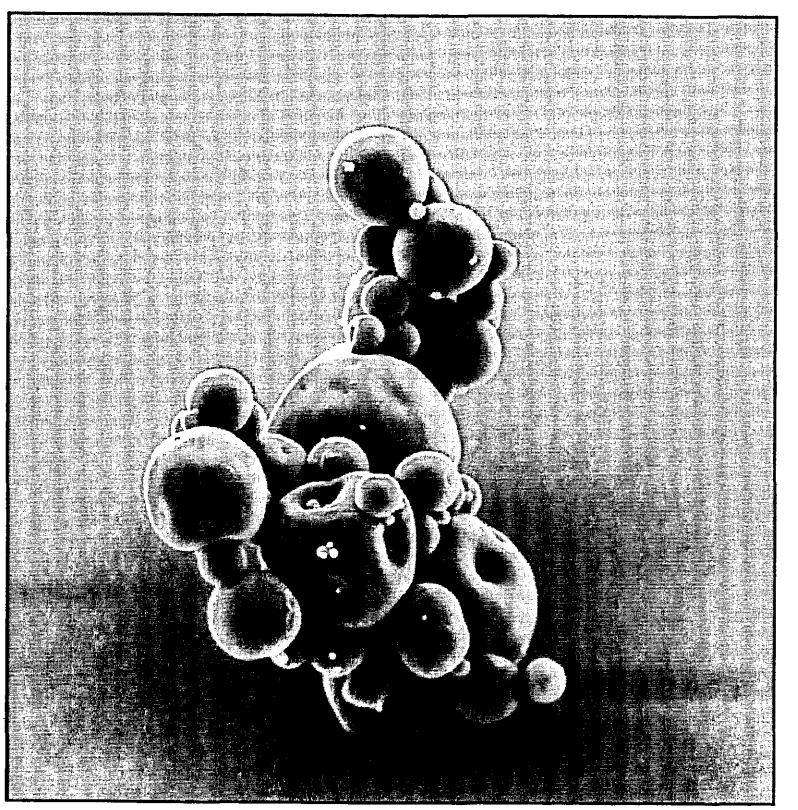
Pearson correlations were calculated to assess the association between two independent parameters; the results were expressed as a coefficient of determination (r^2) and the significance of the correlation was calculated. Groups were compared using the one way ANOVA tests (Armitage et al., 2002) with Tukey's multiple comparison for group-to-group comparisons or with Dunnet's control group comparison test (Hsu, 1996) for samples to control group comparisons. In the case of two samples comparison, Student's t-test along with Fisher's analysis of variance was performed. Statistical evaluations were performed using MINITAB for windows (Release 14.1).

P values <0.05 or <0.01 were considered statistically significant.

The results from ELISA, toxin neutralisation or protection assays were assessed using the principles of parallel-line bioassay analysis. For the ELISA, a log transformation was applied to both the doses and the responses. For the toxin neutralisation and the protection assays, PROBIT transformations (mathematical method of transforming dose-response curve into a straight line) of the proportions of animals surviving at each vaccine dose were plotted against log transformed doses. The linearity and parallelism of the transformed dose-response lines were confirmed and estimates of relative potency (with 95% confidence limits) calculated from the fitted lines. The ED_{50} is defined as the dilution at which 50% of the samples responded and could be estimated from the fitted PROBIT dose-response line (Armitage et al., 2002).

The parallel-line bioassay and the Probit analysis were performed using NIBSC in-house software.

RESULTS



CHAPTER 3: IN VIVO FATE AND UPTAKE OF PLGA MS VACCINES

3-1 Rationale and outline of the study

In this study, *in vivo* uptake of PLGA MS by macrophages (MΦ) and dendritic cells (DCs), as well as biodistribution of a PLGA MS candidate vaccine following sub-cutaneous (s.c.) administration in mice were studied. MΦ and DCs are recognised as professional APCs which play an essential role in the induction of the immune response by efficient Ag presentation to naïve T-cells. Prior to the presentation stage, the foreign molecules are uptaken, supposedly degraded inside the cells and the resulting peptide Ag is transported from the site of infection to the T-cell area of the lymphoid organs (Singh and O'Hagan, 1999). *In vitro* uptake of PLGA MS by MΦ and DCs has been previously demonstrated (Thiele et al., 2003), however, their *in vivo* processing remains unclear. In this chapter the *in vivo* uptake and trafficking of PLGA MS vaccine was investigated. The aim of the study was also to assess the importance of MΦ and DCs in the induction of the immune response. We also wanted to investigate the influence of co-administration of alum and of MS size on the uptake as it was hypothesised that larger MS would not be taken up and that alum would have a depot effect preventing processing of the vaccine. Uptake was investigated by the identification of dual labelled fluorescent MS (F-MS) in excised tissue or peritoneal lavage at fixed time points. The use of the two co-encapsulated fluorophores permits unambiguous co-localisation of the PLGA MS. The phenotype and number of fluorescent cells were determined by flow cytometry using specific markers for macrophages and dendritic cells. The presence of fluorescent beads in tissues was confirmed by fluorescent microscopy on frozen sections. It was possible to follow the uptake

of the fluorescent particles and their transit towards lymphoid organs (MLN and spleen) up to 8 weeks post-immunisation.

3-2 Formulations

Fluorescent microspheres loaded with diphtheria toxoid (DT) were prepared by spray-drying a mixture of DT, fluorescein and rhodamine (see Chapter 2.2). The encapsulation of both fluorochromes was confirmed by fluorescence microscopy (Fig 3.1.a,b,c). The co-encapsulation process did not influence the loading efficiency of the antigen nor the morphological characteristics of the PLGA MS, as confirmed by ELISA (Table 3.1.) and SEM (Figure 3.1.d, e).

Table 3.1. Overview of the PLGA MS formulations used in the localisation studies. Resomer RG503H (28 kDa PLGA 50:50 with uncapped end-group) was used throughout and succinylated gelatine (Physiogel) was co-encapsulated as stabiliser for the encapsulated diphtheria toxoid (DT).

Code	Formulation name	MS size (μm)	Effective DT loading (Lf/mg)
SD503H	Small MS	0.5-10	0.85
F-SD503H	Small F-MS	0.5-10	0.89
CO503H	Large MS	10-60	0.47
F-CO503H	Large F-MS	10-60	0.26

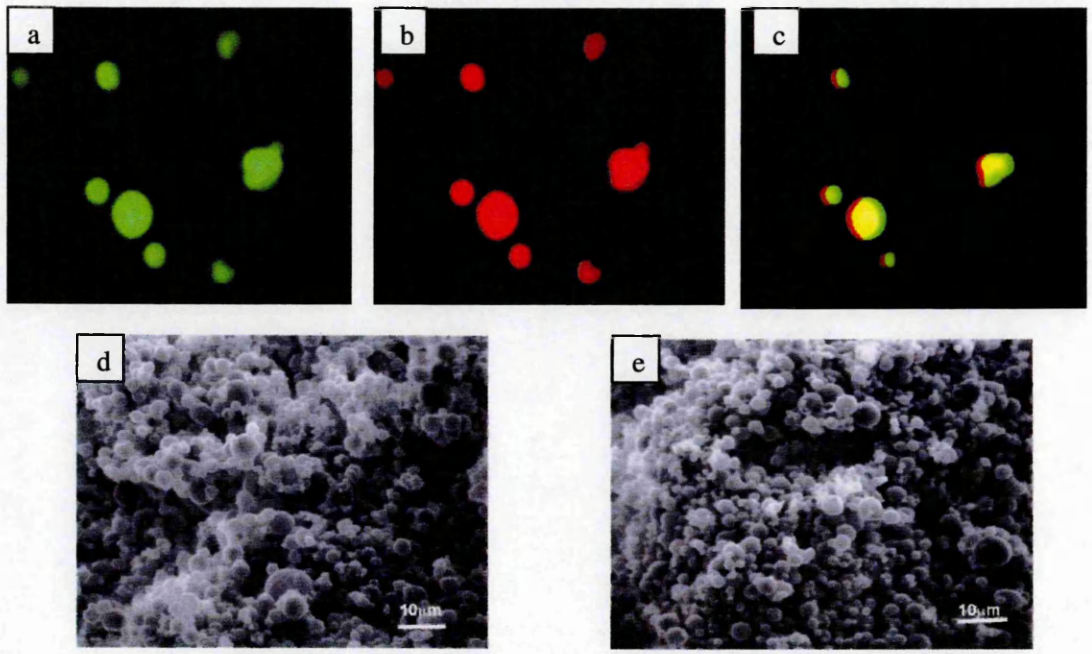


Figure 3.1 Epifluorescence micrographs of dually labelled fluorescent PLGA microspheres (F-MS) (magnification 63x). (a, b): Positives for fluorescein and rhodamine fluorescence emissions, respectively. (c) The yellow colour confirms co-localisation of both fluorophores inside the F-MS. (d, e): Scanning electron micrographs of PLGA MS loaded with DT alone (d) or with a mixture of DT and the fluorophores (e). The morphology of both formulations are comparable, the MS size range is between 0.5 and 10 µm in diameter (scale bar = 10µm).

3-3 Trafficking of F-MS following sub-cutaneous immunisation

F-MS and PS beads could be detected by flow cytometry in high numbers at the injection site (IS), and the numbers of fluorescent particles did not significantly decrease up to 1 week (Fig. 3.2). PS beads were used to standardise the detection technique, as the degradation of the PLGA MS was suspected to induce a substantial decrease in the fluorescence signal. The moderate number of fluorescent particles observed at 10 min and 4 h after injection of F-MS and PS beads, respectively, is probably due to a purification problem during the isolation of cells as the polymer tended to aggregate during the filtration process.

The number of F^+-cells at the IS increased between 4 and 48 h (Figs. 3.2, 3.3.a; Table. 3.2.). This may partly result from the initial burst release of the fluorophores from the PLGA MS with subsequent staining of the basement membrane of the cells. After 4 weeks, the number of F^+-cells at the IS had decreased to background level of the naïve animals (Fig 3.3.a). In the flow cytometry assay, the fluorescence intensity of both the fluorescein (FITC) and rhodamine (PE) decreased ($p<0.05$) (shift to the left in the histogram) between 10 min and 4 weeks, i.e., from 21± 6.2% to 0.03± 0.7% for FITC and from 9.5± 1.5% to 0.15± 0.07% for PE (Fig. 3.3.b).

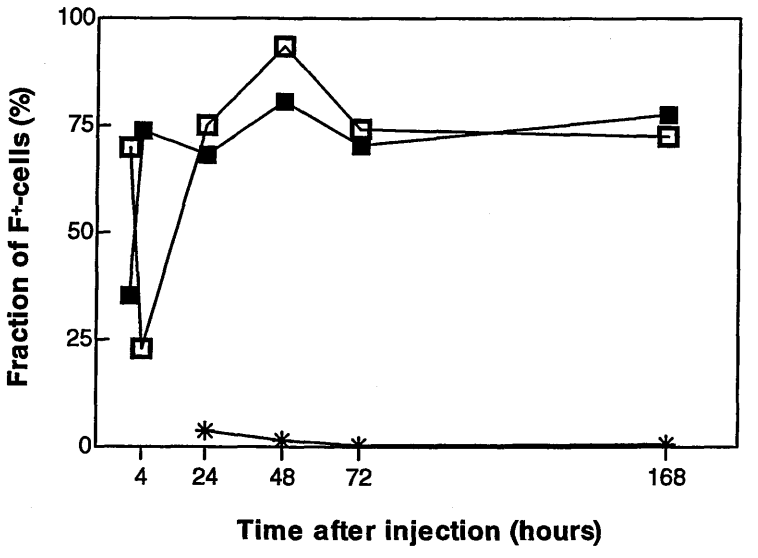


Figure 3.2 Percentage of total fluorescent cells at injection site of (✳) naïve mice and mice treated (s.c.-injection) with (■) F-MS or (□) PS beads. F-MS and PS beads were detected by flow cytometry at the injection site (IS) for up to 1 week post-immunisation. The data are results from one individual animal.

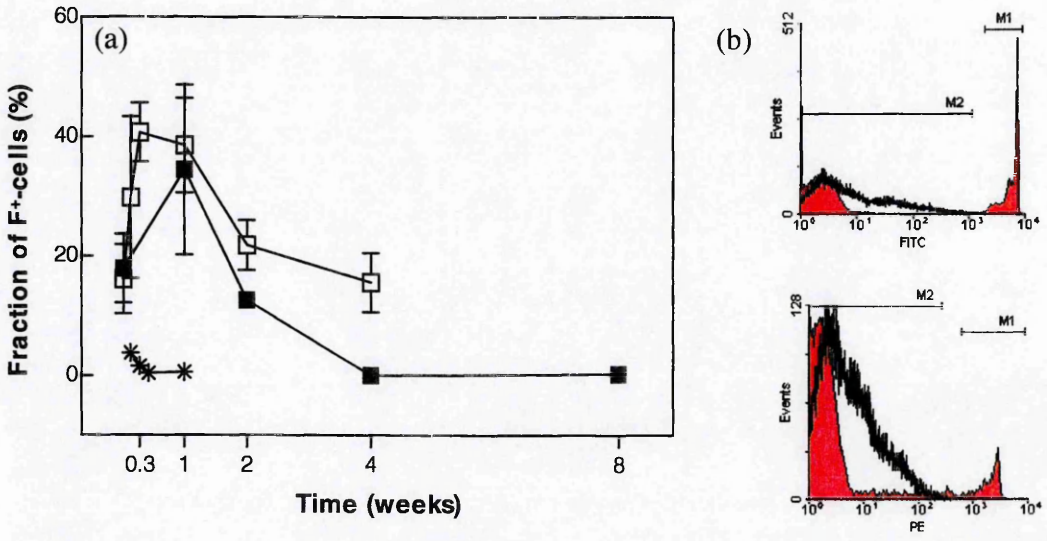


Figure 3.3 F-MS trafficking was followed from the IS to the lymphoid organs (by epifluorescence) up to 8 weeks post-immunisation. (a) Percentage of total F^+-cells at the IS from mice immunised with F-MS. The results are expressed as geometric means ± SEM of 3 individuals on 2 different assays (□: assay 1 ; ■: assay 2). The stars represent the naïve controls. (b) Histogram of fluorescein and rhodamine fluorescence emitted from IS-samples showing the shift in fluorescence intensity that occurred between the time points post-immunisation of 10 min (red) and 4 weeks (black) (with M1 and M2 used as markers for fluorescent intensity higher and lower than 10^3 log unit respectively).

A direct drainage of the F-MS from the injection site towards the mesenteric lymph nodes was observed during dissection, and the confocal microscopy revealed the presence of free and cell-entrapped fluorescent particles in the MLN as early as 10 min post-immunisation. At this early time point, cells collected from the peritoneal lavage were also highly positive for F-MS. The first sign of F-MS in the spleen was observed by fluorescent microscopy at 4 h post-immunisation, but significant levels of co-localised fluorescein and rhodamine were detectable by flow cytometry only after 48 h and 1 week in samples from the MLN and spleen, respectively (Table 3.2.). F-MS with weaker fluorescence were detectable in the spleen up to 4 weeks, but in a significantly lower number as compared to earlier time points ($p<0.05$). F-MS were detectable by FACS inside cells from the peritoneal cavity (PC) at 4 h post-inoculation, and decreased significantly after 1 week ($p<0.05$). Cells from the PC still carried F-MS at 4 weeks post-immunisation.

The kinetics of trafficking of the F-MS was influenced by the presence of alum at early time points (4 h), where alum seemed to retain the F-MS at the injection site and prevent the direct drainage towards the MLN. When the F-MS were dispersed in alum for injection, approx. 70% of F^+-cells remained at the IS at 4 h post-immunisation, against less than 20% for the F-MS-formulation without alum (Figure. 3.4.a). Indeed, at 4 h post-immunisation, tendentiously less F^+-cells were observed by FACS in the MLN (results not shown) and within the PC when F-MS were co-administered with alum, although the difference was not significant (Fig. 3.4.b).

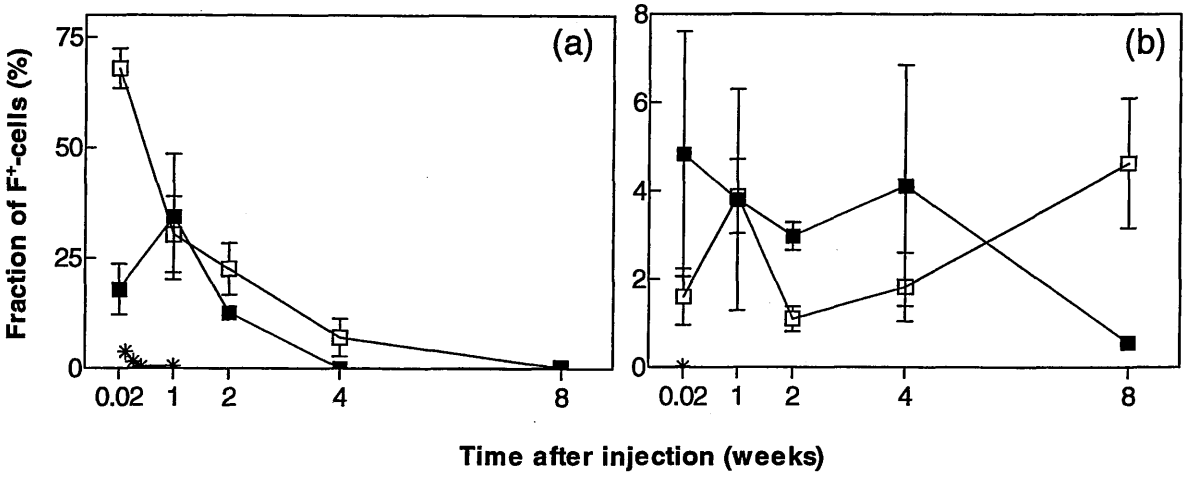


Figure 3.4 Influence of alum on the bio-distribution of F-MS. Percentages of total fluorescent cells from IS (a) or PC (b) of mice immunised with (■) F-MS dispersed in 1% lecithine or (□) F-MS dispersed in alum. The stars represent the naïve controls. The results are expressed as geometric means ± SEM from 3 individual animals.

Table 3.2 Biodistribution of fluorescent microspheres at the IS, the MLN, the peritoneum and the spleen following subcutaneous immunisation of mice. The cells positive for fluorescein and rhodamine were counted by flow cytometry and expressed as a percentage of the total number of cells. The data are presented as geometric mean of triplicate determinations ± standard error of the mean (SEM). Significant differences were calculated at 95% confidence by Student t-test.

Time post-inoculation	Percentage of cells loaded with F-MS in different tissues			
	Injection site	MLN	Peritoneum	Spleen
4 h	11.5 ± 3.60*	0.07 ± 0.02	0.82 ± 0.27*	0.24 ± 0.05
48 h	22.0 ± 10.2	0.14 ± 0.03*	0.55 ± 0.4	0.30 ± 0.11
1 week	28.8 ± 2.75#	0.16 ± 0.05	0.26 ± 0.18#	0.33 ± 0.00*
4 weeks	0.63 ± 0.05#	0.13 ± 0.03	0.24 ± 0.05#	0.11 ± 0.01#,&

* $p<0.05$ compared with control mice immunised with 1% lecithin in PBS

# $p<0.05$ compared with 4 h data

& $p<0.05$ compared with 1 week spleen data

When F-MS dispersed in alum were injected, particle aggregates were observed at the IS, which were not observed with the alum-free formulation. At the end of the study (8 weeks), F-MS could still be observed at the IS, although with a very weak fluorescence; there was also presence of large conglomerate structures at this late time point. Interestingly, at this time point, a significant number F^+-cells was observed in the PC of the F-MS in alum group, whereas no more F^+-cells where detectable in the alum-free group (Fig. 3.4.b). It was very difficult to detect clearly F-MS or F^+-cells in the spleen at any of the time point neither by microscopic observations nor by FACS. However, at 1 week, some of the cells were brighter than the background fluorescence and seemed to have a more granular structure (results not shown).

The kinetics of F-MS at the IS and in the PC were similar for large-sized (10-60 μm) and small-sized (0.5-10 μm) microspheres (Fig 3.5.a), although the number of large-sized F-MS reaching the PC was significantly higher after 8 weeks, where no more small F-MS were detected in the PC (Fig 3.5b). The transport of larger-size MS at later time point was also obvious in MLN and spleen at 8 weeks post-immunisation (Fig 3.6. a,b,c). We also observed that larger sizes of F-MS were phagocytosed and trafficked from the IS to the lymphoid organs and PC, 4 hours after a 2 week booster injection of the F-MS (Fig 3.6. d,e,f).

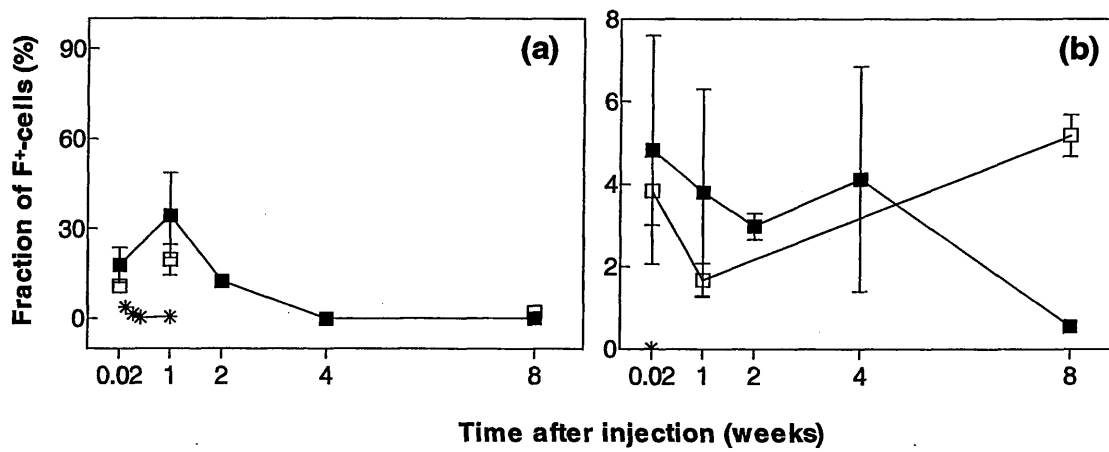


Figure 3.5 Influence of microsphere size on the bio-distribution of F-MS. Percentage of total fluorescent cells at the IS (a) and PC (b) from mice immunised with (■) small-sized (0.5-10 μm) and (□) large-sized (10-60 μm) F-MS. The stars represent the naïve controls. The results are expressed as geometric means ± SEM from 3 individual animals.

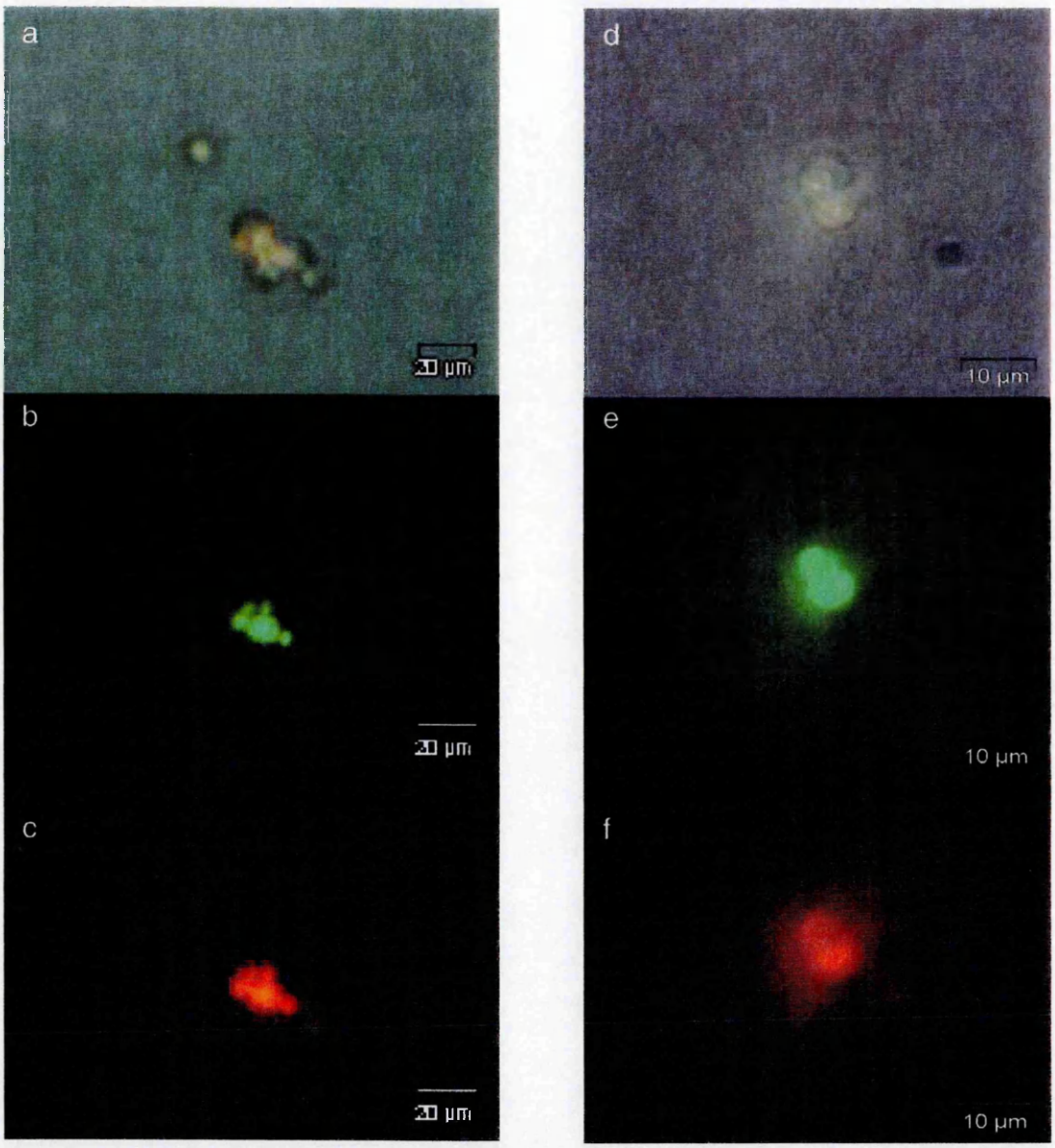


Figure 3.6 Epifluorescence micrographs of cells that had ingested large-sized F-MS *in vivo*: (a, b, c) Cells isolated from the PC 4 h post- a 2 week booster injection of F-MS; (d, e, f) Cells isolated from the spleen of mice 8 weeks post-immunisation with F-MS. The first row of pictures (a, d) is an overlap of bright field and fluorescent images; the second and third rows reveal cells exhibiting fluorescence from fluorescein emission at 488 nm (b, e) and rhodamine emission at 594 nm (c, f), respectively. These micrographs showed that large-sized F-MS were transported after booster dose and during late response to PC and spleen of animals that had previously been injected with F-MS.

3-4 *In vitro* versus *in vivo* uptake of F-MS

Small-sized F-MS were internalised *in vitro* by mouse macrophages (J774 cell line) at a very high ratio as soon as 10 min after exposure. The F-MS quickly adsorbed on the cells and were internalised within 1 h incubation. Even microspheres with a diameter of above 5 μm (Fig 3.7. a,b,c) or above 10 μm were phagocytosed by the macrophages *in vitro*, although the internalisation required longer exposure and incubation time (overnight) (Fig 3.7. d,e,f).

The uptake of the F-MS *in vivo* was studied with cells from the peritoneal cavity as they are known to be mainly of macrophage type. This is the first report demonstrating PLGA MS in the PC following s.c- injection. The cells were collected at 4 h post-injection and studied *ex vivo*, as described in chapter 2.5.6. All the cells contained more than one fluorescent particle, and the ingested particles had diameters of less than 5μm (Fig 3.8.a). This was an interesting observation as the F-MS formulation injected was of a wider size range, i.e., from 0.5 to 10 μm diameter. Internalisation of the F-MS was confirmed by confocal laser light microscopy using the cross-sectioning technique (Fig.3.8.b), as the actin-stained skeleton of the cells was shown to surround the F-MS in both section planes.

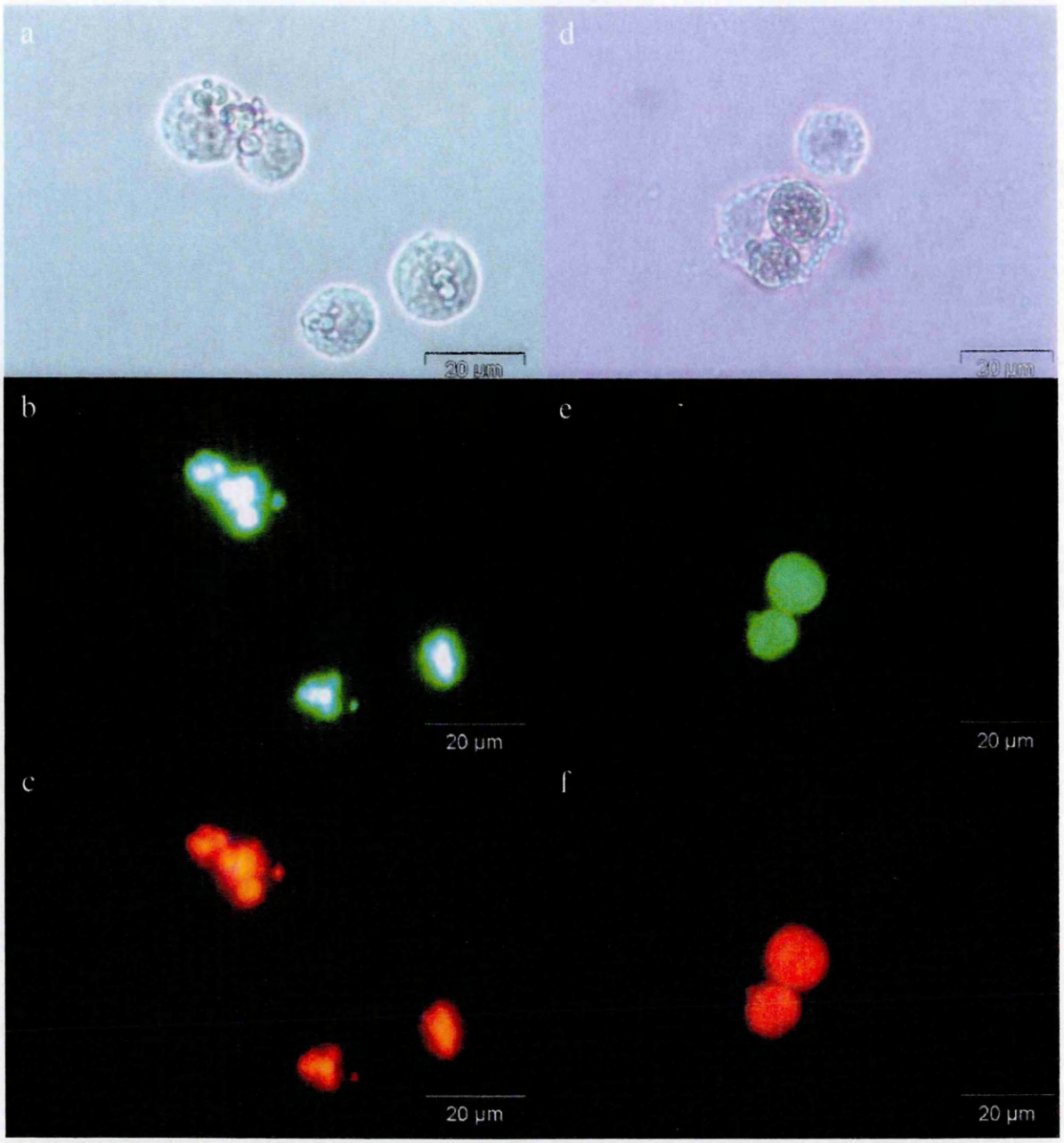


Figure 3.7 Epifluorescence micrographs (20 x) of *in vitro* cultivated macrophages (J774 cell line) that have ingested small-sized (0.5-10 mm) (a,b,c) and large-sized (10-60 mm) (d,e,f) F-MS. The first row of pictures (a, d) is of bright field images; the second and third rows reveal cells exhibiting fluorescence from fluorescein emission at 488 nm (b, e) and rhodamine emission at 594 nm (c, f), respectively. These micrographs provide evidence that F-MS were efficiently ingested by macrophages *in vitro*.

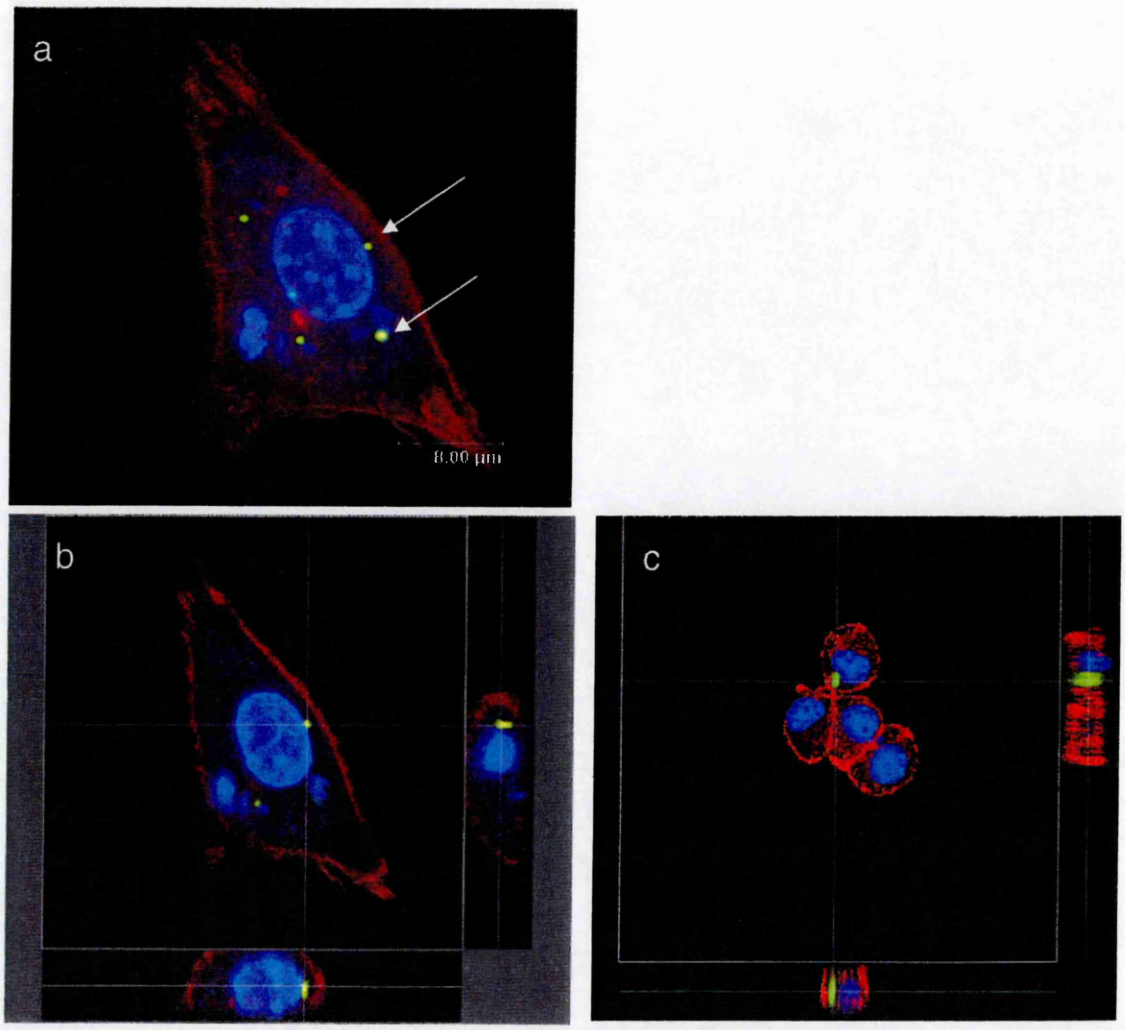


Figure 3.8 Confocal micrographs of cells from the peritoneal cavity collected at 4 h post-immunisation. (a) The cells contained consistently more than one fluorescent particle (size of below 5 µm). (b) The yellowish-greenish F-MS are surrounded by the red skeleton of the cell (in red, nuclei in blue) in both transversal and lateral sections, which proves their internalization. (c) F-MS were internalised into macrophages and dendritic cells from the PC even after injection of a very small volume (4 x 20µl / dose). These micrographs provide evidence that s.c.-injected F-MS were internalised by phagocytic cells from the peritoneal cavity of mice.

The morphology of the cells and the positive staining for either CD11b or CD11c (Table 3.3) suggested that the F-MS were ingested mainly by macrophages ($CD11b^+$, 70-80%) and to a lesser extent by dendritic cells ($CD11c^+$, 2-6%); a significant number of dendritic cells positive for F-MS were observed at the early stages of the study ($p<0.05$) (Table 3.3). The number of dendritic cells present in the peritoneal cavity was significantly higher in the animals immunized with F-MS than in the lecithin control animals. However, the total population of macrophages was similar. The population of macrophages and dendritic cells was higher in both the test and control animals than in the naïve controls. The number of macrophages and dendritic cells positive for F-MS significantly decreased throughout the study ($p<0.05$).

All the previous experiments were performed with a high injection volume of 0.5 ml per animal. When using lower injection volumes, e.g., 4 x 0.02 ml, cells positive for F-MS were still detectable in the PC at 4 h post-immunisation, although in much lower numbers (~0.001% as opposed to ~1% when immunising with 0.5 ml). The internalisation of the F-MS inside antigen presenting cells (APC) from the PC was again confirmed by confocal microscopy (Fig. 3.8.c); in both section planes, the F-MS (yellow) were surrounded by the skeleton of the cell stained with actin (red). Again, the APC generally ingested more than one F-MS, and the size range of ingested particles was very homogeneous. All the F-MS identified inside cells from any tissue samples were all smaller than 5 μm.

Table 3.3. Phenotype and kinetics of cells containing fluorescent microspheres (F-MS) collected from the peritoneal cavity. The phenotype of the cells was assessed using specific surface markers (SM) for macrophages ($CD11b^+$) and dendritic cells ($CD11c^+$). The data are presented as geometric mean of triplicate determinants ± SEM and the significant differences were calculated at 95% confidence by Student's t-test.

Time/ Control group	SM	Cells expressing SM (% of total cells)	Fluorescent cells expressing SM (% of F-MS positive cells)
4 h	CD11b	8.87 ± 1.68 *	81.5 ± 5.11
	CD11c	0.87 ± 0.11 *$	4.00 ± 0.15
48 h	CD11b	2.38 ± 0.54	74.0 ± 12.5
	CD11c	1.89 ± 1.61	6.01 ± 2.17
1 week	CD11b	6.35 ± 3.35	73.4 ± 12.6
	CD11c	0.19 ± 0.08#	2.13 ± 1.35
4 weeks	CD11b	17.3 ± 5.15	71.8 ± 26.2
	CD11c	0.11 ± 0.03#	0.06 ± 0.06#
Lecithin (1%) control (at 4 h)	CD11b	14.7 ± 6.90	-
	CD11c	0.08 ± 0.005	
Naïve control	CD11b	0.73 ± 0.09	-
	CD11c	0.02 ± 0.007	

* $p<0.05$ compared with naïve control
$ $p<0.05$ compared with 1% lecithin control
\# $p<0.05$ compared with 4h results

In an endeavour to observe the cytosolic localisation of the F-MS inside APC, after s.c.-injection, an *in vitro* experiment was first conducted with cultivated macrophages. Noticeably, the F-MS seemed to degrade and/or aggregate during the sample preparation for the transmission electron microscopy (TEM), so that TEM revealed "ghosts" of the F-MS, which were left as fingerprint within the agarose embedding (Fig. 3.9.a). F-MS uptake *in vitro* by the macrophage cell line was previously confirmed by epifluorescence microscopy (Fig. 3.7.). The TEM analysis suggested that the *in vitro* uptake of the F-MS occurred through phagocytosis and invagination within a vacuole (Fig 3.9.b,c). Attempts were then made to identify the localisation of the F-MS inside peritoneal macrophages from mice immunised with F-MS. However, it appeared rather difficult to identify positively F-MS within the isolated and formaldehyde-fixed cells by TEM (Fig 3.9.d). One of the difficulties resided in the low frequency of F-MS positive cells (1%) in the cell population from the PC, which could not be concentrated for TEM. Further, we were unable to identify positively F-MS within the PC-cells, but noticed large cell vacuole suggesting F-MS-like ghost structures within the cells (Fig. 3.9.e,f).

Following s.c. immunisation of mice with MS, cells from the PC were isolated, and their phenotype characterised as described in chapter 2.5.3. The proportion of CD80, CD86, MHCII and dendritic cell (DC) markers expressed on total $CD11b^+$ cells from naïve animals coincided well with published data from the literature (Makala et al., 2002) (Table 3.4.column 2&3). Macrophages ($CD11b^+/CD14^+$) from mice immunised with MS expressed similar levels of CD80 and DC marker as naïve animals, but higher levels of CD86 and lower levels of MHC class II markers (Table 3.4.column 3&4). No statistical analysis could be performed on this data, as only one animal was used for the naïve group.

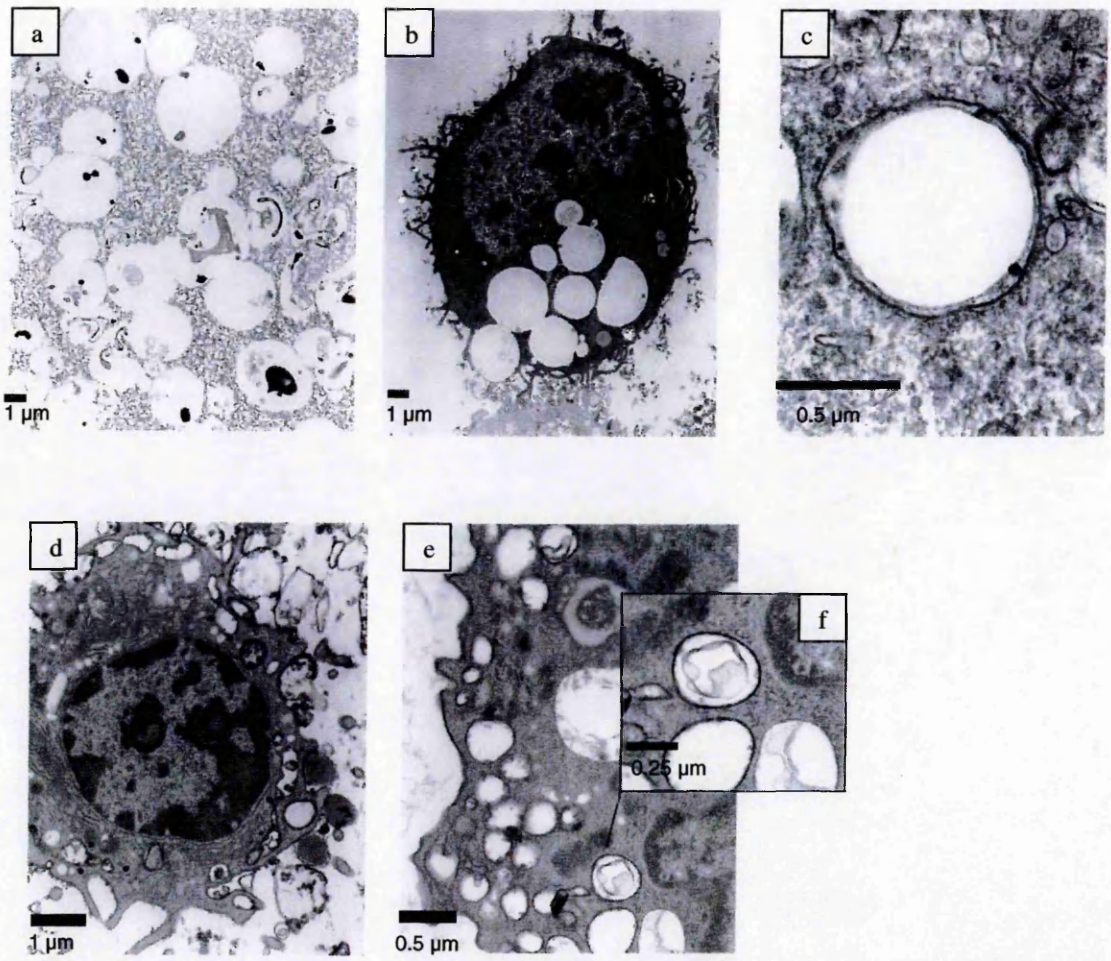


Figure 3.9 Transmission electron micrographs (TEM) of macrophages cultivated *in vitro* and co-incubated with F-MS (b, c) or isolated from the PC of mice previously injected with F-MS (d, e, f). (a) F-MS control embedded in agarose. Notice that there is only a print ("ghost") of the F-MS left after sample preparation for the TEM. (b, c) Cultivated macrophage with ghosts of previously ingested F-MS; the "ghosts" appear to be localised inside a vacuole. (d) Macrophage from the PC of mice previously injected with F-MS. (e, f) F-MS ghosts appear to be located within a macrophage vacuole.

Table 3.4 Phenotype of cells isolated from the PC following *in vivo* uptake. The cells were isolated 4 hours after a booster dose of MS vaccine given 2 weeks post-primary immunisation. Cells were stained with specific markers for macrophages ($CD11b^+$ and $CD14^+$), dendritic cells (DC marker), and for specific APC activation markers (CD80, CD86 and MHC class II).

Surface marker	% of total $CD11b^+$ cells		% of $CD11b^+/CD14^+$ cells	
	Naïve animals	F-MS immunised animals	Naïve animals	F-MS immunised animals
CD80	60.1	42.6 ± 5.44*	97.6	94.1 ± 2.21
CD86	16.7	17.9 ± 5.35	47.2	64.8 ± 3.94
MHC II	70.9	47.5 ± 16.7	73.5	43.7 ± 13.7
DC marker	9.42	6.31 ± 0.82	14.0	11.8 ± 4.51

* $p<0.05$ compared to naïve animals

3-5 *In vitro* versus *in vivo* degradation of F-MS

The first signs of *in vitro* degradation in PBS were observed after 2 weeks, with the majority of F-MS still being intact and brightly fluorescent at that stage (Fig 3.10.a,b). After 3 weeks, a mixed population of degrading and intact MS was observed. Fluorescence was still associated with the degrading particles, although the wavelength was shifted and the fluorescence approached the background yellowish colour (Fig 3.10. c,d). The particulate morphology was largely lost after 4 weeks, and fluorescence was at background level (Fig 3.10. e,f). After 16 weeks, some polymeric residues could still be observed, which were no longer fluorescent (results not shown).

In vivo, the degradation of the F-MS at the IS was monitored in histological sections of the subcutaneous tissue by confocal microscopy. A significant depot of fluorescent particles was observed throughout the whole study period (8 weeks); first signs of polymer degradation appeared from day 7 on, but more advanced particle degradation and morphological changes were obvious only after 4 weeks (Fig 3.11.).

The degradation of F-MS inside phagocytic cells isolated from the PC at 4 h post-immunisation was studied *ex vivo*. A major fraction of phagocytic cells carried more than one F-MS, which had a diameter of less than 5 μm (Fig 3.8.). Bright and nicely spherical F-MS were detected for as long as one week in PC-cells kept in culture. Thereafter, leakage or quenching of the dyes was observed, as the brightness of the particles diminished slowly. No more F-MS were detected after 2 weeks in the PC-cells *ex vivo*; however the cells started to show signs of apoptosis (data not shown).

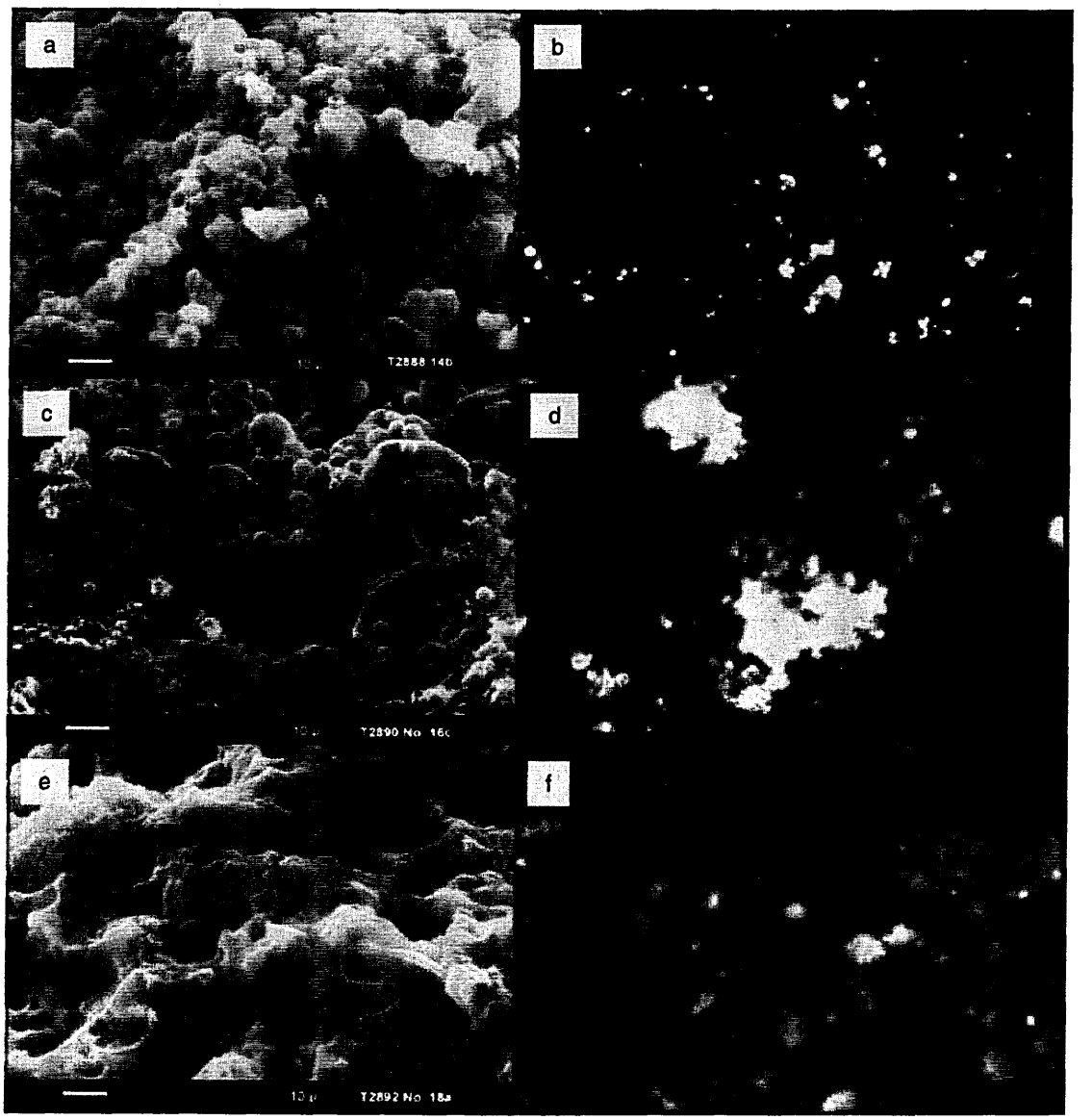


Figure 3.10 Scanning electron and epifluorescence micrographs of the morphological and fluorescence changes during *in vitro* degradation of F-MS after 2 weeks (a, b), 3 weeks (c, d) and 4 weeks (e, f) incubation at 37 °C in PBS of pH 7.4 supplemented with 5% BSA and 0.05% sodium azide (bar scale=10μm).

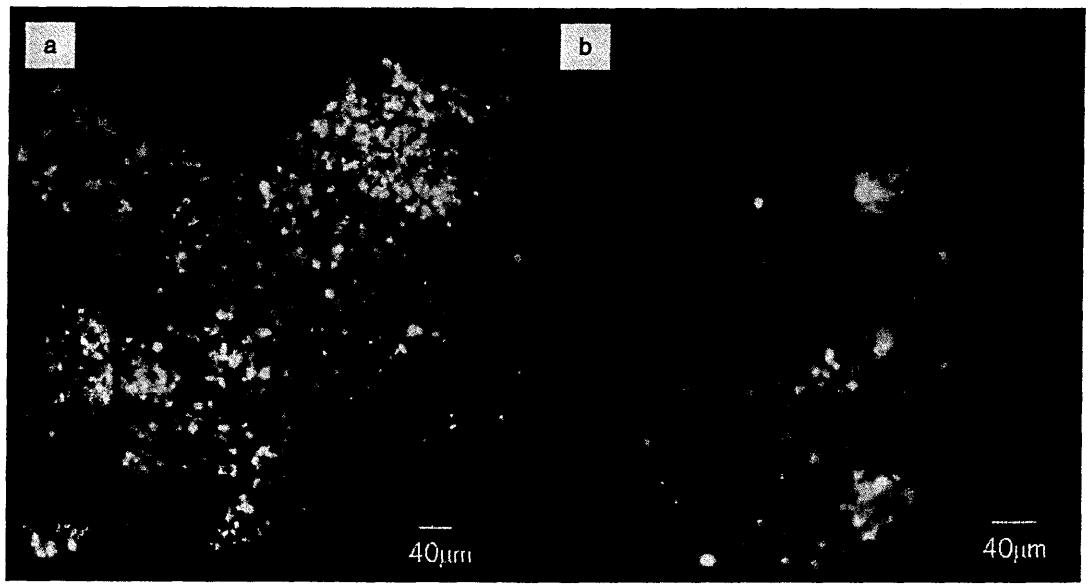


Figure 3.11 Confocal micrographs of frozen sections of the subcutaneous tissue from animals injected s.c. with F-MS. The micrographs show morphological changes of the F-MS during degradation at the injection site at 4 h (a) and 4 weeks (b) post-immunisation

Degradation inside the cells was also observed *in vivo* in cells isolated from PC, MLN and spleen of animals 2 weeks post-immunisation; epifluorescence observations revealed a fluorescent ring surrounding many cells (Fig 3.12.a). A more detailed analysis of spleen cells by confocal microscopy showed that this fluorescent ring was probably due to the degradation of the F-MS inside the cells and leakage of the fluorophores (Fig 3.12.b). Interestingly, mixtures of degrading and intact F-MS could be detected inside phagocytic cells from the PC at this time point (Fig 3.13.).

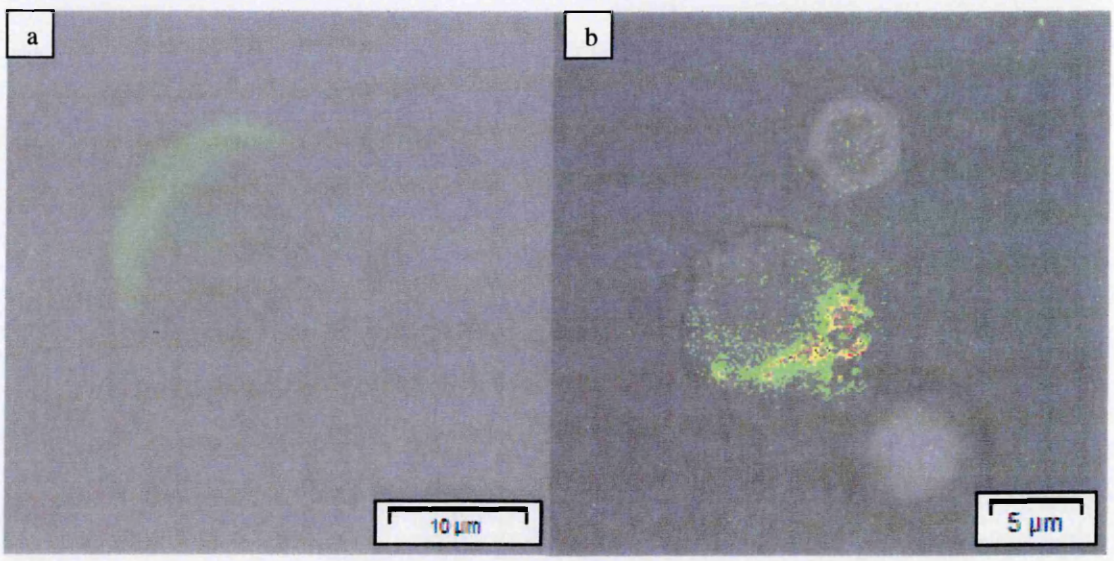


Figure 3.12 F-MS degradation *in vivo*. Spleen cells isolated 2 weeks post-immunisation of mice with F-MS. (a) The epifluorescence micrograph show clearly the fluorescent rings around the cells. (b) The confocal micrograph shows the release of fluorescein (green) and rhodamine (red) from the degrading F-MS inside the cell. Some co-localisation of the dyes is still visible (yellow).

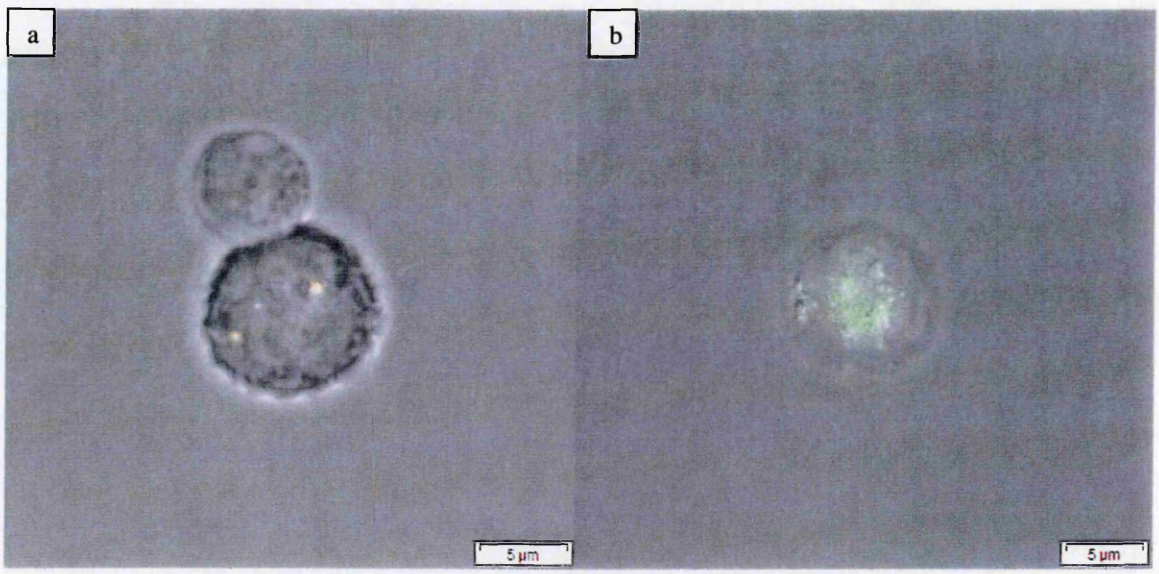


Figure 3.13 Confocal micrographs of cells isolated from the PC at 2 weeks post-immunisation of mice with F-MS. (a) Intact F-MS (yellow) and degrading F-MS (green) were internalised inside the cells. The micrographs suggest that the F-MS were not immediately degraded when internalized by phagocytic cells.

3-6 Inflammatory response induced by F-MS

Differences in the inflammatory response were observed between F-MS and PS beads in the recruitment of phagocytic cells to the IS, with a stronger response for the latter non-immunogenic formulation (Fig. 3.14.). The kinetics of $CD11c^+$ and $CD11b^+$ cells were very similar. It may be hypothesised that the $CD11c^+$ cells were also $CD11b^+$, which is characteristic of the phenotype of peripheral DC. Unfortunately, this hypothesis could not be confirmed with this experiment, as the marking of the cells was made separately using the same conjugated fluorophores. Unfortunately, we could only detect a significant number of $CD11c^+$ cells at the IS at early times when using the single marking technique.

Large-sized F-MS seemed to recruit more $CD11b^+$ cells at the IS than small-sized F-MS throughout the entire experiment (Fig 3.15.a). Up to 4 weeks, the kinetics of $CD11b^+$ cell recruitment was similar in the PC for both the small- and large-sized MS. At 8 weeks, however, more $CD11b^+$ cells were seen in the PC of mice injected with the large-sized F-MS (Fig 3.15.b), whereas the number of $CD11b^+$ cells of animals immunised with the small-sized F-MS was back to the level of the naïve animals (Fig 3.15.b).

The co-injection of alum increased the recruitment of $CD11b^+$ cells at the IS from 2 weeks until the end of the study, where levels of inflammatory cells were not back to the level of naïve animals yet, as opposed to the alum-free formulations (Fig 3.16.a). The number of $CD11b^+$ cells in the PC dramatically increased from 4 to 8 weeks for the formulation with alum, similar to what has been previously seen for the large-sized MS formulation (Fig. 3.15.).

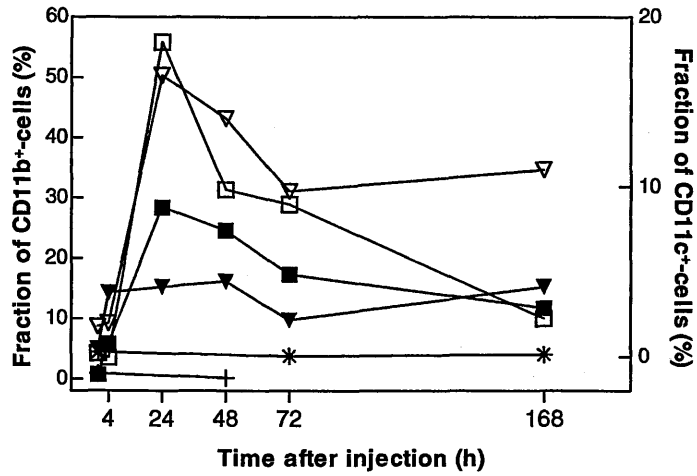


Figure 3.14 Percentage of $CD11b^+$ (square) or $CD11c^+$ (inverted triangle) cells from the IS of mice immunised with F-MS (full symbols) or PS beads (empty symbols). PS beads induced a stronger inflammatory response at the IS than F-MS. The cross and star represent the naïve controls for $CD11b^+$ and $CD11c^+$ cells, respectively. The data are results from one individual animal.

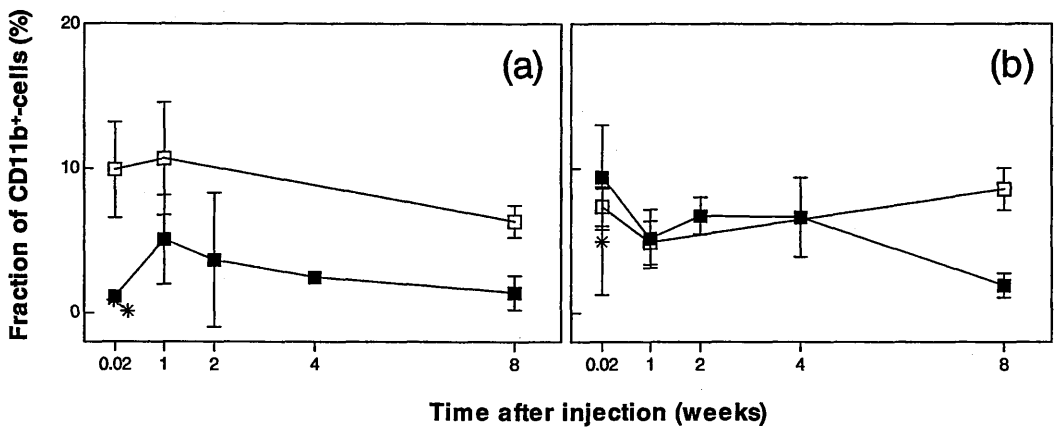


Figure 3.15 Percentage of $CD11b^+$ cells from the IS (a) and the PC (b) of mice immunised with (■) small- and (□) large-sized F-MS. The stars represent naïve animals. Large F-MS formulation induced a stronger inflammatory response. The results are expressed as geometric means ± SEM from 3 individual animals.

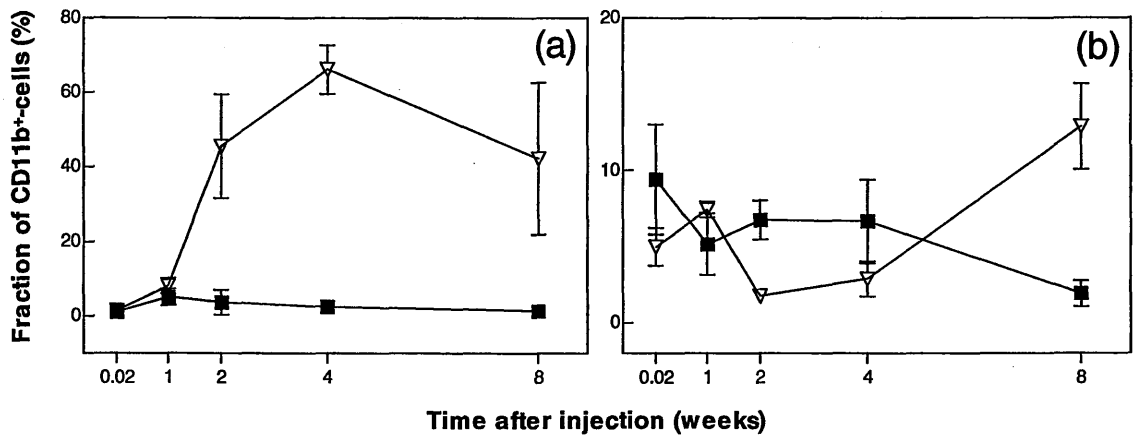


Figure 3.16 Percentage of $CD11b^+$ cells from the IS (a) and the PC (b) of mice immunised with (■) F-MS or (▽) F-MS dispersed in alum. Alum enhanced the inflammatory response. The results are expressed as geometric means ± SEM from 3 individual animals.

CHAPTER 4: IMMUNOLOGICAL PROPERTIES OF PLGA MS VACCINES

4-1 Rationale and outline of the study

This study focused on the characterisation of the biological properties of PLGA MS DT and TT vaccines as a function of their physico-chemical characteristics. PLGA MS vaccine immunogenicity has been assessed previously (Johansen et al., 2000a), however, some critical issues on the importance of formulation parameters such as the MS size or the hydrophobic nature of the polymer remain. The adjuvant efficacy of the microspheres versus their antigen delivery ability has also been questioned and co-administration of the PLGA based vaccine with a potent adjuvant such as aluminium hydroxide has been suggested. Moreover, only a limited number of studies investigated the long term protective efficacy of PLGA MS vaccine administered as a single dose (Johansen et al., 1999) compared to the commercial alum adsorbed vaccine administered with a booster dose schedule.

The variables studied to determine the relationship between formulation and immune response encompassed:

- polymer type with increasing hydrophobicity from RG502H, RG503H to RG503
- MS size, small-sized (0.5μm-10μm diameter) versus large-sized (10μm-60μm diameter)
- DT antigen loading in the PLGA MS, from 0.14 to 9.3 Lf/mg
- antigen immunisation dose, from 1/5 to 1/80 dilution of a single human dose (SHD) equivalent to 5Lf DT/2Lf TT and 0.3Lf DT/0.1Lf TT
- co-administration of various doses of alum, from 1/2 SHD (0.35mg Al^{3+}) to 1/200 SHD (0.0035mg Al^{3+})

The formulations were characterised *in vitro* by measuring their antigen loading and the 24h antigen burst release by ELISA. The biological properties were assessed *in vivo* in mice and guinea-pigs by looking at the antigen specific cellular response by spleen T-cell proliferation and cytokine production and at the total and specific neutralising antibodies by ELISA and toxin neutralisation assays respectively. Long term protection was assessed in both models by direct challenge with diphtheria or tetanus toxin (in guinea-pigs and mice respectively) 24 - 44 weeks post-immunisation with a single dose of MS formulations (with or without alum) against two injections of the commercial alum adsorbed vaccine (alum control vaccine).

4-2 Formulations

Various particulate diphtheria and tetanus vaccine formulations were made using three different type of polymers that varied in their molecular weight and hydrophobicity (PLGA with uncapped or esterified –COOH end-groups). The PLGA MS were prepared by conventional spray-drying to generate small-sized (approx. 0.5-10 μm) particles and by polymer coacervation to produce large-sized (approx. 10-60 μm) particles. Actual antigen loadings in the PLGA MS (Table 4.1.) were varied by microencapsulating different concentrations of aqueous toxoid solutions, as detailed in chapter 2.2. The effect of admixing alum to the toxoid containing PLGA MS was examined with a divalent formulation (co-encapsulated DT and TT), as multivalent PLGA MS vaccines had already produced promising data (Boehm et al., 2002); divalent formulations were also considered more interesting to compare against the current licensed vaccine which always contains at least two components (DT and TT).

Table 4.1. Summary of the MS vaccine formulations used for the immunogenicity study

Formulation code[1]	Antigen[2]	Polymer type[3]	MS Size [4] (μm)	Actual toxoid loading (Lf/mg MS)
Low loading	DT	RG 503H	0.5-10	0.14
Medium loading (SD 503H batch 1)	DT	RG 503H	0.5-10	0.85
High loading	DT	RG 503H	0.5-10	9.31
SD 503H batch 2	DT	RG 503H	0.5-10	2.16
SD 503	DT	RG 503	0.5-10	1.05
SD 502H batch 1	DT	RG 502H	0.5-10	0.60
SD 502H batch 2	DT	RG 502H	0.5-10	0.31
CO 502H	DT	RG 502H	10-60	0.53
CO 503H	DT	RG 503H	10-60	1.64
SD-503H-TT	TT	RG 503H	0.5-10	0.20
MS Divalent	DT &	RG 502H	0.5-10	0.89
	TT			0.43
Alum control	DT &	N/A	N/A	50 Lf/ml
	TT	(1.20 mg/ml Al^{3+})		20 Lf/ml

[1] SD = spray-dried MS; CO = coacervated MS; MS = microspheres

[2] DT = diphtheria toxoid; TT = tetanus toxoid; all MS formulations also contained 4% (nominal) succinylated gelatine (Physiogel) as antigen stabiliser, except for formulation SD502H-batch 1 and MS divalent which contained 5% BSA + 5% trehalose.

[3] RG502H = 14 kDa end-group uncapped PLGA 50:50; RG503H and RG503 = end-group capped and uncapped 35 kDa PLGA 50:50 (hydrophobicity: RG502H<RG503H<RG503)

[4] For SD MS batches, typical particle size distributions were: d(v,s) (10% undersize) = 0.5-0.8 um; d(v,s) (50% undersize) = 2-4 um; d(v,s) (90% undersize) = 7-10 um

For CO MS batches, typical particle size distributions were: d(v,s) (10% undersize) = 10-15 um; d(v,s) (50% undersize) = 20-35 um; d(v,s) (90% undersize) = 35-70 um

4-3 Importance of polymer type, particle size, antigen type and antigen loading for the PLGA MS vaccine properties

The influence of polymer type on the *in vitro* and *in vivo* properties of microencapsulated DT vaccine was assessed using formulations SD502H (batches 1 and 2), SD503H (batches 1and 2) and SD503 increasing in the hydrophobic nature of the polymer. These PLGA MS were of comparable size (small 0.5-10μm diameter), and to enable comparison, the differences in antigen loading between the formulations (from 0.31 to 2.16Lf/mg), were accounted for during statistical analysis of the data.

The *in vitro* 24 h burst release of microencapsulated DT was significantly affected by the polymer type used in the formulation ($p=0.01$) (Fig. 4.1.a). The DT burst release from the RG502H MS was significantly higher than from the RG503H MS which was also higher than from RG503 MS ($p<0.05$) (Fig. 4.1.a). The decrease in burst release from RG502H to RG503H and RG503 MS coincides with the increase in the hydrophobic nature of these polymer types. Anti-DT Ab levels induced in mice 4 weeks post-immunisation with the toxoid-loaded PLGA MS also differed significantly between the polymer types ($p<0.0001$) (Fig. 4.1.b), although those differences did not correlate with the burst release data. Indeed, Ab levels were significantly highest for RG503H MS and significantly lowest for the RG502H MS ($p<0.05$) (Fig. 4.1.b, solid bars).

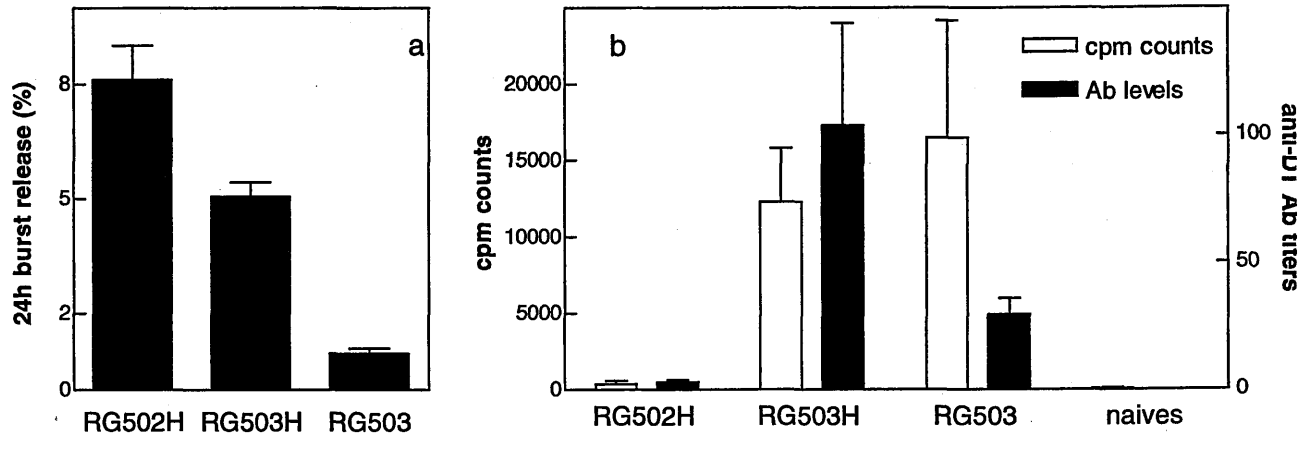


Figure. 4.1. Influence of the polymer type on DT *in vitro* release and *in vivo* immune response. (a) 24 h *in vitro* burst release of DT, expressed in Lf/mg MS, and (b) cellular (cpm counts) and humoral (Ab titers) responses at 4 weeks post-immunisation. The results are expressed as geometric means ± SEM from 3 individual assays and animals respectively. The polymer types RG502H, RG503H and RG503 are further specified in Table 4.1. The immune response appeared to increase with the hydrophobicity of the polymer type. No correlation was observed between burst release and immune response induced.

Similarly, the DT specific cellular response measured from spleen T-cells from the same animals was induced only by the RG503H and RG503 MS, with no significant difference being detected between the two formulations (Fig 4.1.b, empty bars). The immune response was highly improved with increasing hydrophobicity of the polymer type, with higher cellular and Ab responses for SD503H and SD503 than SD502H. This was inversely related to the release of the antigen from the MS during the first 24 hours as SD502H burst release was higher than for SD503H and SD503 formulations.

The influence of MS size on *in vitro* and *in vivo* properties of microencapsulated DT vaccine was assessed using formulations with small-sized MS (SD503H batch 1 and 2) and large-sized MS (CO503H). These were made of the same polymer type (RG503H), and again, the differences in antigen loading between the formulations (from 0.47 to 2.16 Lf/mg), were accounted for during statistical analysis of the data.

Significantly higher 24h *in vitro* burst release was detected with the large-sized (10-60 μm) MS than with the small-sized (0.5-10 μm) MS ($p=0.002$; Fig 4.2.a). Interestingly, only the small-sized PLGA MS elicited a substantial anti-DT Ab level and DT specific cellular response (by spleen T-cell proliferation) in mice 4 weeks post-immunisation (Fig 4.2.b). As seen with the polymer type, the influence of the microsphere size on the response appeared to be inversely related to the antigen release in the first 24h. A lower immune response was measured 4 weeks post-immunisation with larger-sized MS than smaller-sized MS whereas the 24h antigen burst release was higher for the larger ones.

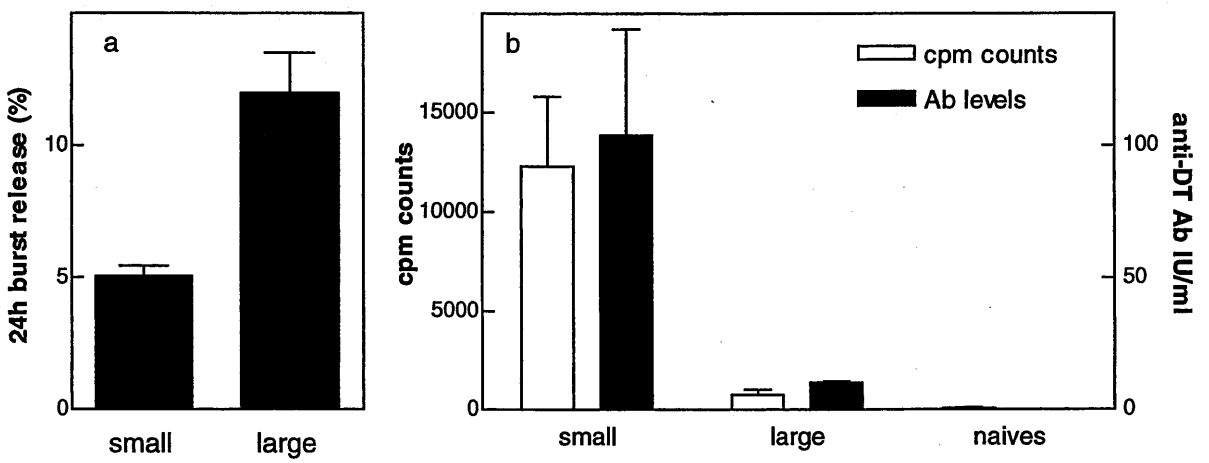


Figure 4.2. Influence of MS size on the DT *in vitro* release and *in vivo* immune response. (a) 24 h *in vitro* burst release of DT, expressed in Lf/mg MS, and (b) cellular (cpm) and humoral (nAb in IU/ml) responses. The results are expressed as geometric means ± SEM from 3 individual assays and animals respectively. "Small" refers to 0.5-10 μm RG503H MS and "large" to 10-60 μm RG503H MS.

The cytokine production induced by *ex vivo* DT restimulation of spleen T-cells from mice immunised with different polymer type or size DT-MS (SD502H, SD503H, SD503, CO502H and CO503H) was assessed at 4 weeks post-immunisation. With the small-sized MS made of RG503H and RG503 polymer types, a substantial level of IL-2 and no inhibition of IFN-γ production was observed, indicating a Th1 type bias response (Table 4.2.). The MS made of RG503H also induced high levels of IL-6 suggesting a mixed Th1/Th2 response (Table 4.2.). The formulation made with polymer RG502H (SD502H and CO502H) and the large-sized CO503H MS clearly inhibited the IFN-γ production. The concomitant induction of IL-10 production with small- and large-sized RG502H MS strongly advocates for a bias towards Th2 type of response with this less hydrophobic polymer type (Table 4.2.). Considering both the cellular response (Fig. 4.1.b) and the IL-2 and IFN-γ production (Table 4.2.), it appears that a Th1 type of response is favoured with increasing hydrophobic MS. This supports the higher cellular response observed with increasing polymer hydrophobicity seen in figure 4.2.

The influence of DT loading inside the MS on the *in vitro* and *in vivo* properties of PLGA MS vaccine was assessed using formulations made with low (0.14), medium (0.85) and high (9.31) antigen loading (Lf/mg). The formulations were designed to differ by a 10 times ratio in their loading and were prepared with the same polymer type (RG503H) and MS size (0.5-10 μm).

The DT-loading in the MS affected the *in vitro* burst release, which surprisingly decreased with increasing antigen loading ($p=0.001$) (Fig. 4.3.). However, the increase in antigen loading correlated neither with an increase in the cellular nor in the humoral response as measured in mice, 4 weeks post-immunisation (Fig. 4.3.). The formulation with the medium loading (0.85

Lf/mg MS) appears to perform better than both low and high loadings (0.14 and 9.31 Lf/mg MS, respectively) (Fig. 4.3.). This may suggest an upper and lower threshold limit in the suitable antigen loading when using this particular type of formulation (small RG503H MS).

Table 4.2. Cytokine profile assessed at 4 weeks post- s.c.- immunisation of mice with small-sized (SD) and large-sized (CO) MS made of RG502H, RG503H and RG503 and loaded with DT. The cytokine production was measured with cells isolated from the spleen and re-stimulated *ex vivo* with DT. The results are expressed in pg/ml as geometric means from 3 individual animals. (Red) represents the up-regulation of the cytokine expression whereas (blue) represents its down-regulation. A balanced Th1/Th2 response was induced by the more hydrophobic polymer type (RG503H and RG503).

Groups	Cytokine production in pg/ml			
	IFNγ	IL-2	IL-6	IL-10
SD502H	-63	33	2512	109
SD503H	51	581	5907	-94
SD503	54	953	527	-316
CO503H	-7	144	4037	-16
CO502H	-81	48	59	141
Naives	47	-80	115	-146

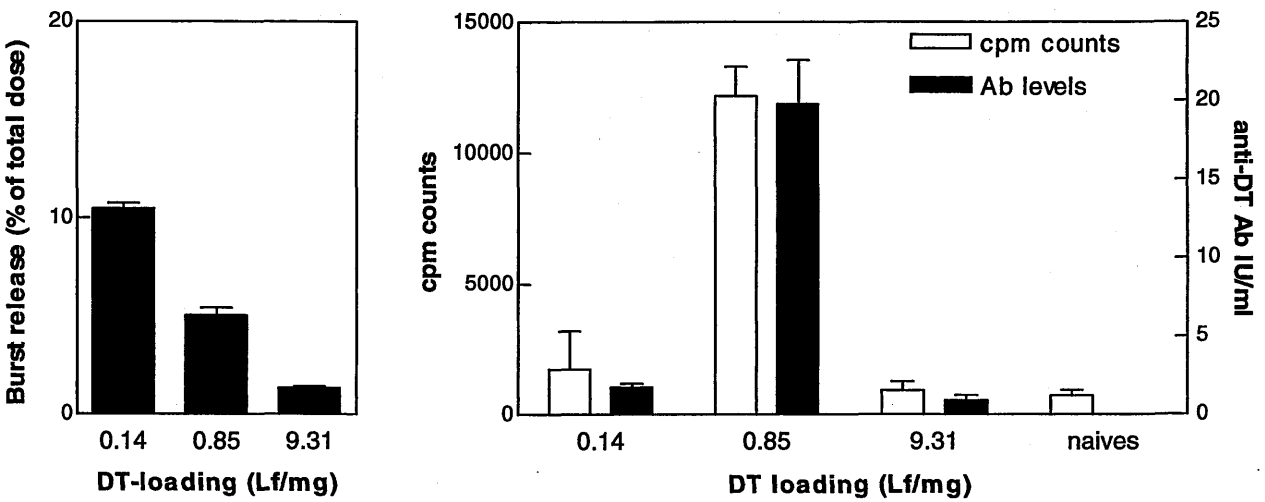


Figure 4.3. Influence of DT-loading in RG503H MS on the *in vitro* release (left panel) within the initial 24 h of incubation (= burst release) and on the cellular (cpm) and humoral (nAb in IU/ml) responses in mice (right panel). The results are expressed as geometric means ± SEM from 3 individual animals.

4-4 Role of co-administration of alum on PLGA MS immunological properties

In this study, the affect of alum on two small-sized MS formulation varying in polymer hydrophobicity (RG502H or RG503H) was investigated. Animals were immunised with single dose MS vaccine, DT-TT MS divalent (RG502H) (guinea-pigs and mice) or mixture of SD503H-DT batch 1 and SD503H-TT (mice only) alone or co-administered with 3 different doses of alum (dose 1, 2 and 3 equivalent to 0.3, 0.03 and 0.003 mg/dose of Al^{3+}, respectively) or with the alum control vaccine administered twice (4 weeks booster; dose 2: 0.03 mg/dose Al^{3+}) (Table 2.2 protocol 2). Total and neutralising Ab levels were measured in guinea-pigs' serum by ELISA and *in vivo* toxin neutralisation respectively. Long term protection against diphtheria toxin was assessed by direct challenge of the same animal one year post-immunisation. Cellular responses were measured in spleen cells from mice, 6 weeks post-immunisation, by antigen specific T-cell proliferation and cytokine production.

Total Ab-responses against both DT and TT encapsulated in RG502H MS divalent vaccine were greatly enhanced in the presence of alum (Fig. 4.4.). The anti-DT response peaked at 16 weeks for all formulations. Thereafter, the Ab-levels decreased in the animals that had received the formulations with the highest doses of alum (MS+alum 1; MS+alum 2; alum-control), although this difference was statistically insignificant because of large variability in the response for the alum control group. In the animals that had received the alum-free MS, the Ab-levels remained constant from week 16 (1.14 IU/ml) till week 44 (1.05 IU/ml), time point at which the study was terminated (Fig. 4.4.a). At the peak of the response, the MS+alum 1 and the alum control vaccines induced significantly higher anti-DT Ab-levels than

the alum-free MS formulation ($p<0.05$). This difference persisted until the end of the study ($p<0.01$) (Fig 4.4.a).

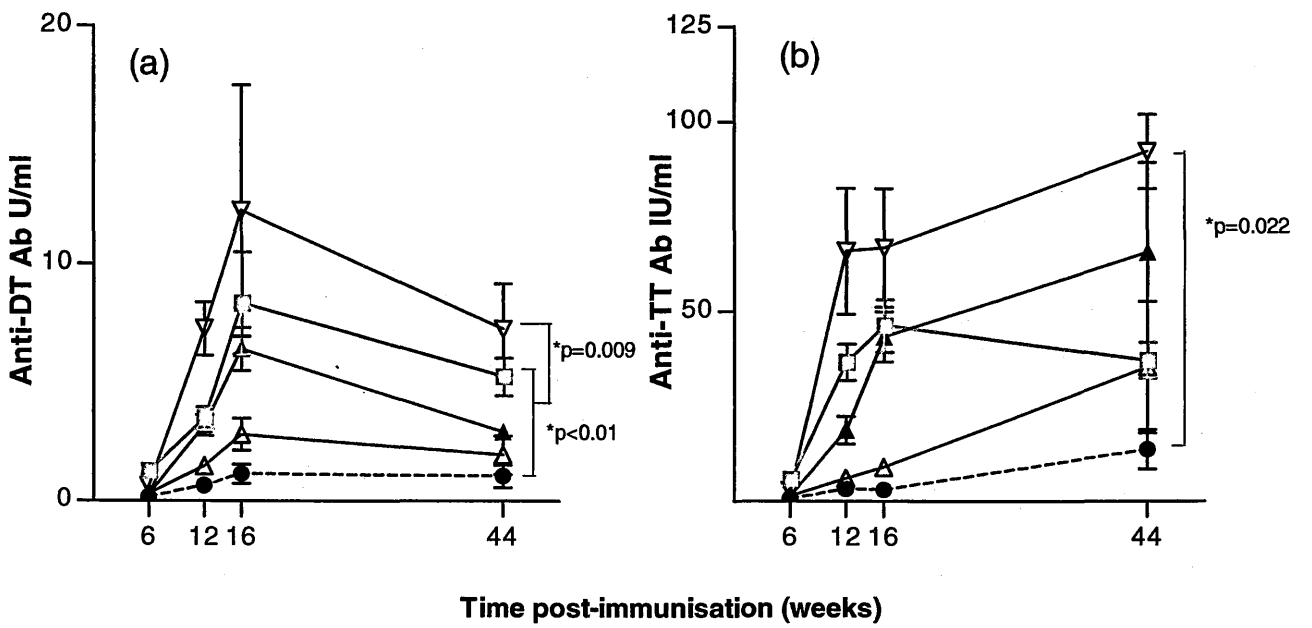


Figure 4.4 Influence of co-admixed alum on the immune response induced by MS-DT/MS-TT. Total anti-DT (a) and anti-TT (b) Ab-titres induced in guinea-pigs immunised with a single injection of alum-free divalent MS (RG502H) (●), divalent MS admixed with 0.3 mg (▽, alum 1), 0.03 mg (▲, alum 2) or 0.003 mg (△, alum 3) Al3+, or immunised with two injections of alum-adsorbed control vaccine (0.03 mg Al3+) (□). The results are expressed as geometric means ± SEM from 8 individual animals. The data show that alum admixed to the MS vaccine strongly enhanced total anti-DT and anti-TT Ab responses.

In contrast to the kinetics of the DT-response, the anti-TT Ab-levels induced by the MS formulations did not decrease from 16 to 44 w, although the Ab-levels diminished tendentiously for the alum-control vaccine (37 IU/ml at 44 w against 46 IU/ml at 16 w). At the end of the study, only the MS vaccine with the highest dose of admixed alum (MS + alum 1) maintained a significantly higher anti-TT antibody response than the alum-free MS ($p=0.022$), and the mean response for the alum control vaccine was twice as high as for the alum-free MS (37 IU/ml versus 14 IU/ml, respectively), although this difference was statistically insignificant because of large variability in the responses (Fig 4.4.b).

The Ab-kinetics demonstrated clearly the boosting properties of the MS vaccine, as a single injection of the MS vaccine, co-administered with or without alum, yielded comparable antibody kinetic profiles as 2 injections of the alum control vaccine (Fig 4.4.). However, the MS vaccine made of RG502H polymer type required co-administration of alum to induce Ab levels similar to those elicited by two injections of the alum control vaccine. Indeed, the alum-free MS vaccine failed to induce anti-DT Ab-levels similar to those of the alum control vaccine ($p<0.01$, Fig 4.4.a). Nonetheless, for all the groups, the Ab titers after 4 weeks were higher than the minimum protective level of 0.1 IU/ml for both antigens.

A clear alum dose - antibody response relationship was observed for total anti-DT and anti-TT Ab levels at 44 w (Fig. 4.5.), i.e., the Ab-production increased with the increase of co-administered alum. There was a better semi-logarithmic correlation (Pearson correlation test) between the two parameters for TT ($r^2=0.997$, $p=0.0014$), than for DT ($r^2=0.892$, $p=0.0554$) (Fig. 4.5.).

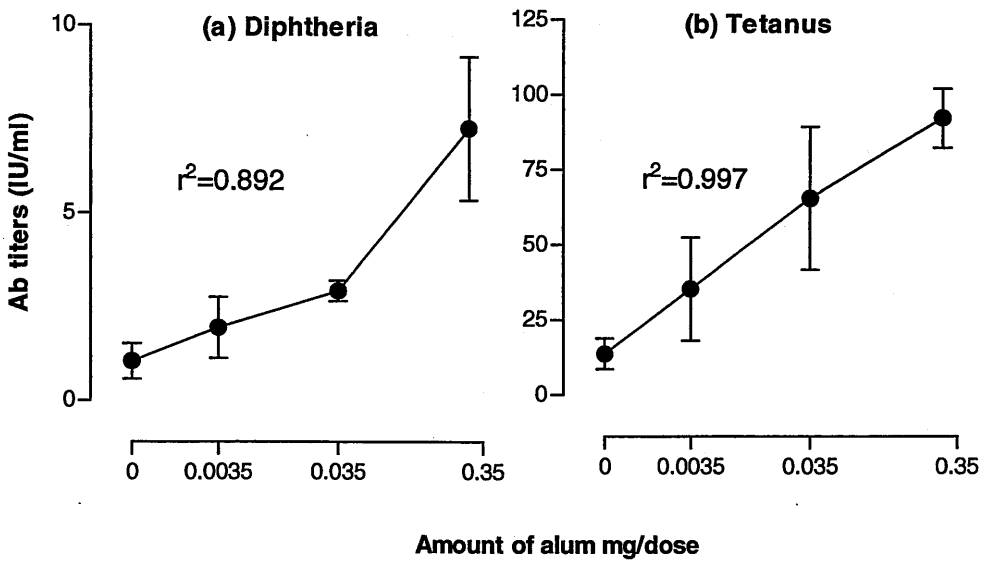


Figure 4.5 Correlation between anti-DT and anti-TT Ab levels and dose of co-admixed alum. Guinea-pigs anti-DT (a) and anti-TT (b) antibody titres at 44 weeks post-immunisation with MS divalent (RG502H) containing admixed alum. The results are expressed as geometric means ± SEM from 3 individual animals. The humoral response depended on the dose of co-administered alum. The coefficient of determination was better for TT (r^2=0.997) than for DT (r^2=0.892).

The cellular response induced by the divalent MS vaccine (RG502H) depended also on the amount of co-admixed alum. This was most pronounced at 6 w post-immunisation where the cellular response was higher with increasing doses of co-admixed alum (Fig 4.6.a and b). However, the MS formulations played a critical role in the adjuvantation of the response. Indeed, the MS-alum mixtures injected once induced a significantly ($p<0.0001$ for both DT and TT) higher cellular response than the alum control vaccine given twice, which contrasts with the findings for the Ab-responses. The alum-free MS given as a single injection induced similar cellular responses as 2 injections of the alum control vaccine ($p=0.122$ for DT; $p=0.065$ for TT) (Fig. 4.6.a,b). Interestingly, placebo MS and alum stimulated a specific cellular response of spleen cells *ex vivo* in animals that had been immunized with the antigen along with both adjuvants (MS+alum) which might indicate a high activation status of the APCs present within the cell samples isolated from those groups (Fig 4.6.c,d).

The observations on the cellular response were supported by the cytokine profile data measured from the same *ex vivo* re-stimulated spleen cells collected from the immunized mice at 2 and 6 weeks post-immunisation (Fig 4.7.).

The IL-2 production (marker for T-cell activation) induced by TT highly depended on the dose of admixed alum in the MS vaccine at 2 and 6 weeks post-immunisation. The IL-2 levels decreased with lower alum doses (Fig 4.7.b). The pattern was a little different for DT as MS vaccines with or without alum induced higher IL-2 production than the alum control (Fig 4.7.a). After 6 weeks, the IL-2 production was antigen-dependant, as no IL-2 was produced in the naïve animals or when placebo MS were injected.

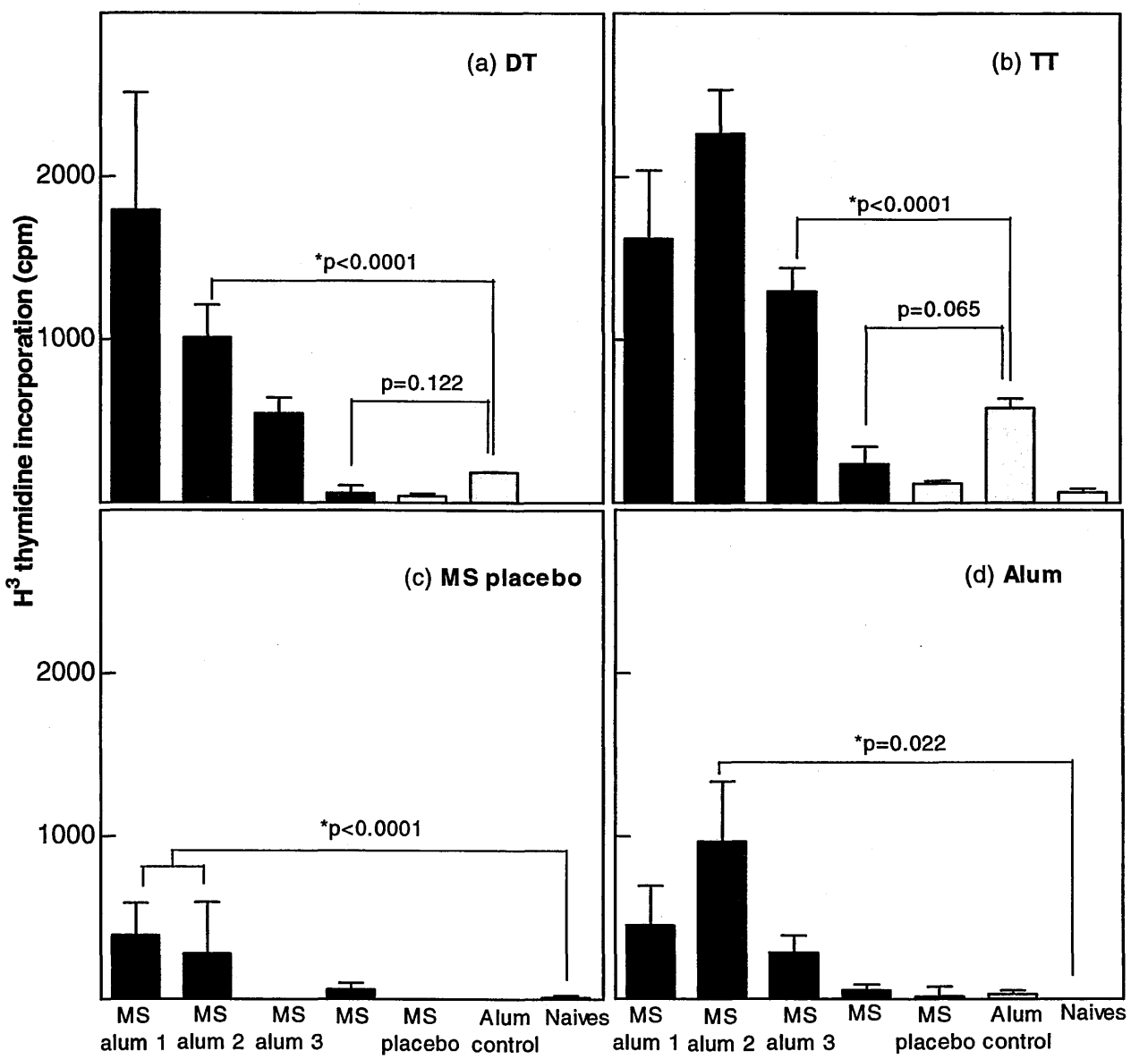


Figure 4.6 Influence of co-admixed alum on the cell proliferation. Mice were immunised s.c. with MS divalent (RG502H), with or without admixed alum, or with alum control vaccine. At 6 weeks post-immunisation, the proliferation of cells isolated from the spleen and re-stimulated *ex vivo* with (a) DT, (b) TT, (c) placebo MS or (d) alum was measured. The results are expressed as geometric means ± SEM from 3 individual animals. Alum exerted a synergetic effect on the cellular response when co-admixed with MS.

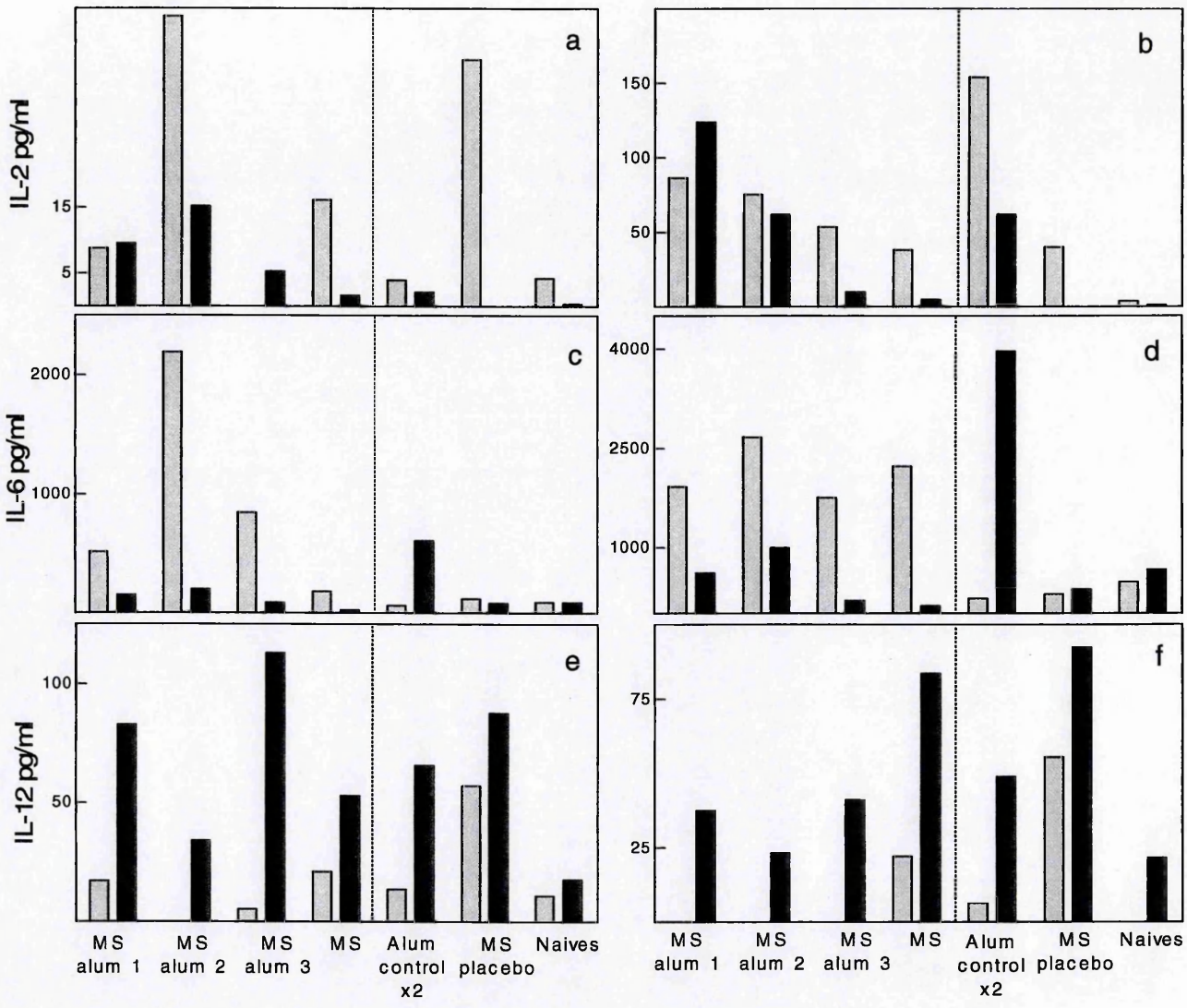


Figure 4.7 Influence of co-admixed alum on splenocyte cytokine production. Cytokine production was measured in isolated spleen cells from immunized and naïve mice upon re-stimulation *ex vivo* with DT (a, c, e) and TT (b, d, f) at 2 weeks (grey bars) and 6 weeks (black bars) post-immunisation with RG502H MS divalent, with or without alum, RG502H MS placebo or 2 doses of the alum control vaccine given as a 4 weeks booster. Naïve animals were used as negative controls. The data are from pooled samples of 5 individual animals and are expressed in pg/ml. Alum increased the inflammatory response (higher IL-6 secretion) and the T-cell activation (higher IL-2 secretion) when co-administrated with MS.

IL-6 (marker of macrophage activation towards MHC class II Ag presentation during Th2 type response) was produced at a very high level (7 fold higher than naïve control) after the booster dose of alum control vaccine (Fig 4.7.c,d, black bars). Conversely, IL-6 was much lower at 6 weeks post-immunisation for animals immunized with MS vaccines (maximum of 2 fold increase in the presence of alum), although at 2 weeks post-immunisation, only the MS vaccine alone and/or MS vaccines co-admixed with alum induced IL-6 production (2 fold higher than naïve control for MS without alum ; 23 fold higher for MS with alum) (Fig 4.7.c,d, grey bars).

An opposite pattern to IL-6 production was observed for IL-12 (marker for macrophage and DC activation towards MHC class I Ag presentation during Th1 response). At 2 weeks post-immunisation, none or very low levels of IL-12 specific to TT re-stimulation were produced in animals that had received the alum control vaccine or the MS co-administered with alum (Fig 4.7.e,f, grey bars). At 6 weeks post-immunisation, the TT-specific IL-12 production was 2 to 4 fold higher in the animals that had received either placebo MS or MS vaccine than in all the other immunized groups; the presence of alum in the vaccine formulation moderated the IL-12 response (Fig 4.7.e,f, black bars). The DT-specific IL-12 production did not seem to be significantly influenced by the presence of alum in the vaccine, neither at 2 nor at 6 weeks post-immunisation, as all the groups of animals induced similar levels (Fig 4.7.e).

Interestingly, the animals immunized with placebo MS generally produced high IL-2 levels (4-13 times higher than naïve control) and IL-12 levels (4-50 times higher than naïve control) at 2 and 2-6 weeks respectively.

IL-12 production was also higher for any of the immunized animals than for the naïve mice when the spleen cells were re-stimulated *ex vivo* with a placebo formulation (lecithine, placebo MS or alum) (data not shown). This was expected from compounds able to induce inflammatory response on their own.

A synergistic effect of alum and the MS formulations was observed only when the MS formulations themselves were weak inducers of cell-mediated immunity (CMI) (i.e., formulation based on RG502H polymer). Indeed, when RG503H polymer was used instead of RG502H, the cellular response was not affected by the admixture of alum ($p= 0.925$ for DT and $p= 0.677$ for TT) (Fig 4.8.). This also held true for the cytokine production as all formulations induced similar IL-2, IFN-γ, IL-4, and IL-6 levels, independent of the presence of co-administered alum and all significantly higher than for the naïve control ($p<0.05$) (data not shown).

Guinea-pigs immunised once with RG502H MS divalent vaccine, with or without alum, or twice with the alum control vaccine were challenged with diphtheria toxin 44 weeks post-immunisation (Fig 4.9.). All the groups showed higher protective responses than naïve animals, but the differences between the immunised groups were statistically insignificant (high variability). Very interestingly, MS vaccines afforded higher protection than the commercial alum control (as measured by ED50), independent of the presence of admixed alum in the formulation; the protection was 3.5-fold higher for the MS alum 1-group (1/2 SHD mg Al^{3+}) and up to 4-fold higher for the formulation without alum (Fig. 4.9.).

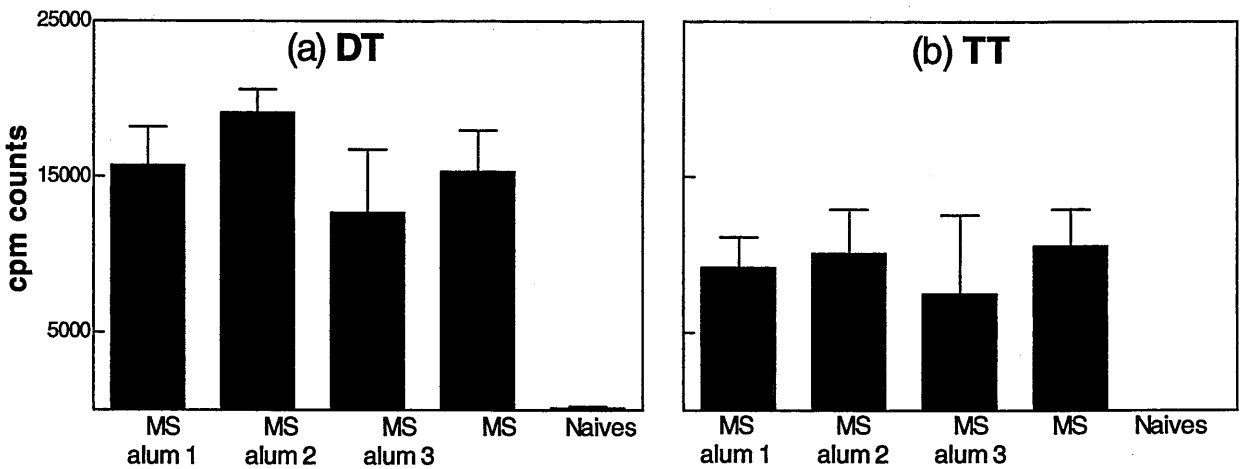


Figure 4.8 Influence of co-admixed alum on cellular response induced by DT and TT encapsulated into PLGA 503H polymer type. Mice were immunized subcutaneously once with a mixture of SD503H-DT and SD503H-TT MS, with or without admixed alum, or twice with alum control vaccine. The T-cell proliferative response was measured at 4 weeks post–immunisation by ex-vivo stimulation of the spleen cells with (a) DT or (b) TT-vaccines. The results are expressed as geometric means ± SEM from 3 individual animals. The co-administration of alum with MS does not exert a synergistic effect on the cellular response when the MS-formulation alone is highly immunogenic (RG 503H polymer type).

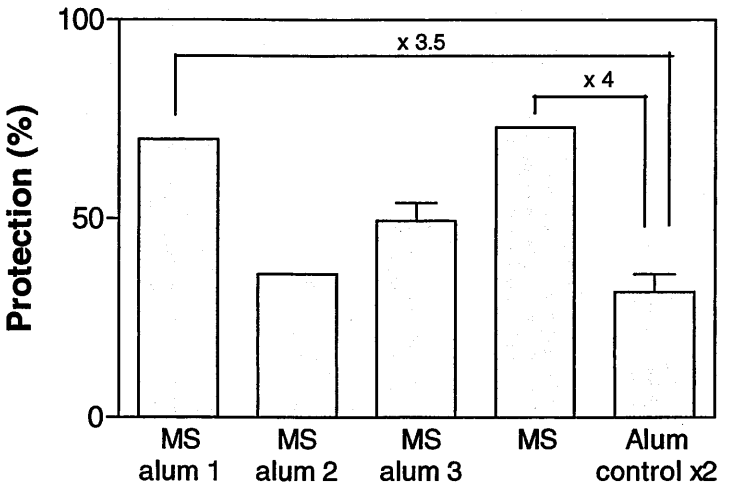


Figure 4.9 Protection against diphtheria toxin, effect of co-administration of MS and alum. Guinea-pigs were immunised with a single injection of RG502H MS divalent, with or without admixed alum, or with two injections of alum control vaccine. The animals were challenged intradermally with diphtheria toxin 44 weeks post-immunisation. Results are expressed as percentage of animals protected at 4mLf toxin/dose (160x MRD). The geometric mean ± SEM from three sets of data from 3 different observers is plotted. No correlation was observed between the protective response and the amount of alum present in the formulation.

The protection data against diphtheria toxin were confirmed by the neutralising antibody levels, which, at the end of the study (44 w), showed no significant differences between MS alone and the alum control vaccine (p=0.867 for DT and p=0.064 for TT) (Table 4.3.). The nAb data did not correlate with the total Ab titers which, as observed previously, were higher for the alum control group than for the MS vaccine group (Fig 4.4.). Moreover, an increase in nAb levels was observed for both Ag between 16 and 44 weeks when the MS were used. The percentage of increase in nAb was inversely proportional to amounts of co-administered alum, for both the DT and TT (Table 4.3.). The increase in nAb levels was 2-3 times higher for MS no alum than MS with alum. Conversely, the nAb titers induced by the alum control vaccine dropped, between weeks 16 and 44, by 50% for DT and by 25% for TT.

For tetanus, none of the immunised animals showed signs of paralysis at the end of the challenge assay, while the non-immunised control animals were affected. Therefore, no group comparison could be made from the challenge with tetanus toxin in mice. However, when looking at the nAb data, there was a significant difference between the MS and the alum control vaccine groups (Table 4.4). The MS vaccine alone induced significantly lower nAb than the alum control (p=0.027). Interestingly, differences appeared in the kinetics of the nAb titres. Only the MS alone or co-injected with the lowest amount of alum maintained the nAb titres constant between weeks 12 and 24 (Table 4.4). Conversely, there was a significant drop in the anti-TT nAb levels for MS co-injected with alum-1 and -2 between those time points. Unfortunately, the 12 weeks nAb level was not measured for the alum control, but one may expect this formulation to perform similarly to the MS alum-2.

Table 4.3. *In vivo* passive neutralisation of diphtheria or tetanus toxins by sera from guinea-pigs immunised with a single injection of RG502H MS vaccine, with or without alum, or with two injections of the alum control vaccine. Neutralising potency was determined on pooled serum from 16 and 44 weeks post-immunisation. Results were expressed in IU/ml, against corresponding anti-DT or TT international standards. Limits were calculated with 95% confidence intervals.

	Anti-DT nAb IU/ml		
Groups	16 weeks	44 weeks	R (nAb ratio between 44 and 16 w)
MS + alum 1	21.0 (8.40-55.0)$^{*, ***}$	53.6 (20.0-134)*	↗ 255%
MS + alum 2	9.20 (3.50-22.9)	24.0 (8.70-58.0)	↗ 255%
MS + alum 3	3.40 (1.50-8.40)	30.0 (12.0-75.0)	↗ 800%
MS no alum	1.00 (0.40-2.60)	7.30 (2.70-18.3)	↗ 700%
Alum control	16.3 (6.30-40.7)*	8.10 (2.70-21.5)	↘ 50%

	Anti-TT nAb IU/ml		
Groups	16 weeks	44 weeks	R
MS + alum 1	88.0 (80.0-123)$^{*, **, ***, \$}$	151 (108-211)$^{*, **, ***, \$}$	↗ 170%
MS + alum 2	45.5 (34.0-64.0)$^{*, ***}$	78.3 (71.0-110)$^{*, ***, \$}$	↗ 170%
MS + alum 3	12.0 (10.9-16.9)*	35.0 (26.9-38.5)*	↗ 290%
MS no alum	4.90 – 10.0	16.9 (15.3-23.6)	↗ 340%
Alum control	42.6 (30.0 -57.0)$^{*, ***}$	31.9 (24.5-41.4)*	↘ 25%

*p<0.05 compared to MS vaccine alone ; **p<0.05 compared to MS + alum 2;

***p<0.05 compared to MS + alum 3 ; $^{\$}$p<0.05 compared to alum control group

Table 4.4. *In vivo* passive neutralisation of tetanus toxins by sera from mice immunised once with MS vaccine alone or co-injected with three different doses of alum, or immunised twice with the alum control vaccine. Neutralising potency was determined on pooled serum from 12 and 24 weeks post-immunisation. Results are expressed in IU/ml, against TT international standard. Limits were derived from ED50 dose response curves, expressed in 95% confidence intervals.

Groups	Anti-TT nAb IU/ml		
	12 weeks		24 weeks
MS alum 1	20.0 (16.7-27.2)*,***	↘	14.3 (12.3-17.2)$^{*,**,***,\$}$
MS alum 2	18.8 (15.7-25.6)*,***	↘	7.42 (6.34-8.90)*
MS alum 3	7.79 (6.66-9.35)*	→	8.14 (5.98-9.77)*
MS no alum	3.67 (3.06-4.29)	→	2.75 (2.02-5.00)
Alum control	N/D	?	7.54 (6.44-9.05)*

*p<0.05 compared to MS vaccine alone; **p<0.05 compared to MS + alum 2; ***p<0.05 compared to MS + alum 3 $^{\$}$p<0.05 compared to alum control group

4-5 Immunogenicity of PLGA MS vaccines versus commercial alum adsorbed control

The previous section demonstrated the ability of MS vaccine alone to induce a long term protective response in guinea-pigs comparable to the commercial alum control. However, in the early response, the level of Ab response was higher for the control vaccine. The next study was designed to investigate further the type of early response (up to 6 weeks) induced by MS vaccine and compared to the commercial alum control. The polymer RG503H and the small-sized MS were selected as the strongest immunogenic formulation from the work performed in section 4.3. A mixture of MS-DT and MS-TT was used to directly compare the immune response induced by PLGA MS vaccine to the divalent commercial DT-TT alum control. Mice were immunised once with a mixture of PLGA MS-DT (SD503H batch 1) and PLGA MS-TT (SD503H-TT) or with DT-TT alum control vaccine at doses ranging from 1/62.5 to 1/10 SHD of DT and TT (Table 2.1). Cellular response and Ab titers were measured by antigen specific spleen T-cell proliferation and ELISA respectively, at 2-6 weeks post-immunisation.

The cellular response induced by MS vaccines was similar for microencapsulated DT and TT (Fig 4.10). However, the response was antigen dose and time dependent. A dose-response relationship (higher response with higher antigen dose) was observed at 2 weeks post-immunisation, which became less obvious after 4 weeks, and vanished after 6 weeks (Fig. 4.10.bars). The cellular response induced by the MS vaccine and alum control did not differ significantly ($p=0.4$ and $p=0.079$ for DT and TT) at 2 weeks post-immunisation, whereas at 4 weeks, the MS vaccine induced a statistically higher cellular response than the equivalent dose of the alum control ($p<0.05$ for both Ag) (Fig. 4.10., bars). It was also noticed that the cellular response induced against DT was slightly impaired when TT was present in the formulation

(admixed or co-encapsulated), although the difference was statistically insignificant (results not shown).

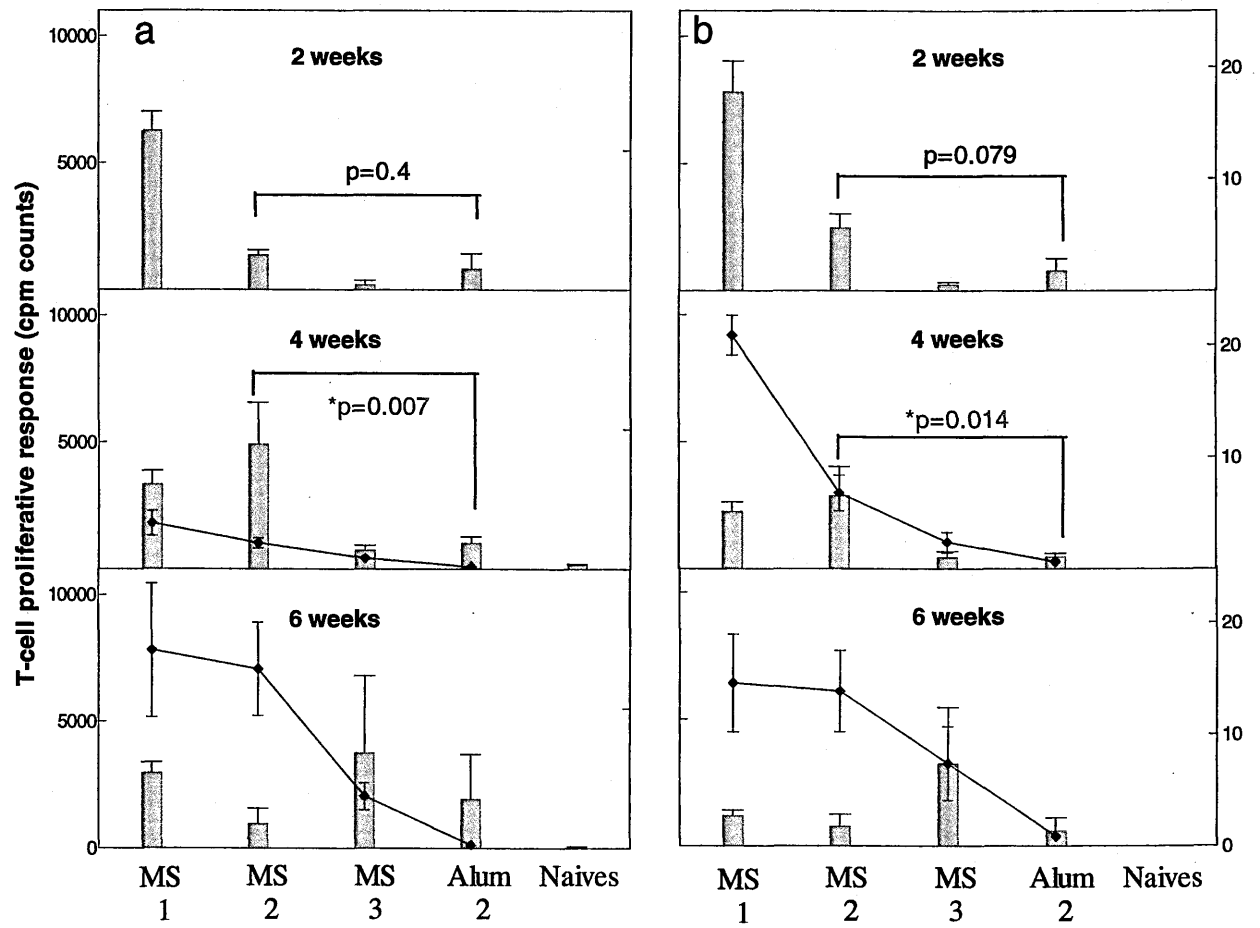


Figure 4.10 Effect of vaccine dose and adjuvant type on the immune responses against DT and TT. Anti-diphtheria (a) and anti-tetanus (b) cellular (cpm counts; bars) and humoral (total Ab in IU/ml; lines) responses in mice at 2, 4 and 6 weeks post-s.c.-immunisation. MS 1, 2 and 3 correspond to the immunisation doses of 2.5, 1 and 0.4 Lf DT and 1, 0.4, and 0.16 Lf TT encapsulated into MS. Alum control 2 corresponds to the immunisation dose of 1 Lf DT and 0.4 Lf TT adsorbed on 0.03 mg alum. The results are expressed as geometric means ± SEM from 3 individual animals. At 4 and 6 weeks, MS vaccine induced higher cellular and humoral responses than equivalent doses of control vaccine; the statistical significance is indicated by the p-values shown at 2 and 4 weeks.

The humoral response was delayed with respect to the cellular response (first detectable Ab measured at 4 weeks post-immunisation) and was proportional to the antigen dose (Fig 4.10., lines). Induction of the humoral response appeared to be slightly slower for microencapsulated DT (lower Ab titres at 4 weeks) than for TT, which confirms previous unpublished data from our laboratory. Importantly, the Ab titres elicited by the MS vaccine were superior to those of the alum control at 4 and 6 weeks post-priming ($p<0.05$).

The cytokine levels were measured at 4 weeks post-immunisation (Fig 4.11.). Here, the MS vaccine induced significantly higher IL-2 (T-cell activation marker) production than the alum control ($p=0.003$ for DT) (Fig. 4.11.). Only the MS vaccines seemed to stimulate a positive IFNγ (Th1 type) production whereas both the MS vaccines and the alum control stimulated a strong IL-6 (Th2 type) production, with a slight tendency towards a dose-response relationship. Detectable levels of IL-10 (Th2 type) upon re-stimulation with antigen were measurable only with the MS vaccines (results not shown). No IL-4 production was detectable within these experimental conditions (results not shown). Due to high assay variability, most of the differences observed were statistically insignificant.

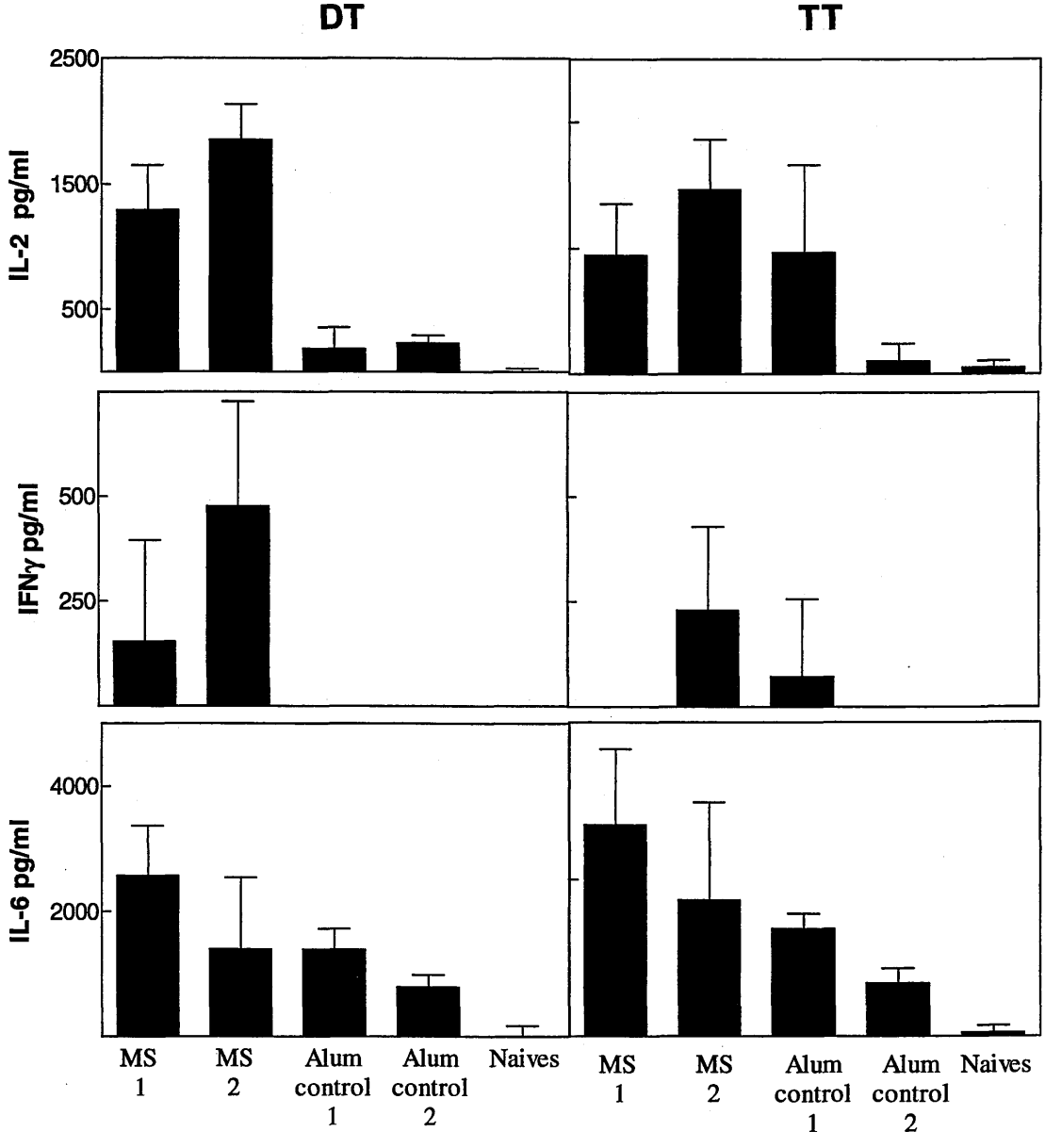


Figure 4.11 Cytokine levels produced by spleen cells from mice immunised subcutaneously with MS vaccines or alum control formulations. The cytokine production was measured 4 weeks post-immunisation in cells isolated from the spleen and re-stimulated *ex vivo* with DT (a) and TT (b). MS or alum control 1 and 2 correspond to 2.5Lf DT/1Lf TT and 1Lf DT/0.4Lf TT either encapsulated in MS or adsorbed on 0.03mg Alum (Control). The results are expressed in pg/ml as geometric means ± SEM from 3 individual animals. MS vaccine induced a mixed Th1/Th2 cytokine profile.

4-6 Sustained protection after a single dose of PLGA MS versus two doses of the alum control vaccine

The previous sections demonstrated the different type of immune response induced by MS vaccine or commercial alum adsorbed vaccine. Indeed, MS vaccines tend to induce a more balanced Th1/Th2 type than the alum control, bias towards a strong Th2 type of reponse. On the long term, MS vaccines were able to induce a protective response comparable to two injections of the alum control without the help of co-administered adjuvant. The next study was designed to investigate the influence of the antigen dose on the long term protective response of MS vaccine alone and to compare it to the dose response induced by the alum control administered twice.

Protection against diphtheria toxin induced by PLGA MS and alum adsorbed vaccines were compared in a long term challenge study in guinea-pigs using a mixture of small-sized RG503H DT and TT MS. The formulation based on RG503H polymer was selected as it induced a strong Ab and protective response independent on the dose of co-administered adjuvant (alum). Animals were immunized once with 4 doses (1/4; 1/10, 1/20, 1/50 SHD) of the MS mixture or with the equivalent doses of the alum control vaccine administered twice with a 4 weeks booster. The total anti-DT Ab production kinetic was measured by ELISA and *in vivo* toxin neutralisation assays respectively. The protection was assessed by direct challenge of the same animals with diphtheria toxin one year post-immunisation.

A single injection of MS vaccine induced a similar Ab profile as two injections of the alum adsorbed control vaccine (Fig. 4.12.). The controlled release properties of the MS appeared to exhibit a booster effect, which was especially apparent with the lower antigen dose ($1/20^{th}$ and $1/50^{th}$ SHD); at 4 weeks, the Ab levels induced by the MS vaccine were comparable to those of the alum control vaccine at 8 weeks, i.e., 4 weeks after the booster dose (Fig 4.12.). The antibody levels sharply decreased between 4 and 8 or 8 and 16 weeks for MS and alum control vaccines, respectively, but remained above the minimum protective level of 0.1 IU/ml up to one year. The boosting effect of the MS vaccine was less pronounced at higher antigen dose ($1/4^{th}$ and $1/10^{th}$ of SHD), as the antibody levels were not raised as high and did not decrease as sharply as observed with the lower doses. Most encouragingly, the antibody levels were maintained almost constant from 16 weeks up to one year. Only at the highest antigen dose ($1/4^{th}$ of SHD), the alum control vaccine afforded higher antibody titres than the MS vaccine until the end of the study ($p<0.01$), which may be ascribed to the very high dose of alum adjuvant present in the control vaccine at this low dilution (0.3mg/ml Al^{3+}) (Fig 4.12.a). For the 1/10 and 1/20 dilution of the antigen, the Ab levels were higher for the alum control vaccine than for the MS vaccine up to 16w post-immunisation. This was probably due to the strong boosting effect of the alum adjuvant with its ability to induce very high level of total Ab in the early phase of the response.

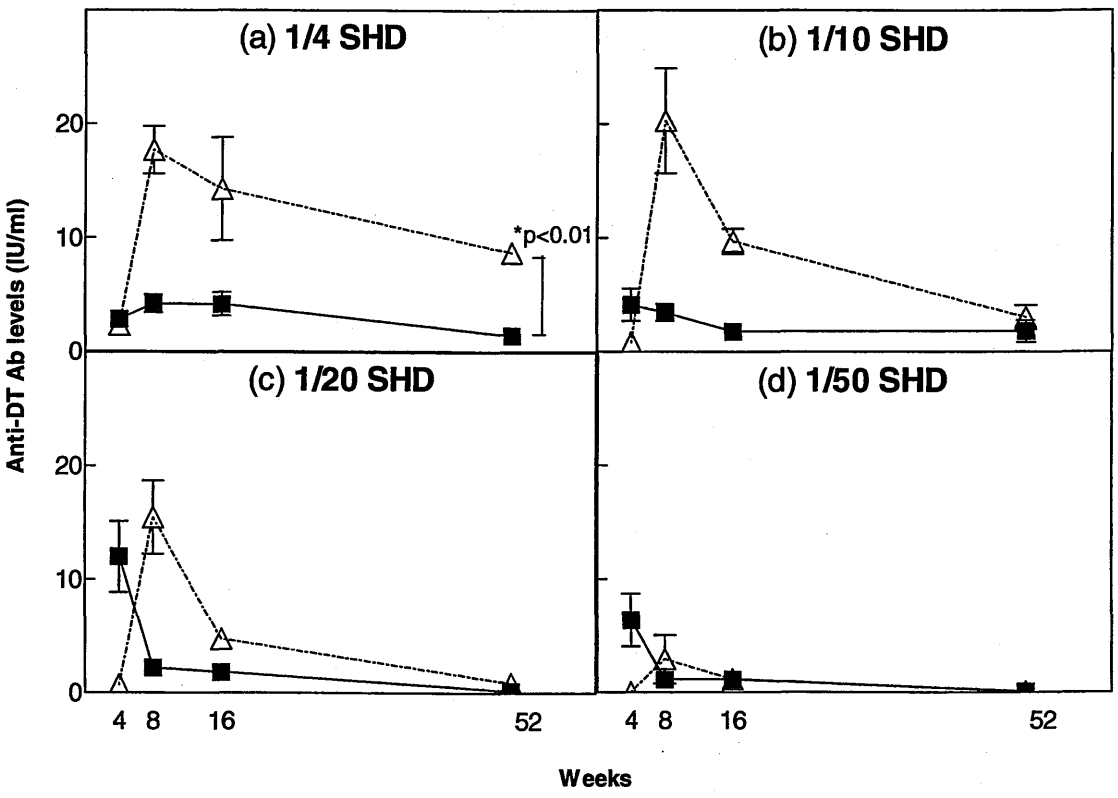


Figure 4.12. Kinetics of anti-DT antibody response in guinea-pigs immunised with MS-DT/MS-TT or alum control. The animals were immunised subcutaneously with (a) 1/4th, (b) 1/10th, (c) 1/20th and (d) 1/50th of a SHD of DT-TT formulated in MS vaccine (■) or adsorbed on alum (△). The MS vaccine was injected once, whereas the alum control vaccine was injected twice at an interval of 4 weeks. The total dose of administered DT was equivalent for the two groups. The data represents the total anti-DT Ab levels measured by ELISA and expressed as geometric means ± SEM from 8 individual animals. MS vaccines could maintain long term anti-DT Ab levels after a single injection.

One year post-immunisation, the animals were challenged intra-dermally with six escalating doses of diphtheria toxin. The MS vaccine gave similar or better protection than the control vaccine (Fig 4.13). At the highest DT vaccine dose ($1/4^{th}$ SHD), the MS vaccine induced 80% protection against a challenge with a toxin dose of 12.8 mLf (12. 8 mLf is equivalent to 640 times the minimum reaction dose (MRD)); this was at least twice as high as the protection conferred by the control vaccine (30%) (Fig 4.13.a, red arrow). It is interesting to note that at the vaccine dose of $1/4^{th}$ of SHD, the protection was higher with MS than with the alum control, whereas total Ab induced with the MS were significantly lower ($p<0.05$) (Fig. 4.12.a). Similarly, at $1/20^{th}$ of SHD of the vaccine and with a toxin dose of 12.8 mLf, the MS vaccine still conferred a 35% protection, as opposed to 25% with the alum control vaccine (Fig 4.13.b).

MS vaccines alone, based on SD503H formulation, could maintain long-term total Ab levels following single injection and confer long-term protection after one year, comparable to 2 doses of the alum control vaccine.

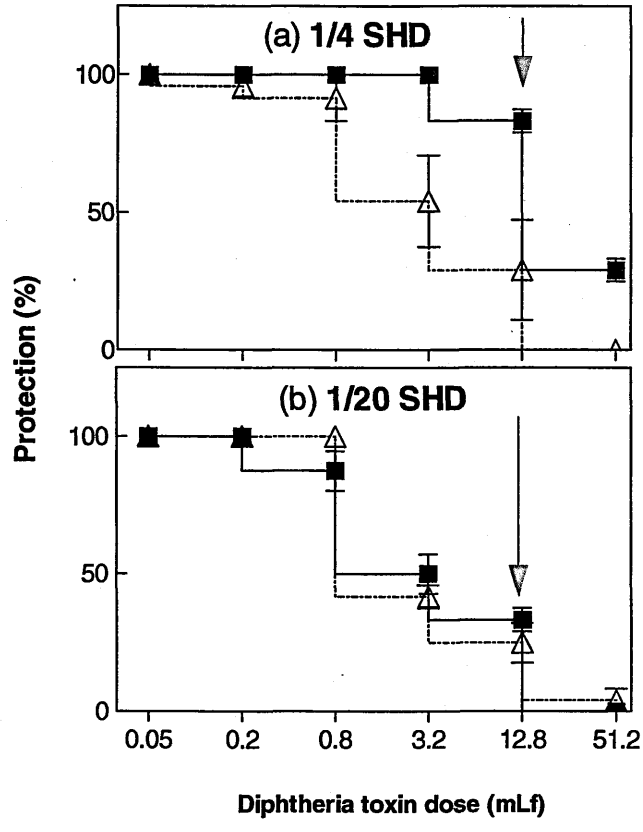


Figure 4.13 Protection against a diphtheria toxin challenge of guinea-pigs immunised with single dose MS-DT/MS-DT or two doses alum control. The animals were first immunised with (a) 1/4th and (b) 1/20th of a SHD in DT-TT-MS vaccine (RG503H) given once (■) or DT-TT alum control vaccine administered twice (△); after 52 weeks, the animals were challenged intra-dermally with six escalating doses of diphtheria toxin (0.05 – 51.2 mLf, equivalent to 2.5-2560 times the minimal reactive dose). Results are expressed as the percentage of animals protected at each toxin challenge dose. The geometric mean ± SEM from 3 set of data from 3 different observers is plotted. MS vaccine given once induced a higher or comparable specific protection after one year than the alum control vaccine given twice

CHAPTER 5: TOWARDS MULTIVALENT PLGA MS VACCINES

5-1 Rationale and outline of the study

Most of the previous work on the characterisation of the formulation was performed on monovalent DT MS vaccine. However, it was shown in chapter 4.5 that a mixture of DT and TT MS vaccine was inducing positive cellular and humoral responses against both Ag. It was also demonstrated that a formulation containing both Ag was inducing protective response against diphtheria and tetanus toxins without the need of co-adjuvant stimulation (chapter 4.4). In this chapter, a more detailed study on the influence of formulation characteristics is described. And in particular the influence of polymer type and particle size on the protective response induced by a mixture of DT- and TT-MS vaccine administered with admixed alum. Secondly, in an endeavour to develop a multivalent vaccine based on biodegradable microspheres, this section investigates the efficiency of a tetravalent co-encapsulated formulation based on DT, TT, PT and PRP-TT antigens. A preliminary study was performed to assess the *in vitro* characteristics of the tetravalent formulation and its capacity to induce a response against each Ag (Boehm et al., 2002). Further, comparison of the tetravalent microsphere formulation with mixtures of monovalent microspheres was performed. Finally, a synthetic liver stage malaria antigen (PfCS) was also microencapsulated, and these malaria antigen MS were admixed to the tetravalent formulation.

5-2 Formulations

Table 5.1. Polymers used for microencapsulation of tetanus and diphtheria toxoids by spray-drying (SD) and coacervation (CO) methods.

Polymer[a]	Code	Loading (Lf/mg)	Mw (kDa)	Size (μm)[c]	Preparation method
PLGA 50:50	502H[b]	0.3 2.0	12	0.5 – 5 (small)	SD
PLA	203	0.25 2.0	23	0.5 – 5 (small)	SD
PLGA 50:50	502H[b]	0.4 1.25	12	10 – 80 (large)	CO
PLA	203	0.4 1.25	23	10 – 80 (large)	CO

[a] *The polymer brand names were Resomer® RG502H and Resomer® RG 203, from Boehringer Ingelheim; for details see under Chapter 2.1.3*

[b] *H-types: PLGA with open carboxylic end-groups*

[c] *For SD MS batches, typical particle size distributions were: d(v,s) (10% undersize) = 0.5-0.8 um; d(v,s) (50% undersize) = 2-4 um; d(v,s) (90% undersize) = 7-10 um*
For CO MS batches, typical particle size distributions were: d(v,s) (10% undersize) = 10-15 um; d(v,s) (50% undersize) = 20-35 um; d(v,s) (90% undersize) = 35-70 um

Table 5.2. Antigen content of the formulations used in the multivalent vaccine study. All MS were of small size and prepared from PLGA 50:50 (502H) by spray-drying.

Formulation	DT[a] Lf/mg	TT[a] Lf/mg	PT[a] μg/mg	Hib-PRP[a] μg/mg	PfCS[a] μg/mg
MS monovalent	2.0	0.3	0.6	0.5	1.5
MS tetravalent, Low loading	0.125	0.05	0.05	0.10	N/A[b]
MS tetravalent, High loading	0.312	0.125	0.125	0.25	N/A[b]

[a] *DT: Diphtheria toxoid; TT: Tetanus toxoid; PT: Pertussis toxoid; Hib-PRP: H. Influenza type b polyribosyl ribitol phosphate; PfCS: Pl. falciparum circumsporozoite protein.* [b] *not applicable*

5-3 Immune response and protection to monovalent DT- and TT-MS vaccines

Guinea-pigs were immunised with monovalent DT-MS or TT-MS of small and large size and made from PLGA 50:50 or PLA (Table 5.1.), or with mixtures of these formulations. All MS formulations were co-administered with 0.035 mg of Al^{3+}/dose (Table 2.2 protocol 5A).

Large sized PLGA MS produced higher anti-TT and anti-DT endpoint (16 weeks) titres than large sized PLA MS ($p<0.05$) (Fig 5.1.a,d) (CO PLGA > CO PLA), and the titers induced by MS vaccines increased more strongly between 8 and 16 weeks for TT (10 - 60%) than for DT (< 10%). Conversely, small sized MS produced similar anti-DT and anti-TT titres for both polymer types (Fig. 5.1.a,d) (SD PLGA = SD PLA). In the mixed MS-size groups, small-sized microspheres (small PLGA MS + small PLA MS) seemed to perform slightly better than the larger particles (large PLGA MS + large PLA MS; small PLGA MS + large PLA MS), although this difference was statistically insignificant (Fig 5.1.b,e).

A single injection of TT and DT in PLGA or PLA MS generated similar or superior Ab levels as compared with an equivalent single dose of alum-adsorbed antigens (1x alum control) (Fig. 5.1. a-d *versus* c,f). As presented in previous section (Chapter 4.5 &4.6), two inoculations of the alum-adsorbed antigens (2x alum control) boosted more prominently the Ab levels than did microspheres injected once ($p < 0.05$, at 8 weeks). However, as expected, the titres of the boosted alum group started to decline after the peak at 8 weeks, and the Ab titres were similar to those attained with some of the MS vaccines after 16 w ($p>0.05$). In this study, a classical boosting effect of the MS vaccines on the antibody titres was not observed, but the animals immunised with MS produced a steady increase in anti-TT and anti-DT Ab throughout the

whole test period of 16 weeks, as did animals immunised with a single dose of the divalent alum control vaccine. Hence, the steady increase in antibody titres may be ascribed to the co-administered alum adjuvant, as seen previously (chapter 4.5.).

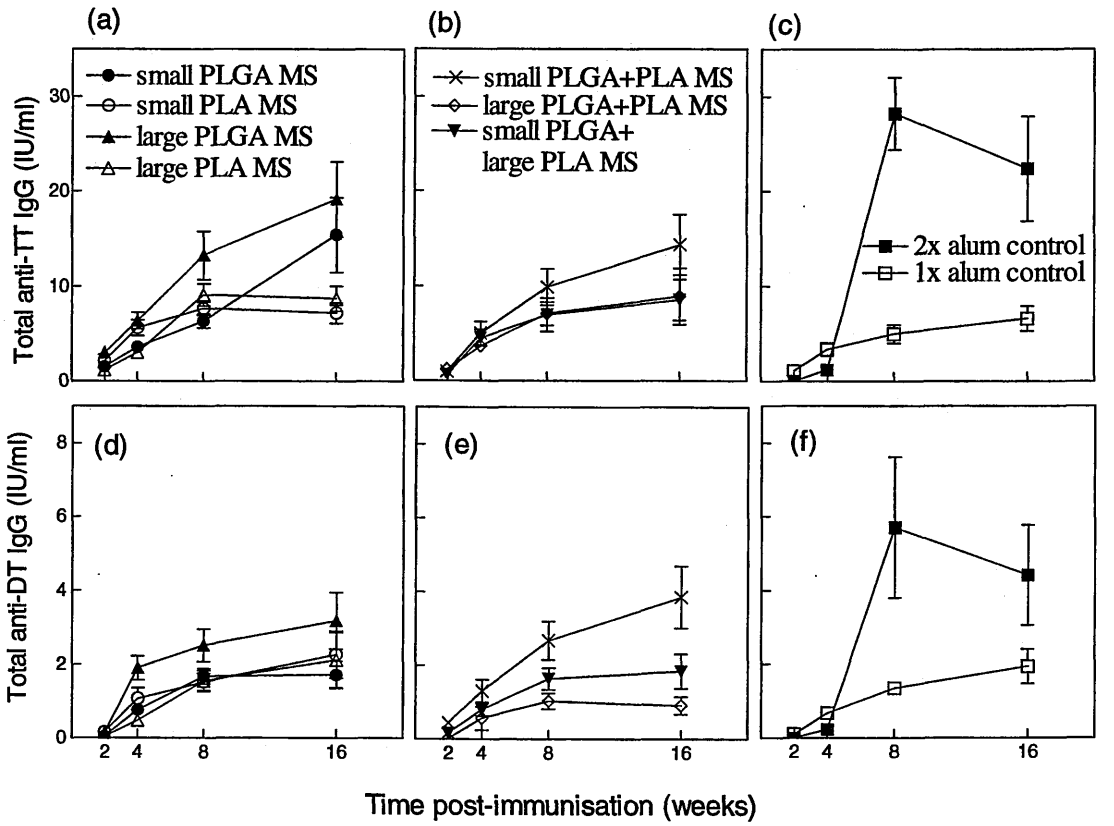


Figure 5.1 Immunogenicity of DT- and TT-MS vaccines as a function of polymer type and MS size. Anti-TT (*upper panel*) and anti-DT (*lower panel*) ELISA antibodies raised in guinea pigs after immunisation with a single injection of experimental MS vaccines (1 Lf TT; 2.5 Lf DT microencapsulated in PLGA 50:50 or PLA, or equivalent dose of alum adsorbed control vaccine injected once (1x alum control) or twice (2x alum control) (0.06mg Al^{3+}/dose) (c & f). For immunisation, the microsphere formulations additionally contained admixed $Al(OH)_3$ (0.035mg Al^{3+}/dose). The microsphere vaccines consisted of either single particle populations (a & d) or blends of particles of different size or polymer type (b & e). Each group represents the geometric mean of IgG titres determined from individual sera (n = 10) at 2, 4, 8 and 16 weeks post-immunisation (± S.E.M). All determinations were repeated once with comparable results. Symbols and corresponding formulations apply to all panels.

Development of single-injection microsphere vaccines has sometimes been directed towards blending of microsphere types with different degradation and antigen release properties to mimic a prime-boost situation. In this study, microsphere mixtures of different polymer types or particle sizes (Fig. 5.1.b,e) generally produced comparable or even slightly lower levels of anti-TT Ab as compared with single microsphere populations ($p > 0.05$). As an exception, the mixture of small PLGA and PLA microspheres induced higher anti-DT Ab serum concentrations than any of the single microsphere formulations ($p < 0.05$). There was a steady increase in anti-TT titers for all microsphere mixtures and in anti-DT titers for two out of three mixtures throughout the test period.

For both single and mixed microsphere populations, the total Ab were mainly of IgG1 subtype with only a low proportion of IgG2 (Table 5.3.). This IgG isotype profile was not notably switched by the polymer type, microsphere size, antigen type, or the type of adjuvant utilised (microspheres or alum). At 16 w, all groups induced similar low levels of anti-DT IgG2 titres (0.11 to 0.16). The levels of IgG1 were higher and slightly influenced by the formulation parameters with higher levels for PLGA versus PLA and large versus small MS ($p<0.05$). These data confirm the differences observed previously in total IgG levels (Fig. 5.1.). Most of the formulations (except large PLGA MS) induced lower anti-DT IgG1 levels than 2 doses of the alum control vaccine ($p<0.05$). At earlier time point (4 w), higher anti-DT IgG2 titres were achieved with PLGA than with PLA MS, and with small than with large PLGA MS ($p<0.05$) (Appendix 1). These data also confirm the results observed in Chapter 4.3. These profiles observed for DT were similar for anti-TT Ab, with low levels of IgG2 and higher levels of IgG1. However, the MS formulations induced significantly higher IgG2 and lower IgG1 levels (except for PLGA formulation) than the alum control vaccine injected twice ($p<0.05$), and the

PLGA MS induced higher IgG1 and IgG2 responses than the PLA MS. As for DT, the large PLGA MS formulation induced higher anti-TT IgG1 titres, but lower IgG2 than the small sized MS.

Table 5.3 Influence of formulation parameters on IgG isotype profile. IgG1 and IgG2 against DT (*upper table*) and TT (*lower table*) were measured by ELISA on individual serum sample from guinea-pigs immunised with experimental MS vaccine or alum adsorbed control vaccines (as described above). The results at 16 weeks post-immunisation are expressed as titers relative to an in house reference used on each ELISA plates. The data represents the geometric mean with 95% CI from pooled samples from 8-10 individual animals.

Formulation	Anti-DT IgG1 titers	Anti-DT IgG2 titers
Small PLGA MS	2.72 (2.34-3.13)**,***	0.14 (0.12-0.16)
Small PLA MS	4.00 (2.93-3.51)***	0.12 (0.11-0.13)
Large PLGA MS	5.35 (4.65-6.19)	0.16 (0.14-0.19)
Large PLA MS	1.78 (1.64-1.94)*,***	0.14 (0.12-0.15)
1x alum control	3.58 (3.00-4.00)***	0.11 (0.09-0.12)
2x alum control	6.07 (5.40-6.83)	0.13 (0.11-0.15)

Formulation	Anti-TT IgG1 titers	Anti-TT IgG2 titers
Small PLGA MS	12.0 (10.4-14.0)**	2.46 (2.14-2.84)***
Small PLA MS	11.5 (10.8-12.3)***	0.76 (0.71-0.81)*,***
Large PLGA MS	21.5 (18.3-25.5)***	1.20 (1.05-1.37)**,***
Large PLA MS	5.51 (5.16-5.89)*,***	0.84 (0.80-0.90)*,***
1x alum control	8.68 (7.83-9.67)***	0.69 (0.62-0.76)
2x alum control	14.9 (154-16.9)	0.61 (0.55-0.67)

*p<0.05 compared to same size, different polymer

**p<0.05 compared to same polymer, different size

***p<0.05 compared to the alum control injected twice

To confirm the presence of functional Ab, pooled sera from selected vaccination groups (small PLGA MS; small PLA MS, large PLGA MS, alum controls) were tested by toxin neutralisation (TN) assays (Fig. 5.2.). For both antigens, nAb against tetanus and diphtheria toxins generally increased between 4 and 16 weeks, demonstrating Ab maturation. Within this time frame, the nAb levels increased from 10 to 1000 times the minimum protective level (0.1 IU/ml for TT and 0.01 IU/ml for DT, as measured by *in vivo* or Vero cell TN assays, respectively). The nAb induced by the MS groups were lower for DT than for TT at 4 weeks ($p<0.0001$), which, as suggested before (Chapter 4.3.), might be a sign of earlier specific B cell maturation for TT. However, from 4 to 8 weeks, there was a much stronger increase in anti-DT nAb (8.3 - 15.4 increase ratio) than in anti-TT nAb (1.1 - 1.6 increase ratios), which suggests a later B cell maturation for DT. For both Ag, nAb generally continued to increase between 8 and 16 weeks ($p<0.05$), with the exception of TT delivered from the large PLGA MS ($p=0.064$) and alum control injected twice ($p=0.541$). At 16 w, no more differences between DT and TT nAb levels were generally observed, except for the group injected with small PLGA MS, which showed lower anti-DT nAb levels ($p=0.006$). No significant differences in anti-DT nAb were observed between the various MS and the alum control injected once; however, nAb induced by the alum control vaccine injected twice were significantly higher than those achieved with all the other formulations injected only once ($p=0.006$). For tetanus, MS vaccines mediated, at 16 weeks post-immunisation, similar anti-TT nAb levels as 2 doses of the alum control ($p=0.1$), while the alum control injected once showed lower nAb than the alum control injected twice ($p<0.05$). As seen before (chapter 4), the nAb induced by MS vaccines are expected to increase further after 16 w and remain high for up to at least 1 year, whereas the nAb in the alum control groups would gradually drop.

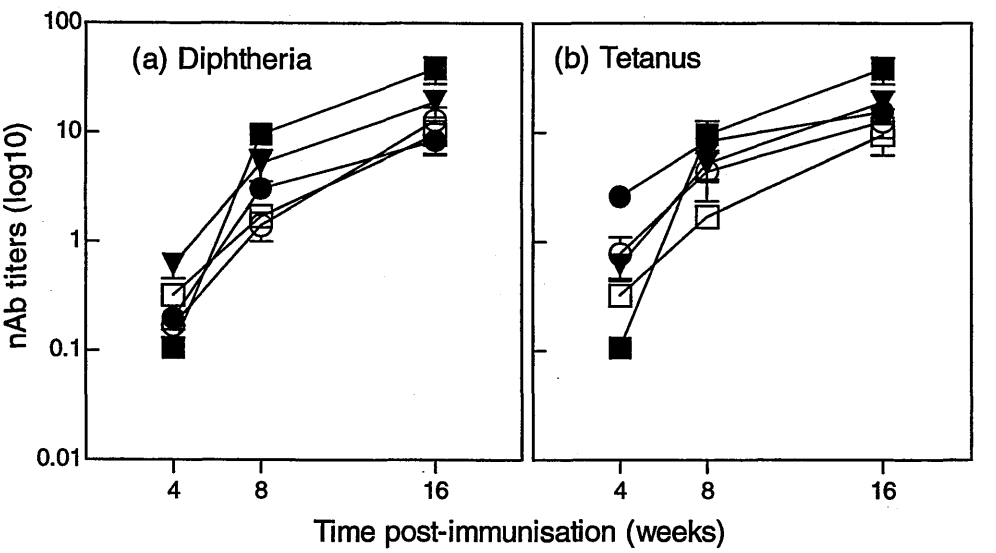


Figure 5.2. Influence of MS size, MS polymer type or adjuvant type on the nAb responses against DT and TT. Toxin-neutralising anti-DT (*left*) and anti-TT (*right*) Ab (nAb) induced in guinea pigs immunised with a single injection of (●) small or (▼) large PLGA MS; (○) large PLA MS or with one [(□) 1x alum control] or two [(■) 2x alum control] injections of alum control vaccines. For tetanus, nAb were measured *in vivo* using pooled sera. For diphtheria, nAb were measured using Vero cells on individual sera. Results are expressed as geometric means with 95% confidence interval. All formulations stimulated maturation of specific antibodies with neutralizing capacity.

The protection efficacy against direct challenge with either diphtheria or tetanus toxins was performed at 6w post-immunisation with selected MS and alum control vaccines (small PLGA, small PLA, large PLGA and 1x alum control) using modified Ph. Eur. assays. As illustrated in Fig. 5.3.a (red arrow), 6 – 19% of the animals immunised with 5 Lf of DT in microspheres were fully protected after a challenge with 4 mLf (equivalent to 64 x MRD). At this toxin dose, no protection was observed in animals that had received an equivalent single dose of the alum control vaccine. Similarly, at the lower challenge toxin doses, DT in microspheres protected the animals equally well or better than the alum control. An effect of different microsphere types on the protection of animals was not noticeable at the generally used immunisation dose of 5 Lf. However, when animals were immunised with only 0.6 Lf of DT in microspheres, the small PLGA MS tended to protect better than the large PLGA MS, the small PLA MS) and the alum control; at 0.8 μLf toxin, protection was 40% with the small PLGA MS *versus* 6% for both the large PLGA and small PLA MS and 19% for the alum control (Fig. 5.3.b, red arrow).

Protection against tetanus toxin challenge was achieved in all animals immunised with 0.2 Lf of tetanus vaccines, irrespective of the formulation used (Fig. 5.3.c). At a lower vaccine dose (0.05 Lf TT), the MS formulations protected notably better than the alum-based licensed vaccine, i.e., approx. 50 - 60% *versus* 10% protection, respectively. No difference in protection was observed with varying particle characteristics.

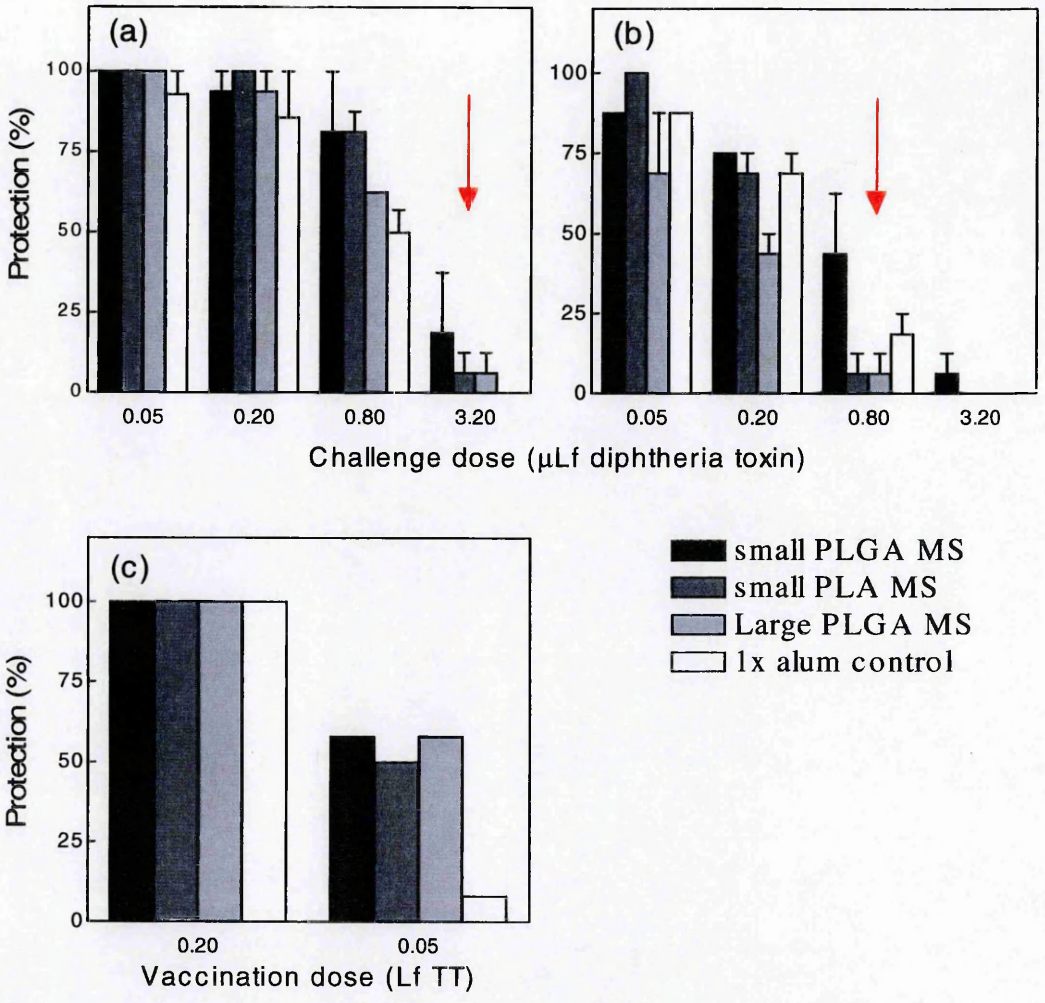


Figure 5.3. Influence of MS size, MS polymer type or adjuvant type on the protective responses against direct challenge with Dtx and Ttx. Protection study by direct challenge in guinea pigs (*a* and *b*) or mice (*c*) inoculated with different doses of diphtheria (*a* and *b*) or tetanus (*c*) toxins six weeks after immunisation. For diphtheria, animals were immunised once with 5.0 (*a*) or 0.6 (*b*) Lf DT encapsulated in MS or adsorbed on alum and challenged with four different doses of toxin. For tetanus (*c*), animals were immunised once with 0.2 or 0.05 Lf TT encapsulated in MS or adsorbed on alum and challenged with 50 x PD_{50}. The results are expressed as a percentage of protected animals. Note that diphtheria protection is illustrated as a function of the diphtheria toxin challenge dose, whereas tetanus protection is illustrated as a function of the tetanus toxoid vaccine dose. Microsphere vaccines induced higher potency than control vaccine when challenged with diphtheria or tetanus toxins.

5-4 Immune response and protection to tetravalent MS vaccines

First, monovalent MS formulations were prepared with each containing either DT, TT, aP or Hib antigen. The formulations were characterised *in vitro* (Table 5.2.) and *in vivo* to assess the response against DT and TT after consecutive mixing of the monovalent preparations (Fig. 5.4. and Table 5.4.). The results indicated that the mixture of the two formulations containing diphtheria or tetanus toxoid (DT-MS + TT-MS) produced significantly higher titers of total IgG against diphtheria (Fig. 5.4.a; $p=0.004$) than did the monovalent diphtheria formulation (DT-MS) (Fig. 5.4.a). The admixing of microencapsulated aP antigen (DT-MS + TT-MS + aP-MS) and Hib antigen (DT-MS + TT-MS + aP-MS + Hib-MS) did not further improve the immunogenicity of the DT-MS + TT-MS blend. For tetanus toxoid, non-significant changes in the total IgG were observed as a function of admixed formulations (Fig.5.4.b). For both antigens, the kinetics showed a sharp increase between four and eight weeks after vaccination, after which the titers rose more slowly or persisted at constant levels.

To test the functional significance of consecutive mixing of monovalent formulations on the generated immune response, 16 w sera were analysed for toxin-neutralising antibodies. All formulations induced protective levels of diphtheria- and tetanus-specific antibodies (Table 5.4.). In accordance with total IgG, the DT-MS + TT-MS mixture produced slightly higher anti-DT nAb titers than DT-MS alone or the triple (DT-MS + TT-MS + aP-MS) or quadruple (DT-MS + TT-MS + aP-MS + Hib-MS) mixtures. Only minor differences were observed with respect to tetanus-specific neutralisation as a function of the number of injected antigens in MS, but two injections of the alum control vaccine produced significantly higher anti-TT and tendentiously higher anti-DT neutralisation titres than any of the MS-based vaccines.

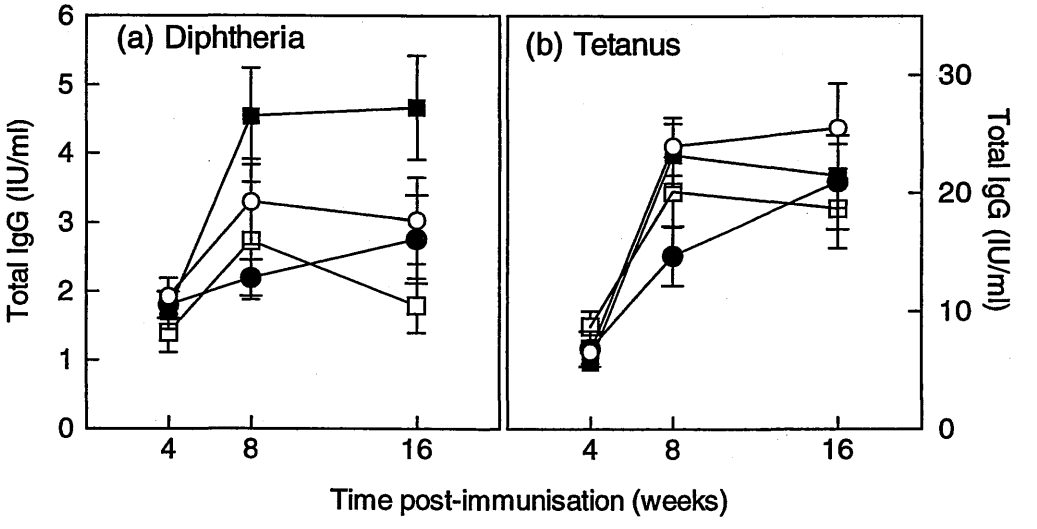


Figure 5.4 Effect of co-administration of multiple encapsulated antigens on the anti-DT and anti-TT humoral responses. Guinea pigs (n=10) were vaccinated once subcutaneously with a single MS formulation (□: DT-MS or TT-MS), or a mixture of two (■: DT-MS + TT-MS), three (○:DT-MS + TT-MS + aP-MS), or four (●:DT-MS + TT-MS + aP-MS + Hib-MS) MS formulations. The data were calculated as the geometric mean ± SEM. The results are representative of two individual ELISA experiments. Mixtures of monovalent MS formulations induced strong anti-DT (a) and anti-TT (b) Ab responses.

In the direct challenge test with diphtheria toxin at 16 weeks post-vaccination, the protection improved with increasing number of MS formulations in the mixture (Fig. 5.5.). Moreover, the protection was higher in guinea-pigs vaccinated once with MS formulations than in the alum control group that had received two doses of the alum adsorbed vaccine (Fig.5.5.red arrow). Excellent protection against tetanus toxin challenge was already shown previously (Chapter 5.1.) for the DT-MS + TT-MS mixture.

As an alternative to the approach of mixing monovalent MS formulations, we co-encapsulated DT, TT, aP and Hib antigens into a single MS formulation, henceforth referred to as DT-TT-aP-Hib-MS. As presented previously (study on admixed alum ; Chapter 4.), co-encapsulation of DT and TT induced protective humoral and cellular responses against both Ag. Here, we prepared two MS formulations containing all four antigens at high and low loading, respectively (Table 5.2.) to investigate the influence of the amount of injected MS (at identical antigen dose) on the immune response. Total Ab (Fig.5.6.) and nAb (Table 5.4.) against DT and TT were measured by ELISA and Vero cell assay, respectively. High titres of persistent total Ab were raised in guinea pigs after a single administration of both the low and high loaded tetravalent MS vaccines, as compared with the tetravalent alum control vaccine administered once ($p>0.05$) (Fig.5.6.); nonetheless, the highest Ab titres were attained with the tetravalent alum control vaccine injected twice ($p<0.0001$, data not shown). Both MS types with low and high loading produced comparable results to each other in terms of kinetics and maximum titres ($p > 0.05$, as analysed on antibody titres at 16 weeks), and both induced a protective level of specific antibodies (>0.1 IU/ml for TT and >0.01 IU/ml for DT) against DT and TT (Table 5.4.,DT-TT-aP-Hib-MS).

Table 5.4. *In vivo* neutralisation of tetanus and diphtheria toxin with sera from guinea-pigs immunised with multiple encapsulated antigens. Neutralising potency was determined *in vivo* on pooled sera from guinea pigs bled 16 weeks after vaccination with MS and alum control vaccines. Results are expressed in IU/ml, against the international standard with 95% confidence intervals (CI).

Experimental group	Anti-diphtheria antitoxin potency IU/ml (95% CI)		Anti-tetanus antitoxin potency IU/ml (95% CI)	
DT-MS or TT-MS	4.95	(2.90-7.92)	9.60	(7.38-10.56)
DT-MS + TT-MS	6.97	(2.32-18.8)	13.68	(12.44-19.15)
DT-MS + TT-MS + aP-MS	4.50	(1.50-12.1)	16.72	(15.20-23.40)*
DT-MS + TT-MS + aP-MS + Hib-MS	4.12	(1.38-11.1)	12.60	(9.70-13.90)
2x DT-TT-aP-Hib-alum	12.7	(6.33-38.0)*	656	(48.74-82.37)**
DT-TT-aP-Hib-MS [a]	2.00	(1.00-6.00)	11.52	(8.86-14.98)

* $p<0.05$ when compared to DT-TT-aP-Hib-MS ; ** $p<0.05$ when compared to all MS formulations ; [a] High loading

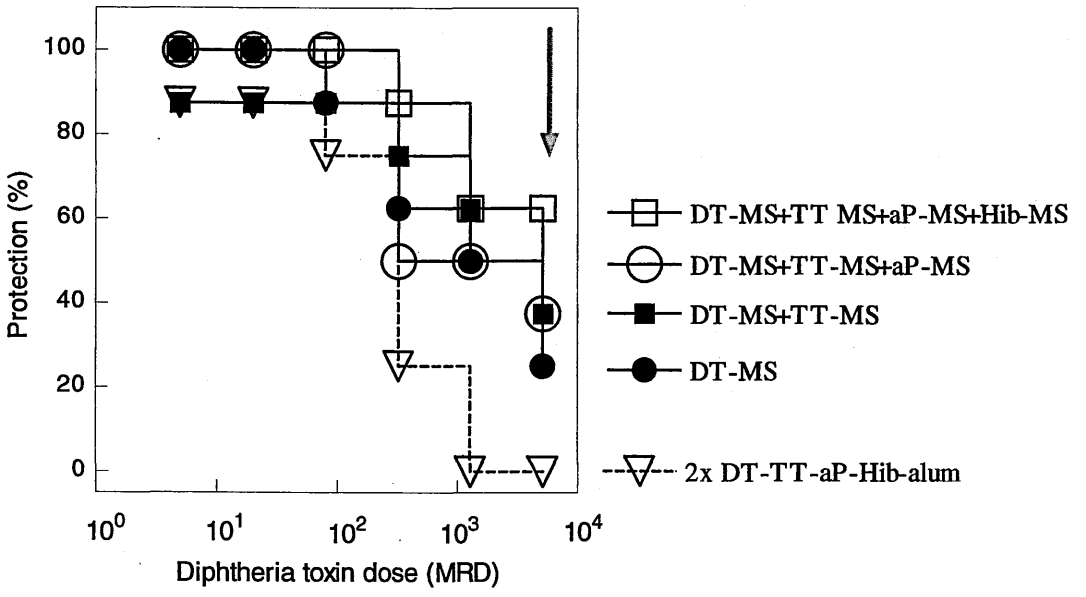


Figure 5.5 Effect of co-administration of multiple encapsulated antigens on the protective immunity against diphtheria toxin. Guinea-pigs were vaccinated with monovalent DT-MS, or mixtures of monovalent MS formulations, or the tetravalent alum-adsorbed control vaccine. At 16 weeks after vaccination, the animals were challenged with increasing amounts of diphtheria toxin (2.5-2560 MRD). The toxin was administered intradermally into both flanks of the animals, and the skin reaction was observed for two days. Results are expressed as percentage of animals protected at each toxin dose. Mixtures of monovalent MS formulations induced strong protective immunity against diphtheria toxin challenge.

The tetravalent MS formulation also elicited high titers of IgG against attenuated pertussis toxin (Fig. 5.6.c) and Hib (Fig. 5.6.d), which were comparable to those induced with the alum control vaccine administered once or twice ($p>0.05$). Whereas the anti-DT and anti-TT antibodies typically declined after 4 weeks in animals vaccinated with the alum control, the anti-Hib and anti-PT antibodies unexpectedly increased steadily until 8 and 16 weeks, respectively. Interestingly, no difference in total anti-PT and anti-Hib Ab levels was observed at 16 w between the single and double injections of the alum control vaccine ($p>0.05$) (data not shown).

Co-encapsulation of the four antigens into a single MS population did not influence the antigenicity of DT or TT, as no significant difference in total Ab (Fig 5.7.) and nAb (Table 5.4.) was observed between the tetravalent and the DT-MS + TT-MS mixture. Compared with the quadruple mixture (DT-MS + TT-MS + aP-MS + Hib-MS), the tetravalent DT-TT-aP-Hib-MS produced similar total IgG against both diphtheria ($p=0.248$) and tetanus ($p=0.669$) toxoids at 16 weeks (Fig 5.7.). Further, the titres of anti-pertussis and anti-Hib antibodies were similar between the tetravalent DT-TT-aP-Hib-MS and the quadruple mixture of monovalent MS formulations (DT-MS + TT-MS + aP-MS + Hib-MS) ($p=0.2$ for PT and $p=0.85$ for Hib) (Fig. 5.8.).

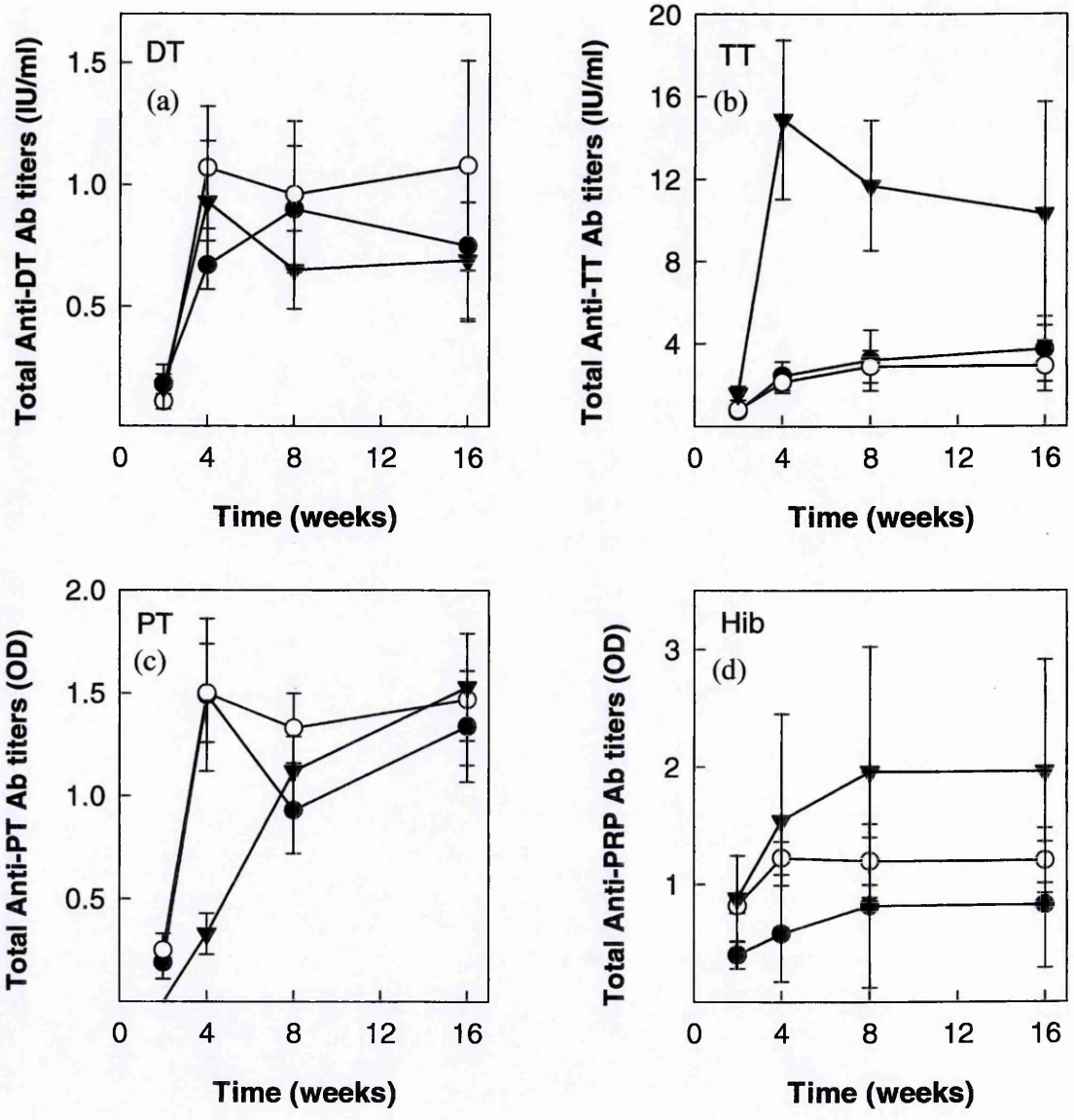


Figure 5.6 Effect of co-encapsulation of multiple antigens on the anti-DT, -TT, -PT and –PRP total Ab responses. Anti-DT (a), anti-TT (b), anti-aP (c) and anti-PRP (d) antibodies measured by ELISA in sera from guinea pigs immunised with tetravalent MS or alum control vaccines injected once. ▼: 1x alum-based control vaccine; ○: MS vaccine with high loading; ●: MS vaccine with low loading. Tetravalent MS formulations induced high Ab levels against all four antigens.

The tetravalent DT-TT-aP-Hib-MS formulation also induced protective levels of antibodies against both diphtheria and tetanus, which were not significantly different from those induced by the tetravalent DT-MS + TT-MS + aP-MS + Hib-MS mixture ($p=0.175$ and $p=0.816$ for anti-DT and anti-TT nAb in 16 week sera, respectively) (Table 5.4.). However, the protection efficacy in the challenge assay with diphtheria toxin appeared to be higher for the MS mixture than for the co-encapsulated antigens, with 62.5% protected animals versus 25% at the highest dose of toxin (Fig. 5.9. red arrow). Whereas the tetravalent DT-TT-aP-Hib-MS produced similar titres of total and neutralising IgG against diphtheria as the DT-MS + TT-MS + aP-MS + Hib-MS (Fig. 5.8., $p=0.284$; Table 5.4., $p=0.48$), the protection efficacy was lower for the tetravalent formulation. Moreover, the tetravalent DT-TT-aP-Hib-MS provided better protection against diphtheria toxin than the equivalent dose of a conventional alum-adsorbed vaccine administered twice. This observation is inconsistent with the anti-DT nAb data, where the alum control vaccine induced a significantly higher anti-DT nAb level than the tetravalent DT-TT-aP-Hib-MS ($p<0.05$, Table 5.4.).

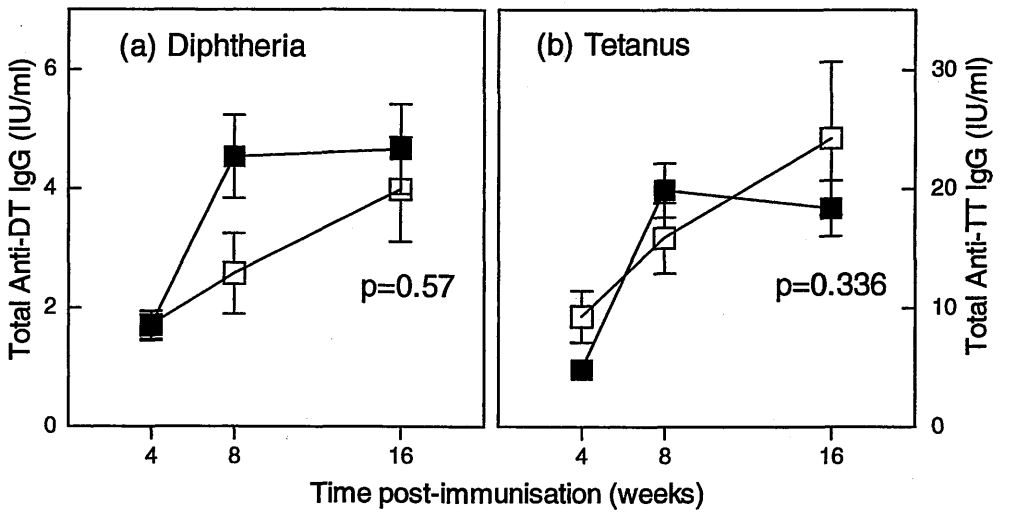


Figure 5.7 Influence of DT and TT co-encaspulation versus mixture of monovalent on the anti-DT and -TT total Ab response. Total anti-DT (a) and anti-TT (b) Ab responses. Guinea pigs (n=10) were vaccinated once subcutaneously with: (□) tetravalent DT-TT-aP-Hib-MS or (■) DT-MS + TT-MS divalent MS mixture. The data were calculated as the geometric mean ± SEM. The results are representative of two individual ELISA experiments. Tetravalent co-encapsulated antigens (DT-TT-aP-Hib-MS) induced similar IgG levels against DT and TT than the divalent mixture of DT-MS + TT-MS.

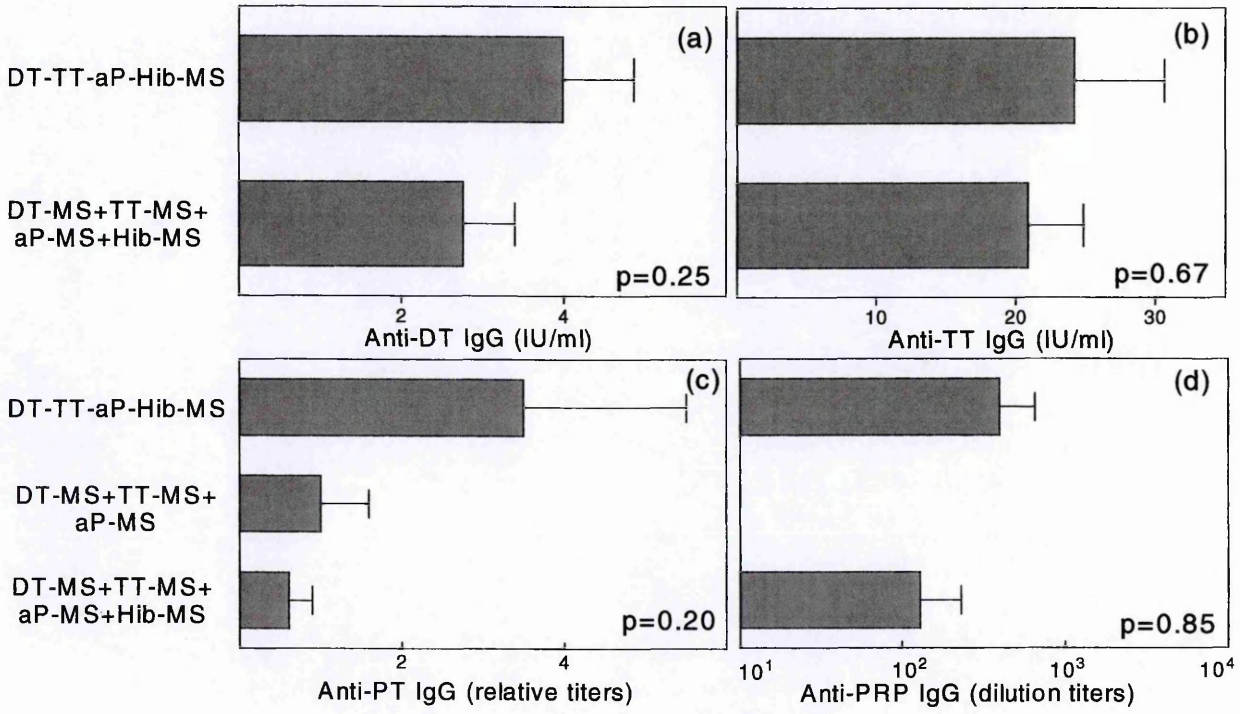


Figure 5.8. Total anti-DT, -TT, -PT and -PRP responses against co-encapsulated antigens versus individually encapsulated antigens in MS. Total IgG against diphtheria (a), tetanus (b), pertussis (c) and Hib (d) antigens measured by ELISA in sera from animals immunised with tetravalent DT-TT-aP-Hib-MS or mixture(s) of monovalent MS (DT-MS + TT-MS + aP-MS and DT-MS + TT-MS + aP-MS + Hib-MS). The results are expressed as geometric means ± SEM and are representative of two individual experiments.

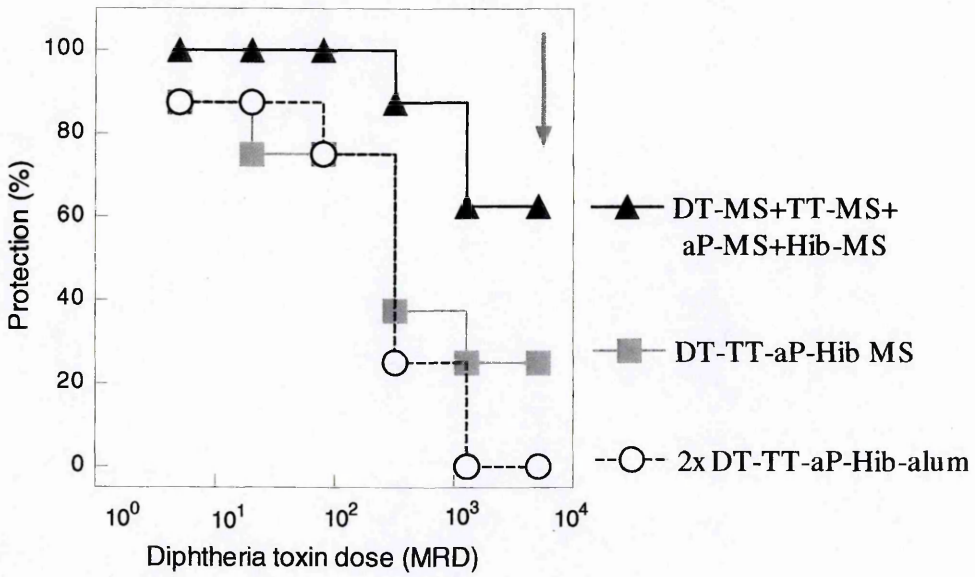


Figure 5.9. Influence of co-encapsulation versus mixture of monovalent encapsulated antigens on the protective immunity against diphtheria toxin. Guinea pigs were vaccinated with tetravalent co-encapsulated or individually encapsulated antigens in MS, or with the alum control vaccine given twice. At 16 weeks after vaccination, the animals were challenged with increasing amounts of diphtheria toxin (2.5-2560 MRD). The toxin was administered intradermally into both flanks of the animals, and the skin reaction was observed for two days. Results are expressed as percentage of animals protected at each challenge toxin dose. Multivalent co-encapsulated antigens induced a potent protective response against diphtheria toxin with more than 50% of the animals protected at the highest challenge toxin dose.

5-5 Influence of admixed malaria antigen-containing MS on the tetravalent DT-TT-aP-Hib-MS vaccine efficacy

In an additional experiment, we combined the tetravalent DT-TT-aP-Hib-MS with a monovalent PfCS-containing MS formulation (PfCS-MS). The presence of the synthetic malaria antigen mediated significantly higher total Ab levels for both diphtheria (Fig. 5.10.a) and tetanus (Fig. 5.10.b) toxoids, when compared with the PfCS-free MS vaccine. A similar effect was observed for diphtheria-toxin neutralisation (Fig. 5.10.c and 5.10.d) and protection (Fig. 5.10.e). At 10^3 and 10^4 MRD diphtheria toxin challenge dose, the licensed alum adsorbed vaccine injected twice was not protective, whereas the tetravalent DT-TT-aP-Hib-MS with or without admixed monovalent PfCS-MS induced approximately 44 and 25% protection, respectively (Fig. 5.10.e, red arrows). Similar results were obtained by mixing monovalent DT-MS or TT-MS with the monovalent PfCS-MS (results not shown).

Further, the capacity of monovalent PfCS-MS to generate anti-PfCS Ab was examined. When injected alone, PfCS-MS induced strong anti-PfCS Ab response in both guinea-pigs and mice (Fig. 5.11. a,b). However, in the guinea-pig model, the addition of DT-TT-aP-Hib-MS to the PfCS-MS seemed to lower the Ab level against PfCS, although the difference was marginally not statistically significant ($p=0.051$) (Fig.5.11a). In BALB/c mice, the combination of the tetravalent DT-TT-aP-Hib-MS and the monovalent PfCS-MS produced 5-10-fold higher IgG titres than the monovalent PfCS-MS alone, with the titres being in the range of 10^4-10^5 ELISA units. It is to note that the mouse is a more sensitive animal model for testing of PfCS-related immunity.

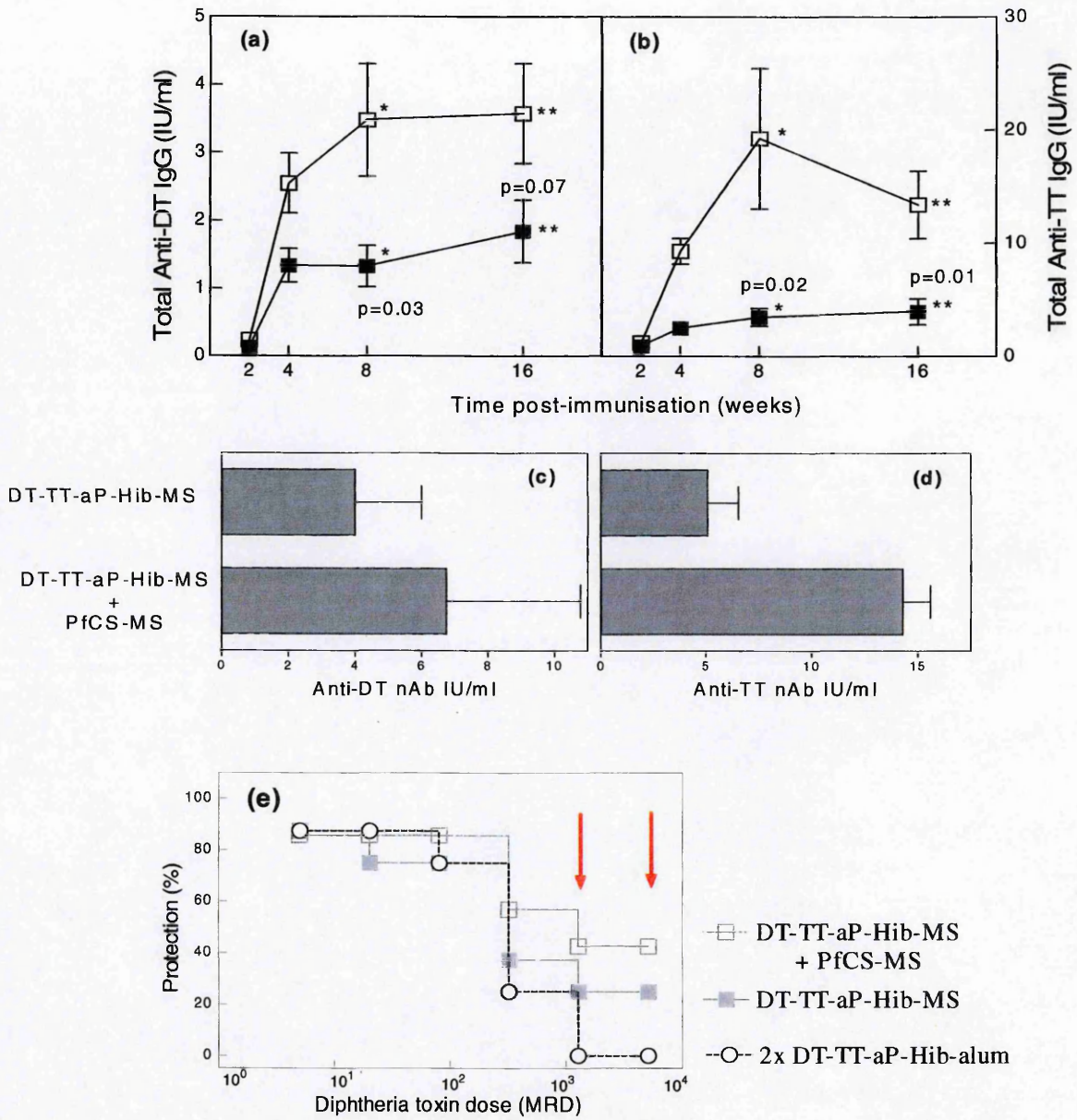


Figure 5.10. Effect of combining the tetravalent DT-TT-aP-Hib-MS with monovalent PfCS-containing MS on the immune responses. Guinea pigs were vaccinated once with the tetravalent DT-TT-aP-Hib-MS in the presence (□) or absence (■) of a monovalent PfCS-MS. Total IgG against diphtheria (a) and tetanus (b) was measured in sera obtained at 4-16 weeks after vaccination. Neutralising antibodies against diphtheria (c) and tetanus (d) toxins were measured on pooled sera samples from 16 weeks. The results are expressed as geometric mean ± SEM. The effect of PfCS on the protection induced by MS vaccines against a diphtheria challenge was tested against the alum control vaccine (empty circle) injected twice on a four weeks interval (e). Results were expressed as percentages of protected animals.

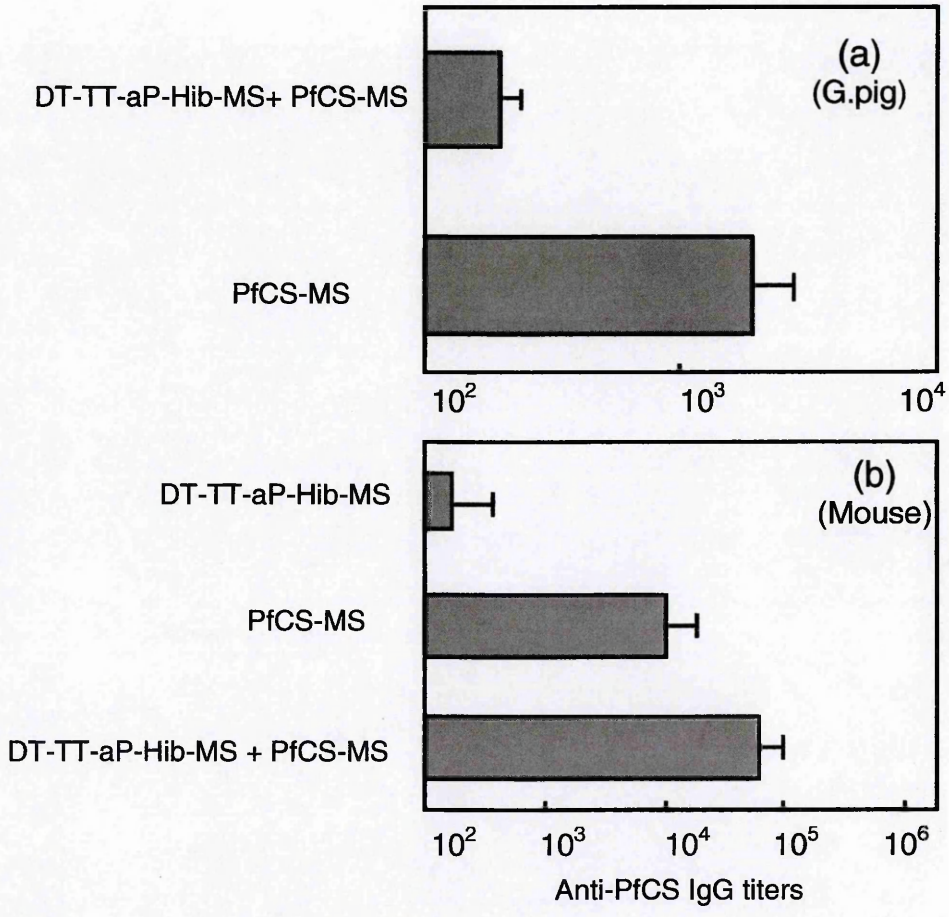
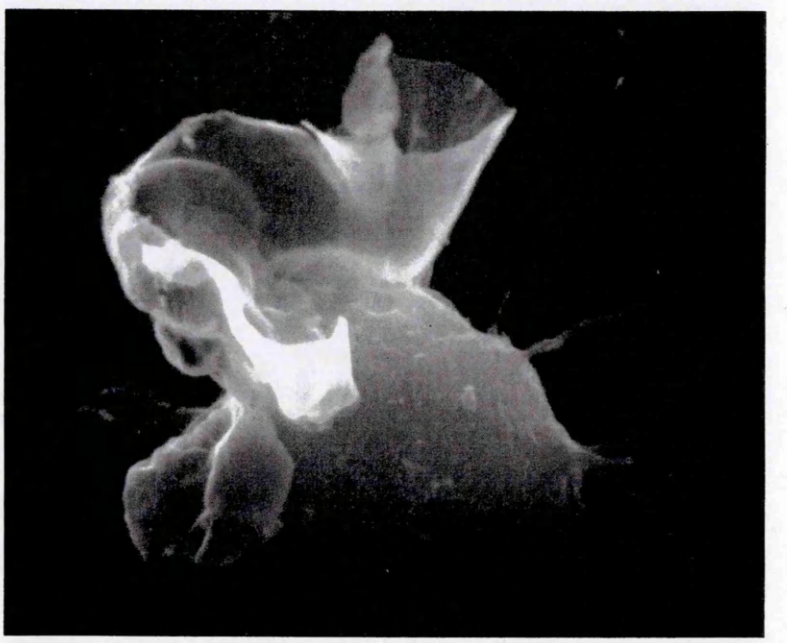


Figure 5.11 Influence of co-administration of tetravalent MS vaccine on PfCS immune response. Immunogenicity of microencapsulated PfCS in guinea pigs (a) and BALB/c mice (b). Animals were vaccinated subcutaneously with PfCS-MS alone or in combination with tetravalent DT-TT-aP-Hib-MS; a group of control animals was injected with PfCS-free DT-TT-aP-Hib-MS. Total anti-PfCS IgG was measured in guinea pigs at 16 weeks (n=8-10) or in mice at 12 weeks (n=5) after vaccination. The results are expressed as geometric mean ± SEM and are representative of two experiments.

DISCUSSION



CHAPTER 6: DISCUSSION

6-1 Adjuvant mechanisms of PLGA microsphere vaccines

Delivery of antigens from PLGA particles greatly enhances the immune response, which reflects the adjuvant properties of this type of delivery system (O'Hagan and Singh, 2003). PLGA microspheres (MS) are also able to induce and sustain a level of immune response similar to that induced by the conventional aluminium hydroxide adjuvant (Chapter 4.5.) (Thomasin et al., 1996; Singh et al., 1998b; Men et al., 1995). The immuno-stimulatory effect of PLGA MS has not yet been fully understood, although some obvious contributing mechanisms have been suggested (Johansen et al., 2000c): (i) the controlled released of antigen prolongs the contact between antigen and the immunocompetent cells; (ii) the efficient uptake of MS by phagocytic cells represents a targeting process and accumulates antigens inside antigen presenting cells (APC); (iii) the processing and prolonged presentation of antigen released from ingested PLGA MS by antigen presenting cells.

In this study, the presence of MS at the injection site for up to 4 weeks post-immunisation supports the concept of a formulation depot and controlled antigen release. The MS depot at the injection site warranted a prolonged uptake of intact MS and subsequent long-term trafficking of the particles towards lymphoid organs. Moreover, when the PLGA MS vaccine was administered s.c. with alum, 50% more particles remained at the injection site for up to 1 week post-immunisation as compared to PLGA MS alone. These findings are in agreement with the suggested depot effect afforded by alum, which according to Holt et al appeared to

last only for a maximum of 14 days and which mediated an Ab response similar to that achieved when the depot was left *in situ* for only 7 days (Holt, 1950). The prolonged localisation of PLGA MS vaccine at the injection site was also demonstrated by Gupta *et al.* (Gupta et al., 1996), who also found that their PLGA MS retained the entrapped radio-labeled antigen for a less extended period than did the alum adsorbate.

Following s.c. injection, MS were detected within the lympoid tissues such as the draining lymph nodes and the spleen. The presence of MS at the site of T-cell selection and maturation supports the "geographical" concept of site-dependent immune reactivity (Zinkernagel et al., 1997) and active uptake and transportation of the MS by competent APCs. The number of MS at the injection site dropped slowly up to 1 week whereas a direct drainage of free MS through the lymphatic canals lasted only for a maximum of 1 day, suggesting clearance through active transportation of the MS from the injection site. Indeed, MS were ingested by phagocytic cells at the injection site, which played a leading role in the transport of the particles to principal lymphoid organs, thereby providing a continuous supply of particles and antigen. The MS retained their gross morphology inside the cells for at least 2 weeks. Thus, MS degradation and antigen release inside the cells must have lasted for more than 2 weeks. MS were observed in the spleen and the cells from the peritoneal cavity as long as 4 weeks following s.c. injection. This suggests long-term trafficking of the particles inside phagocytic cells from the injection site to lymphoid organs (Fig. 6.1: schematic diagram of the *in vivo* trafficking of F-MS).

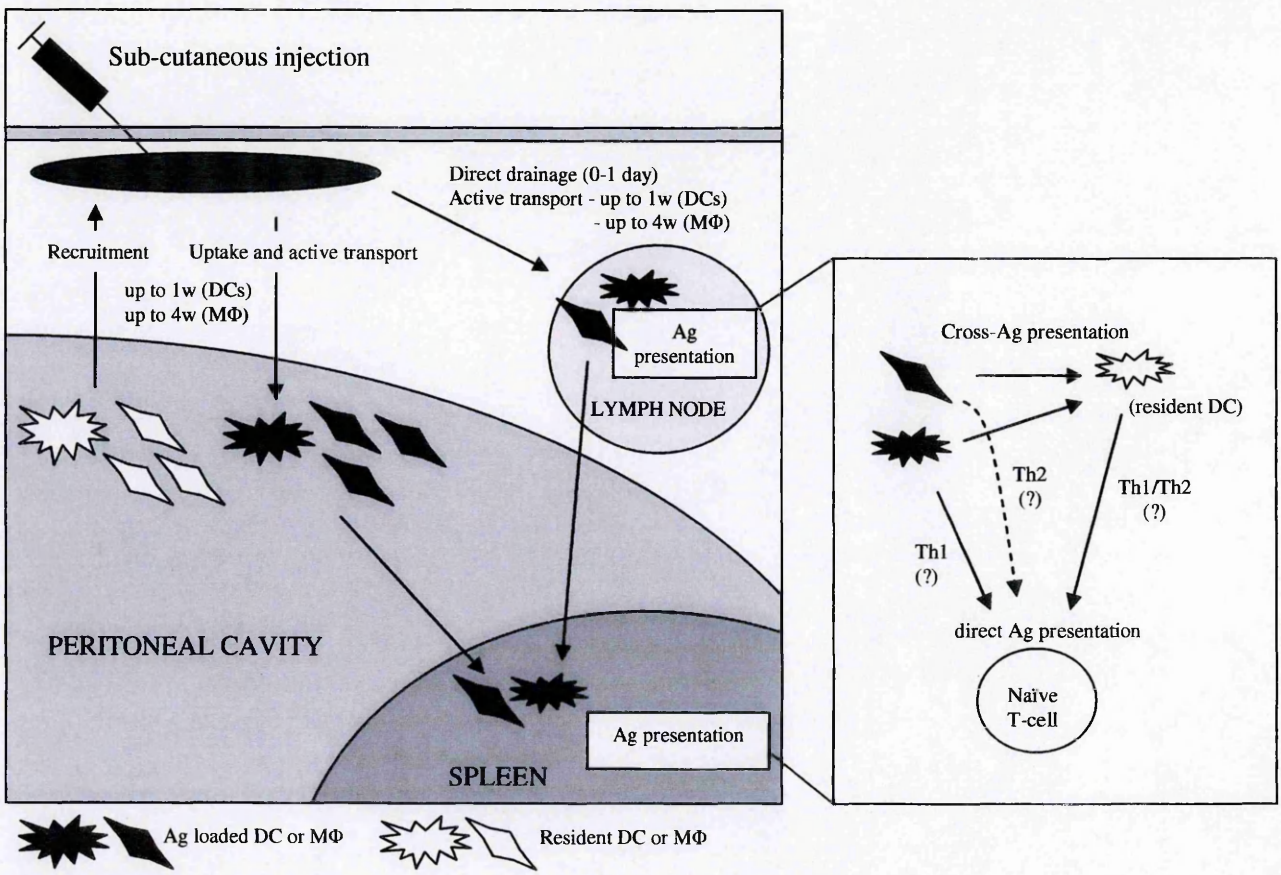


Figure 6.1 Antigen presenting cells (APCs) migration following a sub-cutaneous injection. It has to be noted that the transport of APCs through blood vessels from the IS or MLN to the spleen from 1 week post-immunisation is not represented there. Further details are shown in the schematic diagrams in appendix 2.

The internalised particles were less than 5 μm in diameter. This finding agrees with previous studies which observed a similar threshold size in the biodistribution of particles following mucosal and systemic routes of immunisation (Damge et al., 1996; Desai et al., 1996; Eldridge et al., 1991; Jani et al., 1990; Gupta et al., 1996; Tabata et al., 1996; McClean et al., 1998). Larger sized particles were not detected inside lymphoid organs before 4 weeks; however, entrapment of larger sized MS and enhanced transportation inside cells were observed at later time points. A possible explanation for this phenomenon may be an antibody mediated (Fc-fragment) uptake of larger particles. Indeed, anti-diphtheria specific antibodies were detected in the blood serum 2 weeks after primary immunisation. It may be reasonable to assume that these anti-DT antibodies bound to the DT present at the surface of the MS; this enhanced opsonisation may facilitate further MS uptake and trafficking, even of larger particles.

Data presented in this study emphasize the key role of macrophages in the uptake and transport of the PLGA particles (Harmsen et al., 1985). PLGA MS have been observed inside macrophages of the peritoneal cavity following intra-peritoneal injection, and inside the dendritic cells of the skin following intradermal aministration (Newman et al., 2002). In the present study, MS were localised inside macrophages taken from the injection site and peritoneal cavity following s.c. injection, and also inside dendritic cells from the lymphoid organs. Characterisation of the phenotype of the phagocytic cells from the peritoneal cavity revealed that MS stimulated activation of macrophages, as indicated by an increase in the CD86 marker. However, their ability in presenting antigen via MHC class II pathway seemed to be diminished, as indicated by a lower expression of the MHC class II molecule. It has been shown that MS can escape the MHC class II pathway and mediate MHC class I presentation and CD8 cytotoxic T cell activation (Newman et al., 2000). This study shows the degradation

of PLGA MS and release of microencapsulated fluorescent dyes directly inside the cytoplasm of the cells (Fig. 3.12.) allowing availability of the Ag present in the cytoplasm to be presented via the MHC class I route. Then, the decrease in MHC class II protein complexes might well be counter-balanced by an increase in MHC class I molecules expression, although this was not measured in this study. The hypothesis will be that PLGA MS induce maturation of macrophages so that they become competent APC, as shown *in vitro* with alum (Zinkernagel et al., 1997; Rimaniol et al., 2004). Further, we have also shown that PLGA MS involve dendritic cells in the early stages of the response (Table 3.3.). These observations may support the theory of APC-APC interaction and antigen presentation before final presentation to naïve T-cells (Knight et al., 2001; Carbone et al., 2004) (Fig. 6.1). Accordingly, the macrophages would ingest and transport the MS from the injection site to MLN or spleen and further transfer the particles to dendritic cells, along with activation signals, leading to antigen presentation to naïve T-cells. The reason for the more balanced Th1/Th2-response conferred by PLGA MS might also reside in the fact that both the macrophages and dendritic cells appear to be associated with the processing of these particulate vaccine formulations (Fig. 6.1.).

After s.c. administration of a booster dose of PLGA MS, the size and number of MS ingested by phagocytic cells increased substantially (Fig. 3.6.). This phenomenon is probably a consequence of the aforementioned mechanisms underlying long-term particle phagocytosis and transport: (i) the long-term depot of MS at the injection site, (ii) production of antigen-specific antibodies, (iii) enhanced opsonisation of the particles through antigen specific antibodies that recognise remaining antigens on the particle surface, and (iv) macrophage activation.

6-2 Formulation is everything: essential role of polymer type and particle size.

The hydrophobicity of microparticles has been shown to be a determinant factor in phagocytosis (Tabata and Ikada, 1988a; van Oss, 1978; Tabata and Ikada, 1994). This was supported also by data presented here with PS beads, which recruited more inflammatory cells at the injection site than the less hydrophobic antigen-containing PLGA MS (Chapter 3), and which were also ingested more efficiently by macrophages *in vitro* than the PLGA MS. (Appendix 3 shows micrograph pictures of the *in vitro* uptake of PS beads by macrophages cell line. The uptake of PS beads is more efficient than for PLGA MS when compared with Figure 3.7., with at least a 10 fold difference in the number of particles being uptaken). When considering particle surface hydrophobicity, the presence of microencapsulated materials (antigens, dyes) on the particle surface must not be disregarded. Presence of hydrophilic molecules may alter the hydrophobic property of the particles and, thereby, reduce their uptake by professional phagocytic cells. This could well explain the higher IL-12 production observed with the placebo PLGA MS as opposed to DT-containing PLGA MS (Fig. 4.7.). It was also observed in this study that the microencapsulation of hydrophilic fluorescent dyes greatly lowered the cellular response (Appendix 4 shows the influence of the co-encapsulation of fluorescent dyes on the degree of immune response induced against DT). By varying the PLGA type, only the slightly more hydrophobic PLGA50:50 MS made from RG503H and RG503 induced a T-cell memory response, as measured at 4 weeks post-immunisation (Figure 4.1.) while the quite hydrophilic RG502H MS did not mediate such response. The inflammatory response was also higher for the RG503H MS than for the RG502H MS (Conway et al., 1997).

The priming potential of PLGA and PLA MS for anti-TT and anti-DT Ab was similar over 2 - 8 weeks. However, considering previous work as well as the *in vitro* release and degradation properties of these polymeric MS (Thomasin et al., 1996; Men et al., 1995), it was expected that the two polymer types will produce different kinetics of immune response, with PLA MS giving a delay in Ab production and maturation. As this was only partly observed, other characteristics such as particle hydrophobicity, enhanced particle uptake and antigen processing by antigen-presenting cells (Tabata and Ikada, 1988b) might have compensated for the slower degradation and release properties of PLA MS. Although the PLGA MS performed immunologically better than the PLA MS, it might be of interest to extend the duration of antigen presentation using the more hydrophobic PLA MS. The choice of polymer type may be crucial to obtain efficient prophylaxis or therapy with encapsulated antigens or protein therapeutics that require particular dosing frequencies and intervals (Johansen et al., 1999; Partidos et al., 1994).

Besides polymer type, the MS size also appeared to be of some importance for the efficacy of the MS vaccines, although solely for the RG502H MS and for up to 4 weeks, where the smaller sized MS induced stronger inflammatory signals (cytokines) than the larger sized ones (Table 4.2). Thus, particle size might be a secondary limiting parameter for immunological efficacy. As observed in the trafficking study, both small- and large-sized RG503H MS were cleared at similar rates from the injection site. Conversely, the large-sized RG503H MS appear to recruit more inflammatory cells at the injection site and in the peritoneal cavity after 8 weeks than the small-sized MS (Fig. 3.15.). However, only small-sized RG503H MS induced

T-cell activation at 4 weeks, which was not attributable to a less efficient particle uptake and clearance of the large-sized MS from the injection site. The large-sized MS did not seem to be transported towards lymphoid organs before the time span between 4 and 8 weeks. This may suggest two different mechanisms of particle uptake by macrophages. Up to a certain threshold size of approximately 5 μm, which incidentally covers also the dimensions of most pathogens, macrophages transport and degrade foreign particulates for presentation to naïve T-cells. However, if the foreign object is above such threshold size, but still small enough for being phagocytosed, it will get quickly degraded, thereby inducing probably apoptosis of the phagocytic cells and clearance of the particles via blood vessels into the liver and kidney (Djaldetti et al., 2002; Flarend et al., 1997). This theory would explain the similar kinetics of clearance observed for both small- and large-sized MS from the injection site, on the one side, and the difference in inducing early T-cell maturation between the small- and large-sized MS, on the other side. Indeed, at early time points, only the small-sized RG503H MS induced memory T-cells, as T-cell maturation seems to require uptake and re-localisation of Ag in lymphoid organs. It would be interesting to substantiate further this hypothesis by performing a long-term cellular response study to assess the effect of the observed late re-localisation of larger particles after 8 weeks post-immunisation.

One of the early hypotheses for MS-based, single-injection vaccines was that they should consist of a blend of MS-types with different polymer erosion and antigen release kinetics (Dutton et al., 1999; Singh et al., 1998b; Men et al., 1995). It was postulated that the formulation should contain two to three types of MS, i.e., small and fast-releasing particles that could be taken up readily by antigen-presenting cells for priming of the immune response, and one or two other MS populations for antigen release at different time points to boost and

sustain the Ab response. The latter depot-forming MS should also be sufficiently large to avoid uptake by antigen presenting cells. However, the present investigation supports recent observations (Johansen et al., 1999; Johansen et al., 2000a) that the former concept may not be optimal. First of all, no real depot effect was observed with larger particles as opposed to the alum adjuvant (chapter 3.3, Fig 3.4.). Furthermore, single MS populations generally generated a stronger immune response during 16 weeks than mixtures of two MS types. Only one specific MS mixture, composed of small PLGA and PLA particles, produced higher anti-DT Ab titres than did PLGA or PLA MS (small or large) alone (Fig. 5.1.). So, considering the technological challenge of producing and blending two or more MS populations of different sizes, a single MS type consisting of small particles may be preferable.

6-3 Efficacy of PLGA MS vaccines with and without co-administration of aluminium hydroxide

One objective in the development of new vaccine delivery systems has been the replacement of alum adjuvants to improve safety of current vaccines. Previous studies have shown that PLGA MS vaccines performed as efficiently as the conventional alum-adsorbed vaccines in inducing high levels of Ab (Gupta et al., 1998a; Johansen et al., 1999; Singh et al., 1992; Men et al., 1995) and conferring protection (Kersten et al., 1996; Kissel et al., 1997b; Singh et al., 1997b; Singh et al., 1998b). Co-administration of aluminum hydroxide and DT-TT PLGA MS in parenteral vaccination appeared to improve further their immunological responses (Johansen et al., 2000a). In this study, the influence of admixed alum on the immune response induced by PLGA MS vaccines was investigated.

The amount of admixed alum in DT- and TT-containing PLGA50:50 MS (RG502H) enhanced significantly the antigen-specific humoral response. Total Ab levels measured at the end of the experiments (16-44 w) were always significantly higher in the animals that had received the alum-adsorbed control vaccine or the mixture of DT-MS + TT-MS + alum than in those injected with the MS vaccine alone. The high Ab levels were shown to result from the enhancement of the early humoral response by the alum adjuvant (Fig.4.4.), which was likely mediated by the strong inflammatory response triggered by alum at the injection site for up to 4 weeks (Fig 3.16.). Because of the nature of the alum-adsorbed vaccine, the protein Ag cannot apparently escape the MHC class II presentation pathway, which is in contrast to the MS vaccines (chapter 3.5, Figure 3.12) (Newman et al., 2000). With the alum-adsorbed vaccines, the response is focused exclusively on the Th2 pathway, with an increase in Th2 signals promoting B-cell clone expansion and maturation into memory B cells. However, memory B-cells require multiple activation signals in the form of booster doses to maintain high levels of circulating Ab. As seen with the 2 doses schedule of the alum-adsorbate vaccine (injections at 0 and 4 weeks), the Ab levels increased substantially up to 16 weeks and then sharply decreased up to 1 year post-immunisation, which contrasts the sustained Ab levels induced by MS vaccines. This suggests non-availability of Ag from the alum-adsorbate for longer times, especially within the lymphoid organs. In this context, the role of alum remains somewhat elusive, particularly when administered together with MS vaccine. As observed in the trafficking study, it was very difficult to detect any MS within the lymphoid tissues when the MS vaccine was co-administered with alum, as opposed to the traceability of the MS when injected alone.

As expected, admixed alum in MS vaccines strongly promoted the immune response and directed it towards a Th2 type response, with increased levels of IgG1, but undetectable levels of IgG2a antibodies (Bomford, 1980a; Bomford, 1980b). This immunological pattern depended on the amount of admixed alum and differed kinetically for the two antigens. Both the antibody and cellular responses peaked at 4 weeks for tetanus and at 6 weeks for diphtheria. Interestingly, the cellular response against both DT and TT was quite strong upon administration of the RG503H MS which was not affected by the co-administration of alum. No significant cellular response was mediated by the more hydrophilic RG502H MS unless alum was co-administrated. Aluminium played an important role in the IL-2 production and activation of precursor (Th0) and inflammatory T-cells (Th1) in the early response. In comparison to alum (1 and 2 injections), the administration of MS suppressed the production of IL-6 and favored IL-12 synthesis at 6 weeks post-immunisation. MS vaccines induced more balanced cellular and humoral responses with higher levels of IL-12 and lower levels of IL-6 than did the aluminium vaccines, which strongly triggered the response towards a Th2 type (high levels of IL-6, low levels of IL-12).

Interestingly, the boosting property of a single injection of MS vaccines becomes obvious when the Ab response induced by the MS vaccines injected alone was compared to that elicited by alum-containing vaccines (PLGA MS vaccine with admixed alum and control alum-adsorbed vaccine given twice). Indeed, Ab levels induced by PLGA MS vaccines alone remained constant between 16 w and the end of the study (up to 1 year), whereas the initially high Ab levels mediated by all alum-containing formulations dropped sharply over this time. This stable level of Ab production, strongly suggests intrinsic adjuvant properties of the MS, probably due to sustained antigen release and related Ab affinity maturation (Eldridge et al.,

1991). Nonetheless, the alum-based prime-boost procedure appeared to be superior to the single injection of MS for tetanus, but not for diphtheria toxin neutralisation at 44 weeks post-immunisation (Table 4.3.). Total and neutralizing anti-TT Ab levels depended greatly on the presence of alum, whereas alum did not considerably affect the levels of functional anti-DT Ab. This demonstrates an antigen-specific sensitivity of the response with a bias towards Th2 response with tetanus and a more balanced Th1/Th2 response with diphtheria toxoid (Comoy et al., 1997).

Despite the relatively low levels of total anti-TT and anti-DT Ab induced by the MS vaccines alone, a single dose of DT-MS + TT-MS vaccine, without admixed alum, was sufficient to induce a long-term (one year) protective response in animal models. While alum enhanced the inflammatory response (Fig. 3.16.) and antigen persistency at the injection site (Fig.3.14.), resulting in the production of cytokines that triggered the production of antibodies (Fig.4.12.), the PLGA MS acted as a delivery system (Fig. 3.12. and 3.13.), providing antigen presenting cells with sustained low doses of antigen directly at the site of naïve T-cells and triggering the production of specific neutralising antibodies (Table 4.3.). The combination of alum and MS has helped to induce high antibody levels, although a single administration of DT-MS + TT-MS on its own was sufficient to maintain protection over nearly a year (Fig 4.4 & 4.12.). Indeed, a single injection of MS vaccine induced superior protective response than 2 doses of the control vaccine one year post-immunisation (Fig. 4.9 & 4.13).

The influence of alum on the innate inflammatory response became more apparent when different amounts of alum were co-administered with MS vaccine, as the inflammatory cytokine production, along with inhibition of Th1 specific cytokines, depended on the amount of administered alum. The observed hyper inflammatory reaction caused the formation of large granulomatous nodules at the injection site of animals immunized with mixtures of MS and alum for up to 4 weeks post-immunisation. Such high inflammatory response might be detrimental and cause serious complications such as alum contact allergy, as observed in the same children immunized with combined alum and pertussis components (PT, FHA, PRN, FIM 2 and 3) in the DTaP alum-adsorbate vaccine (Bergfors et al., 2003). Arthus-type and other adverse reactions have also occurred in the presence of high levels of circulating Ab (Relyveld et al., 1979; Rappuoli, 1990), which might be a limiting factor for using multiple antigens combined with alum (Offit et al., 2002).

Even though PLGA MS vaccines alone were capable to stimulate a protective response, total Ab levels induced by MS adjuvanted with alum were lower than those elicited with the conventional alum-adsorbed vaccine. This suggests that alum enhances the production of polyclonal Ab against multiple non-functional epitopes (Dr. R. Zurbrigen personal communication 2004). On the other hand, high circulating Ab may be beneficial for long-lasting protection, as protection from diphtheria and tetanus diseases in humans is mediated by circulating Ab, defined by levels of >0.1 IU/ml or >0.01IU/ml when measured by ELISA or toxin neutralization assays (WHO Expended Programme on Immunization, 1993b; WHO Expended Programme on Immunization, 1993a). Alum appeared to slow down particle uptake by phagocytes, which enhanced the Th2-type presentation, B-cell maturation and Ab production. Under these considerations, the combination of MS and aluminium hydroxide may

increase the percentage of protected individuals. Nonetheless, the substitution of alum by new adjuvants or antigen delivery systems or lowering the amount of injected alum by 10 - 50% of the maximal dose allowed (1.2mg/SHD Al^{3+}) (European Pharmacopoeia, 2005e) in a vaccine formulation such as PLGA MS is desirable to avoid alum-related adverse effects. However, high levels of circulating Abs might be detrimental for eliciting responses against other antigens. Moreover, adjuvant-adjuvant interactions may be of concern as little is known so far on safety and efficacy of combined adjuvants or delivery systems (Sesardic and Dobbelaer, 2004). Even if a particular novel adjuvant or delivery system is used alone, interaction with a previously injected adjuvant from a former vaccination may still take place. The concern might be even greater for slow-release PLGA-based vaccines, as PLGA would remain at least 4 weeks, or even substantially longer depending on the PLGA type, at the injection site (Sesardic and Corbel, 1997). One alternative would be to combine a maximum of antigens within the same formulation. However, interaction between antigens remains a principal concern regarding combination vaccines, especially when the formulation contains a novel adjuvant or delivery system.

6-4 Multivalent vaccines based on PLGA slow release technology

Currently up to twelve vaccine antigens are included in the routine childhood immunisation, with multiple doses of most required for protection. If given separately, this would imply approximately 70 injections (http://www.cdc.gov/mmr/). Fortunately, combination vaccines are available, which reduce distress to the vaccinees, increase their compliance and the overall vaccination coverage. Penta- and hexavalent vaccines against diphtheria, tetanus, pertussis, *Haemophilus influenzae* type b, polio and hepatitis B virus are already on the market

(Aristegui et al., 2003a), and an heptavalent childhood vaccine associated with two warfare agents has even been tested in animals (Griffiths et al., 2001). Issues that should be considered when combining vaccine components include the current childhood vaccination schedule, compatibility of components, availability of antigens for the targeted diseases, route of delivery, safety, immunogenicity and efficacy (Yeh and Ward, 2001; Yeh et al., 2001). Many combinations are as efficacious as the separate vaccines, but the increasing number of antigens could theoretically pose problems with respect to efficacy (Clemens et al., 1996) and safety (Elliman and Bedford, 2003). Immune response against TT, DT or Hib have already been shown to be compromised when administered in combinations (Dagan et al., 1998; Gabutti et al., 2004). A key question to be solved is also the capacity of the human organism to deal efficiently with several antigens at the same time (Offit et al., 2002; Dagan et al., 2004).

As a first step in the investigation of multivalent PLGA MS vaccines, the immune response induced by a divalent DT-TT-PLGA MS vaccine was studied. The divalent formulation was either administered as a mixture of monovalent DT-MS + TT-MS or as a single MS preparation with both antigens being co-encapsulated (DT-TT-MS). These experiments were carried out to assess the feasibility of multivalent MS technology.

Although one objective of this study was the development of MS vaccines eliciting sustained immune responses, ELISA Ab responses were monitored during a 16 weeks period. This relatively short study period appeared sufficient for long-term extrapolation, because persistence of toxin-neutralising anti-TT and anti-DT Ab over a year had previously been demonstrated with similar formulations (chapters 4.4. and 4.5.) and with divalent DT-TT-MS vaccine (Table 4.3.) and also through data in the literature (Gupta et al., 1997; Johansen et al.,

2000b; Singh et al., 1997b; Singh et al., 1998b). In some of these previous experiments, microencapsulated DT or TT revealed maximum Ab titers between week 8 and 16 post-immunisation, which did not change substantially up to one year. Furthermore, the long-term potential of TT-MS has been demonstrated in mice and guinea pigs by boosting the animals a year after priming (Gupta et al., 1997). The booster injection after 1 year elevated the titers of nAb to a higher level in animals injected once with TT-MS than in those immunised twice with TT in alum. It was expected that similar Ab-response kinetics will persists in the present investigation, with differences between the formulations being reflected by the maximum Ab levels in the initial phase of Ab production (Johansen et al., 1999).

All divalent MS vaccine formulations generated strong Ab responses against both DT and TT, and the titres substantially increased during the study period of 16 weeks (Fig. 5.1.). Interestingly, PLGA MS produced a more TT-skewed Ab response than PLA particles, i.e., a higher ratio of anti-TT over anti-DT ELISA Ab titres with PLGA as compared to PLA MS. As shown in chapter 3, PLA MS, being slightly more hydrophobic than PLGA MS, were probably more efficiently ingested than the PLGA MS, thereby enhancing the Th1 response, which appeared to be more important for the anti-DT response. Indeed, as shown in chapter 4, DT induced a more balanced cellular-to- humoral (Th1/Th2) response than TT. Differences in antigen-release kinetics or antigen stability of DT and TT as well as of the two antigens in PLGA and PLA MS may have caused the steady increase in anti-TT Ab over 16 weeks, on one side, and profile flattening of anti-DT Ab after eight weeks, on the other side (Fig. 5.1.). Thanks to the well-known robustness of TT and its high molecular weight (approx. 150 KDa versus 60 kDa for DT), it would be considered logical that TT releases more slowly from the MS than DT.

Different MS preparations (0.5-10μm small- and 10-60μm large-sized PLGA and PLA MS) mediated similar initial kinetics with continuously increasing ELISA Ab titers. For TT, the kinetics of Ab-titers even suggested a further increase beyond 16 weeks, at least in some of the immunisation groups. The reason for the important continuous increase in Ab, particularly against TT, as compared to the more instant rise towards a plateau in the earlier investigations (Johansen et al., 1999), was very likely related to the presence of admixed alum. As shown previously (chapters 3.3. and 4.4.), alum had the ability to prime the humoral response against the antigen better than MS alone by activating a larger number of precursor lymphocytes. As a consequence, less antigen is required to boost Ab when alum is used as a co-adjuvant.

In this study, the experimental divalent MS vaccines provided better protection against diphtheria and tetanus upon challenges in animal models than did a single injection of a comparable licenced alum-adsorbed divalent vaccine (Fig. 5.3.). The comparison was performed according to a modified Ph. Eur. control test that is a requirement for batch release of DT/TT vaccines (challenged at 6 weeks instead of 4 weeks post-immunisation). Those assays determine vaccine potency against a reference vaccine calibrated in International Units (IU) within defined specifications (European Pharmacopoeia, 2005a; European Pharmacopoeia, 2005b). Differences in protection between MS preparations were observed mainly for diphtheria. At low vaccine dose (0.6 Lf; approx. 1/80 of a single human dose), fast degrading small-sized PLGA MS induced better protection than slowly degrading small-sized PLA MS. Protection against DT probably requires a subtle balance between circulating Ab and cytotoxic T-cells, which are actively involved in the DTH reaction measured by the potency challenge assay. It is a general notion, that nAb are the major effectors in the immunological defence against intruding toxins and that cellular responses induced

post-infection or vaccination play a critical role in the activation and specification of antibody-secreting cells. Hence, the reason why the licensed alum-based control vaccine, injected once, protected less efficiently than the MS vaccines 6 weeks post-immunisation, despite comparable antibody responses, might be due to a better stimulation of the cellular response by the MS (Scheifele et al., 2001). The favorable cellular response that was induced by the small-sized MS is probably due to a prompt particle uptake by antigen-presenting cells, wherein the fast degrading particles liberate antigens for processing and MHC class-I and II presentation (Ma et al., 1998; Moore et al., 1995; Newman et al., 1998; Men et al., 1997). Subsequent slow antigen release is likely to facilitate the maturation of memory T- and B-cells. For TT, a good agreement between protection and Ab data was observed, harmonising the fact that protection against tetanus is mainly mediated by humoral response. However, efficacy differences among the various MS formulations were not detected in the protection assay, probably because of the limited discriminative power of the challenge assay in animal models.

In summary, all divalent DT-TT-MS vaccines, co-injected with admixed alum, induced functional antibodies against TT and DT and produced protective immunity against tetanus and diphtheria by direct challenge with toxins. The observation that the immunological performance of the MS formulations did not crucially depend on the physico-chemical properties of the MS, i.e., the polymer type and MS size, suggests that the co-admixed alum possibly represented a limiting factor for the discrimination between intrinsic parameters of the formulations.

The development of new multivalent vaccine delivery systems has to address the feasibility of combining as many antigens as there are in current childhood combination vaccines (DT, TT, aP or DT, TT, aP, Hib). Previously, the combination of a trivalent alum-adsorbed DT-TT-aP vaccine with a tetanus toxoid-conjugated Hib vaccine caused no immunological interference in guinea pigs, whereas the tetravalent alum adsorbed DT-TT-aP-Hib vaccine produced significantly lower IgG antibodies to pertussis toxin and filamentous haemagglutinin than the DT-TT-aP-adsorbate alone (Gupta et al., 1999). Therefore, the mixing of vaccine components prior to inoculation may be advantageous over the combining of all antigens in one vaccine formulation. Physically separated antigens would exclude any potential physico-chemical interactions during storage and upon application. Nevertheless, relative immunological dominance of one or several antigens or skewing of the innate and adaptive immune responses toward a type that favors some antigens still remains an issue to be addressed in any new vaccine formulation. Any type of new combination vaccine in development has to possess advantageous properties over a mixture of the individual components before clinical testing can be considered (Vidor et al., 1999).

In Chapter 5.4., the immunological properties of a tetravalent (DT, TT, aP and Hib) MS vaccine were evaluated and, particularly, the effect of co-encapsulating the antigens *versus* mixing the monovalent formulations. When monovalent formulations were combined, the immunogenicity of tetanus toxoid was not significantly affected by adding other MS that contained DT, aP or Hib antigens, although some improvement in toxin neutralisation was observed when the mixture contained DT and aP antigens. For diphtheria-specific immunity, the mixing of TT-MS and DT-MS significantly improved the immunogenicity and specific protection against diphtheria toxin, whereas admixture of aP-MS slightly decreased the anti-

DT immunogenicity and neutralisation capacity as compared to DT-MS+TT-MS (Fig. 5.4 & Table 5.4.). This finding is consistent with published results showing that co-injection of acellular pertussis (aP) and DT decreases the immunogenicity of diphtheria toxoid, whereas whole-cell pertussis (wP) increases the DT-immunogenicity (Sesardic et al., 1999; Tiru et al., 2000). By the same token, DT-TT-wP-alum, but not DT-TT-aP-alum, was combined with Hib without immunological implications (Halperin et al., 1999; Pichichero et al., 1997), and a rise in Hib cases in vaccinated children has been ascribed partly to the use of combined DT-TT-aP-Hib-alum preparations (Trotter et al., 2003). This adjuvant effect of whole-cell pertussis vaccines has been well documented for a long time, whereby the effect might be mediated through co-stimulatory signals induced by the whole-cell pertussis, or perhaps solely through the particulate nature of the vaccine. In case of the latter, the substitution of the cell with another particulate carrier, e.g., polymeric MS, might yield a comparable adjuvant effect. In accordance with this concept, Mazzantini and co-workers immunised mice with recombinant BCG expressing non-toxic fragments of the diphtheria or tetanus toxin (Mazzantini et al., 2004). They found a reciprocal adjuvant effect of the two vaccines leading to more effective immune response against both diseases. It is therefore important to monitor the immunogenicity and reactogenicity of antigens in combination vaccines carefully, and it might become necessary to change the nominal amount of certain antigens in novel antigen-delivery systems (Capiau et al., 2003).

The tetravalent DT-TT-aP-Hib-MS formulation and the different mixtures of monovalent MS produced very similar tetanus-specific immune responses, indicating that tetanus toxoid is a relatively stable antigen that can be safely combined. For diphtheria, a synergic effect appeared when combining DT with TT in MS, with a rise in immunogenicity and potency.

One possible reason for this is the sharing of helper epitopes, which improves the priming of the immune response and demonstrates the strength of tetanus toxoid helper epitopes, which are often used as universal epitopes for other sub-unit and peptide vaccines (Diethelm-Okita et al., 2000); efficient generation of antibody responses and B-cell memory requires CD4 T-cell help (Gray et al., 1996) (European Pharmacopoeia, 2005c; European Pharmacopoeia, 2005d). Furthermore, the enhanced immunogenicity of co-encapsulated protein antigens may also be related to a stabilising effect the antigens exerted on each other within the polymeric matrix (Boehm et al., 2002).

One might expect, from a dose-response perspective, tetanus-toxoid conjugated Hib antigen to improve the tetanus-specific immune response by using a TT-Hib combination vaccine. This was not evident in the present investigation, which agrees with some (Shams and Heron, 1999) and contradicts other reports (Gupta et al., 1999). Upon vaccinating infants with a combination of a tetravalent tetanus-conjugated pneumococcal vaccine and a DT-TT-aP-Hib-alum vaccine, both anti-Hib and anti-tetanus antibody concentrations were inversely related to the tetanus toxoid content of the conjugate and the vaccine. This phenomenon was interpreted as interference by the common tetanus toxoid carrier protein. Furthermore, whereas an increase in anti-tetanus responses in the presence of Hib was observed in mice (Redhead et al., 1994), several laboratories found little or no effect on anti-tetanus responses in guinea pigs with DT-TT-aP-Hib than with in DT-TT-aP (Winsnes et al., 2004; Gupta et al., 1999).

Protection against intruding toxin is commonly attributed to neutralising antibodies, whereas cellular responses induced post-infection or vaccination mainly play a role in the activation and clonal expansion of specific antibody-secreting cells. Hence, it was surprising to note that the alum control vaccine produced apparent lower diphtheria protection than the MS

formulations, despite generating higher levels of neutralising antibodies. Within the context of the divalent formulation, we ascribed this phenomenon, at least partly, to the experimental set-up with the challenge being performed at six weeks after priming. In the later study, the challenge was therefore postponed to week 16 or even week 52 to obtain full benefit of the prime-boost regime with aluminium hydroxide (booster injection at four weeks). Nonetheless, the alum control vaccine still produced less protection (Figs. 4.9 & 4.13. and 5.5 & 5.9.). An alternative explanation may be that the protection assay for diphtheria reflects more a cellular response (delayed-type hypersensitivity) and that MS stimulate such cellular responses better than aluminium (Scheifele et al., 2001). The cellular response was probably induced by the fraction of small MS that were promptly and avidly taken up by antigen-presenting cells, wherein the particles released antigens for processing and MHC class-restricted presentation (Men et al., 1999a; Moore et al., 1995; Newman et al., 1998; Ma et al., 1998; Men et al., 1997).

Combination of conventional childhood vaccines with a malaria protective antigen would be extremely valuable, because of the high prevalence of malaria, particularly in sub-Saharan areas, where vaccination coverage is sub-optimal. This study explored therefore the potential of combining the tetravalent DT-TT-aP-Hib-MS vaccine with microencapsulated liver stage malaria antigen from the *Plasmodium falciparum* circumsporozoite protein (PfCS-MS). The PfCS antigen used in this study was shown to stimulate specific humoral and cellular immunity in mice when encapsulated in PLGA MS (Men et al., 1999b; Men et al., 1996; Men et al., 1997) and has already gone through successful clinical testing (Roggero et al., 1999; Lopez et al., 2001). The blending of PfCS-MS with DT-TT-aP-Hib-MS improved the diphtheria- and tetanus-specific immunity. One possible explanation could be that the higher

number of particles administered (three *versus* two milligram MS) resulted in increased pro-inflammatory stimulation, leading to a stronger commitment of a higher number of antigen-presenting cells. However, the fact that the immune response against TT did not increase when the monovalent MS formulations (DT-, TT-, aP- and Hib-MS) were mixed together, highly increasing the number of MS administered (Fig. 5.4.), does not support this hypothesis. The malaria antigen itself may have mediated the observed effect, thanks to the provision of CD4 T-helper epitopes for the toxoids.

Immunisation of guinea pigs with PfCS-MS induced only low levels of anti-PfCS antibodies, with the titers being several orders of magnitude lower than in BALB/c mice. It is unclear if this species-related difference is due to allelic restriction or an insufficient antigen dose in guinea pigs. Several PfCS epitopes, which are recognized in BALB/c mice ($H\text{-}2D^d$) and men (HLA-A*021), are unknown for their recognition in guinea pigs. Furthermore, the presence of the tetravalent DT-TT-aP-Hib-MS vaccine significantly decreased the immunogenicity of the PfCS-MS in guinea pigs, but enhanced it in mice. From these experiments it is difficult to conclude on the mechanisms of immunological interaction between PfCS and the other antigens; and it was not possible to compare with other studies as no studies on the combination of malarial antigens with DT, TT, aP or Hib vaccines have been previously reported. Nonetheless, the results reveal that depending on the specific antigens used in a study, the experimental design and animal model must be carefully planned to assure haplotype matching, without compromising the quality of the pre-clinical read-out for any of the antigens under study.

6-5 Implications of slow release PLGA MS vaccines for quality control

A key issue in the production and development of novel vaccines is maintenance of consistency within the formulation, thus ensuring the safety and the efficacy of every batch. Batch-to-batch reproducibility needs to be monitored (Sesardic and Corbel, 1997), and predefined quality criteria have to be met before vaccine formulations can reach the clinical development stage (Sesardic and Dobbelaer, 2004).

In this work, SEM and fluorescence microscopy were essential tools to evaluate formulation and process parameters, to monitor formulation consistency, batch-to- batch reproducibility and MS *in vitro* degradation.

Formulation greatly influenced the structure of the MS. The morphology of the beads were first assessed by SEM. Microspheres may possess regular spherical morphologies with smooth and essentially non-porous surfaces (PLA-MS), or may possess a lot of internals pores (PLGA-MS), or the particles may be collapsed (coacervation) or coalesced (spray-drying), all depending on particular formulation parameters (as shown in SEM micrographs in Appendix 5). Size was influenced by the fabrication method. Small-sized MS (0.5-10μm diameter) were produced by spray–drying, while coacervation produced larger particles (10-60μm diameter) (the SEM micrographs in Annex 5 show the difference in size according to the fabrication technique). MS-degradation was also followed *in vitro* by SEM and epifluorescence microscopy using fluorescent MS (F-MS). The MS gradually disintegrated over the 4 week incubation period with corresponding loss of fluorescence intensity and increased aggregation of degraded polymer (Fig.3.10). These data gave essential information on the methodological approach to study the physical properties of PLGA microspheres when used as potential new

vaccine delivery. As reported before, SEM was an effective characterisation technique highlighting differences in size, morphology and batch to-batch variability (Hockley et al., 1999).

One of the leading controversies in the development of slow release vaccines has been the accurate measurement of active antigen content inside MS (Gupta et al., 1997; Xing et al., 1996b). In this study, a simple extraction /filtration technique was used (Chapter 2.3.3). Only the immunogenic antigen, as opposed to total protein content, was measured using neutralizing anti-TT and anti-DT monoclonal antibodies. Based on such experimentally determined antigen contents, there was a good correlation in the immune responses between the control and MS vaccine doses. We also observed a good reproducibility of the immunological response induced by MS from different batches with different experimentally determined antigen contents and between identical batches used within different experiments (Tables 4.1 & 5.2).

Safety is a major concern with new vaccine delivery systems. Even though the polymers used have been extensively studied and are widely used in humans, the safety of slowly released antigens has not yet been studied specifically. In our study, we observed the development of granulomatous nodules at the site of injection of mice immunized with the RG503H MS. Although a wide variability between animals was noticed, 70-100% of mice developed nodules when immunised with 2.5 Lf of DT-MS vaccine (corresponding to approx. 2.5 mg of MS), but less than 30% when immunized with 1 Lf of TT-MS (corresponding to 1 mg of MS). The nodules appeared 2 weeks post-immunisation and cleared spontaneously after 4 weeks. Nodules were larger when alum was co-administered and, in some cases where the highest amount of admixed alum was used, led to necrosis of the skin. No such reactions were

observed in guinea-pigs. Furthermore, no granulomateous reaction was observed with the RG502H MS, which coincides with a lower efficiency of this MS-type in the induction of the immune response. As reported by others (Gupta et al., 1993), a certain degree of inflammatory response seems to be required to induce a protective response. Granuloma formation has also been observed previously with aluminium, as well as the prolonged persistence of a sub-cutaneous nodules in children after vaccination with alum-adsorbed vaccines (Erdohazi and Newman, 1971; Bergfors et al., 2003). However, thanks to the biodegradability of the PLGA and PLA MS, the formulation shall eventually disappear from the injection site and may not cause permanent nodules, an issue that still needs to be addressed (Gupta et al., 1997).

To assess the efficacy or potency of a vaccine, the induced immune response or protection has to be measured and compared to an established reference. For slow release vaccine delivery systems, two different aspects may have to be addressed: (i) the potency of the vaccine and (ii) its efficacy after a single administration. In this study, we have shown that a conventional alum-adsorbed vaccine induced significantly higher total anti-DT Ab levels than corresponding PLGA MS vaccine at all time points; very importantly, however, the protective potency was superior with the slow-release MS vaccine. This emphasizes that simple serology assay measuring total Ab levels may not be sufficient to predict the efficacy of the PLGA MS vaccines and that a protection experiment performed earlier than 12 weeks post-immunisation is probably premature to assess the efficacy of MS-based vaccines (Sesardic and Corbel, 1997). Moreover, neutralising Ab correlated well with total ELISA-Ab data for up to 16 weeks post-immunisation. This could be misleading as at later time points (e.g., at one year), the nAb levels had dropped for the alum control vaccine injected twice, which is consistent with the results of the direct protection experiment.

T-cell proliferative response may give a good indication of the efficacy of MS vaccines to induce maturation of memory cells and could prevail over the long term protection assay, which is not practical for batch release use. However, this assay would need to be validated, i.e., correlated with long-term efficacy of high and low potency batches. For such validation, the conventional alum-adsorbed vaccine would not be a good reference, as it does not stimulate a high T-cell proliferative response.

It is noticeable that *in vitro* testing of MS vaccines, as for most other vaccines, has strict limitations for predicting the mode or extent of immunological actions *in vivo*. For example, our data did not reveal any relationship between the 24 h burst release and the extent of the early immune response induced. These findings corroborate previous observations where MS vaccines, incubated *in vitro* for 24 h and injected into guinea-pigs after removal of the supernatant (Sasiak et al., 2000), remained immunogenic without showing a relationship between the extent of *in vitro* burst release and the degree of immune response. Initially released Ag does not seem to be phagocytosed efficiently by APC, processed and presented to naïve T-cells. However, in the presence of alum, initially released Ag could well have adsorbed directly on alum for uptake by APC and further processing. SEM pictures have shown the presence of a coating on the surface of the spheres (data not shown) when alum was present in the formulation. This could well explain the presence of large conglomerates observed *in vivo* after injection of MS dispersed in alum solution. Alum seemed to be adsorbed on the surface of the MS and act as a link between other MS, creating a MS-alum particulate network. Thus, the particulate nature of the vaccine formulation is critical for the initiation of the immune response (Djaldetti et al., 2002).

The limitations of *in vitro* testing to predict *in vivo* outcome has been further evidenced by the *in vitro* uptake of MS by macrophages [e.g., by J774 cells or primary cultured cells (data not shown)], where the influence of particle size on the trafficking of the phagocytosed MS could not been determined. *In vivo*, only MS that were smaller than 5µm were efficiently transported towards lymphoid organs at early time points, which is necessary for Ag presentation. *In vitro*, macrophages ingested efficiently all small- and large-sized MS even though some kinetic differences were observed between particles sizes. *In vitro* particle uptake studies with macrophages or possibly also with dendritic cells can, therefore, provide only limited information on the faith of the MS in the early phase of the immune response. Additional stimulation factors present at the injection site and relevant for the innate response must play an important role in promoting the transport of the ingested particles. The *in vitro* uptake of MS does not *per se* provide essential information on the efficacy of the vaccine. It would therefore be interesting to develop an *in vitro* test for MS phagocytosis combined with an assay of cell activation in the presence of appropriate activation factors. Such a test associated with re-stimulation of T-cell clones specific for a defined Ag and characterised for their Th1 or Th2 immuno-type ($CD4^+$ and $CD8^+$ T-cells) could provide valuable information about the efficacy of MS vaccines. Indeed, such *in vitro* tests would not only allow us to assess Ag-release from the particles inside the cell, but also define the pathway of Ag-presentation.

CONCLUSION

This study on the trafficking and immunological properties of PLGA MS after sub-cutaneous administration in mice and guinea pigs confirmed previously postulated adjuvant mechanisms of PLGA MS as vaccine delivery system. Phagocytic cells ingested very efficiently the PLGA MS of a size of less than 5 μm in diameter. Phagocytosed antigen-containing PLGA MS slowly released the antigen inside APCs, which was facilitated and controlled, to some extent, by the biodegradation of the PLGA material. This process afforded a prolonged Ag presentation by APCs. This study also emphasised the importance of peritoneal macrophages as both professional scavengers in the first line of defense of innate immunity and active transporters of particulate materials to lymph nodes and spleen, where specific immune responses are induced. However, according to those findings, the efficiency of these immunological events depended on the stimulation of a strong innate inflammatory response by the PLGA MS, which was influenced by the hydrophobic nature of the polymer or the co-administration of the synergistically acting alum adjuvant. Therefore, in this study it was possible to demonstrate that MS may represent a safe and efficient alternative to alum as adjuvant for toxoid vaccines, provided that the polymer type and MS size are carefully optimized. Although PLGA MS may cause mild adverse effects, as observed with conventional alum adsorbates, it is expected that long-term protection should be achievable with a lower number of injections, e.g., 1-2 injections with PLGA MS instead of 3-4 with alum adsorbates.

Considering all previously published data and the results of this thesis it appears that the most essential pre-validation testing required for DT- and TT-containing PLGA MS vaccines has been performed in animals both in terms of biological safety and immunological efficacy. The next step in further investigating this new type of vaccine delivery system would be the safety testing in humans (phase I Clinical trial), which of course requires validated MS manufacturing under aseptic and GMP-conditions. Obviously, such MS manufacturing can only be done in a specialised industrial production plant.

A multivalent, single-injection vaccine containing ideally three to five antigens would be a major achievement in the vaccine field and should also open new paths in the fight against diseases for which vaccines are not yet available. The medical advantage of such a multivalent formulation would be the reduction of the number of vaccination sessions required during early childhood, with considerable socio-economic benefits, especially in the developing countries. This study has shown that high, persistent and protective levels of antibodies can be achieved after a single injection of multivalent childhood vaccines (DT+TT+aP+Hib) in biodegradable and biocompatible PLGA microspheres. Very interestingly, the multivalent MS-based childhood vaccine possessed increased immunogenicity when it was combined with MS-based synthetic malaria antigen derived from *Plasmodium falciparum*.

Taken together, biodegradable microspheres appear to have a realistic potential as delivery system for combination vaccines. Nonetheless, it remains to be seen how multivalent MS-based vaccines will perform in humans, and whether one single inoculation is sufficient or two injections are necessary. The prime advantage of such MS-based vaccine would indeed be the reduction of the number of vaccination sessions required during early childhood with related considerable socio-economic benefits. A breakthrough in the development of single-injection particulate vaccines might significantly improve the global vaccination coverage, simplify immunisation programmes, and be an important milestone for developing countries in which health conditions are sometimes poor, and many individuals do not return for their booster vaccination doses.

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APPENDICES

APPENDIX 1: IgG1 and IgG2a isotype levels at 4 and 8 weeks post-immunisation of guinea-pigs with DT-TT-MS or alum control

Table A.1 Influence of formulation parameters on IgG isotype profile, 4 weeks post-immunisation

Formulation	Anti-DT IgG1 titers	Anti-DT IgG2 titers
Small PLGA MS	10.47 (9.00-12.30)*	0.41 (0.36-0.46)*,**
Small PLA MS	2.90 (2.53-3.34)	0.07 (0.06-0.08)
Large PLGA MS	5.39 (4.87-5.98)	0.18 (0.16-0.20)
Large PLA MS	5.71 (5.16-6.35)	0.30 (0.27-0.33)
1x alum control	5.05 (4.35-5.92)	0.15 (0.13-0.17)
2x alum control	ND	ND

Formulation	Anti-TT IgG1 titers	Anti-TT IgG2 titers
Small PLGA MS	9.92 (9.39-10.49)	0.66 (0.63-0.70)
Small PLA MS	10.28 (9.73-10.88)	1.10 (1.03-1.17)*,**
Large PLGA MS	29.76 (21.47-42.30)	1.65 (1.14-2.30)**
Large PLA MS	14.53 (10.45-20.12)	0.67 (0.45-0.94)
1x alum control	8.97 (8.49-9.48)	0.52 (0.48-0.56)
2x alum control	ND	ND

*$p<0.05$ compared to same size, different polymer
**$p<0.05$ compared to same polymer, different size

Table A.2 Influence of formulation parameters on IgG isotype profile, 8 weeks post-immunisation

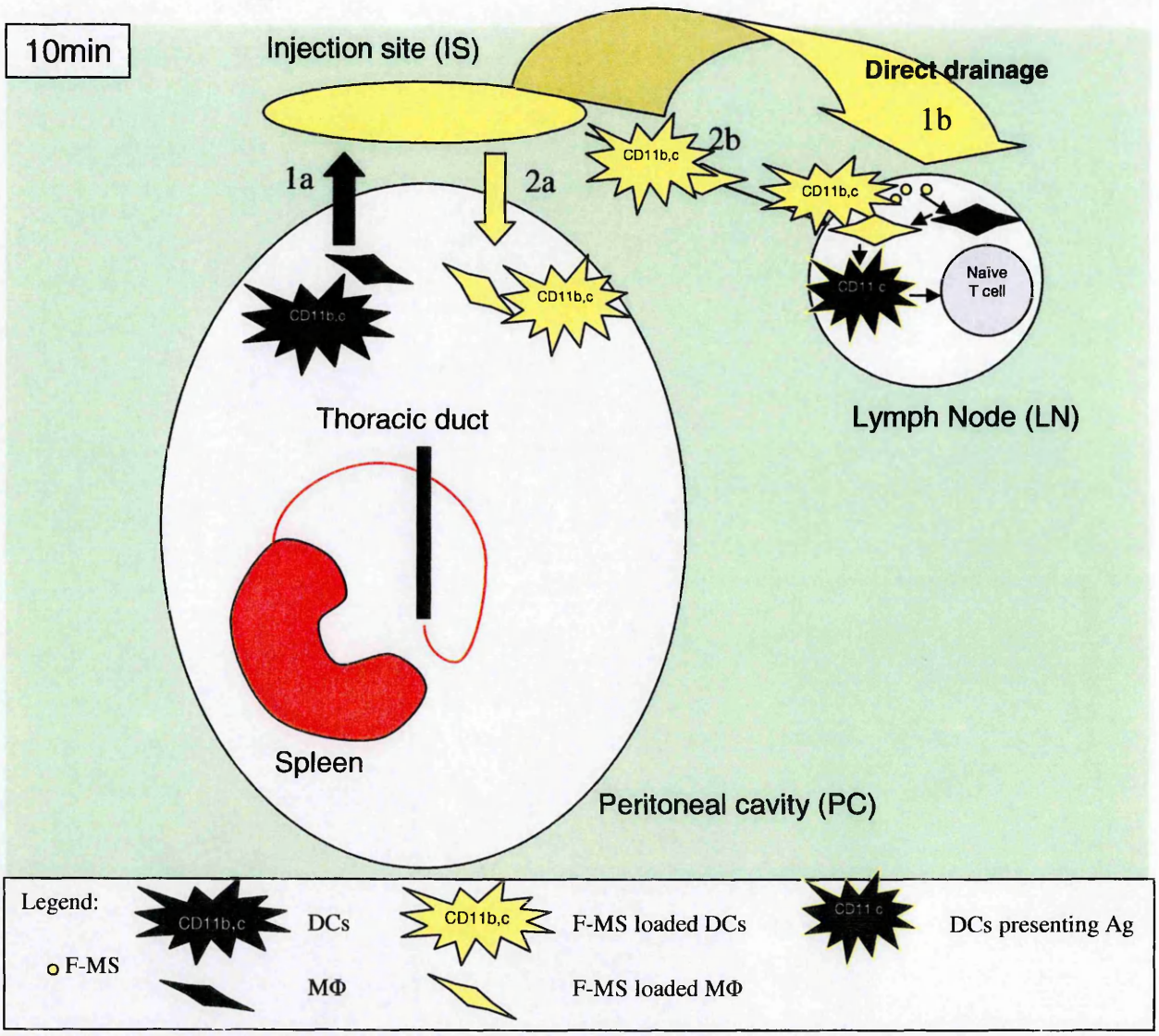
Formulation	Anti-DT IgG1 titers	Anti-DT IgG2 titers
Small PLGA MS	1.38 (0.72-3.49)	0.08 (0.05-0.14)*
Small PLA MS	1.362 (0.961-1.962)	0.03 (0.01-0.05)
Large PLGA MS	2.45 (1.18-7.17)	0.11 (0.06-0.18)
Large PLA MS	1.359 (0.959-1.957)	0.03 (0.01-0.05)
1x alum control	3.85 (1.92-10.60)	0.14 (0.08-0.24)
2x alum control	1.37 (0.71-3.47)	0.04 (0.02-0.07)

Formulation	Anti-TT IgG1 titers	Anti-TT IgG2 titers
Small PLGA MS	4.12 (3.76-4.52)	0.50 (0.46-0.55)
Small PLA MS	5.61 (4.92-6.46)*	0.53 (0.47-0.59)
Large PLGA MS	6.82 (6.17-7.56)**	0.55 (0.51-0.60)
Large PLA MS	5.63 (4.94-6.49)	0.55 (0.50-0.62)
1x alum control	15.81 (14.43-17.35)	0.62 (0.57-0.68)
2x alum control	2.76 (2.52-3.06)	0.35 (0.31-0.39)

*$p<0.05$ compared to same size, different polymer
**$p<0.05$ compared to same polymer, different size

IgG1 and IgG2 against DT (upper table) and TT (lower table) were measured by ELISA on individual serum sample from guinea-pigs immunised with experimental MS vaccine or alum adsorbed control vaccines (as described above). The results are expressed as titers relative to an in house reference used on each ELISA plates. The data represents the geometric mean with 95% Cl from pooled samples from 8-10 individual animals.

APPENDIX 2: Phagocytic cells trafficking from 10min to 4 weeks post- sub-cutaneous immunisation



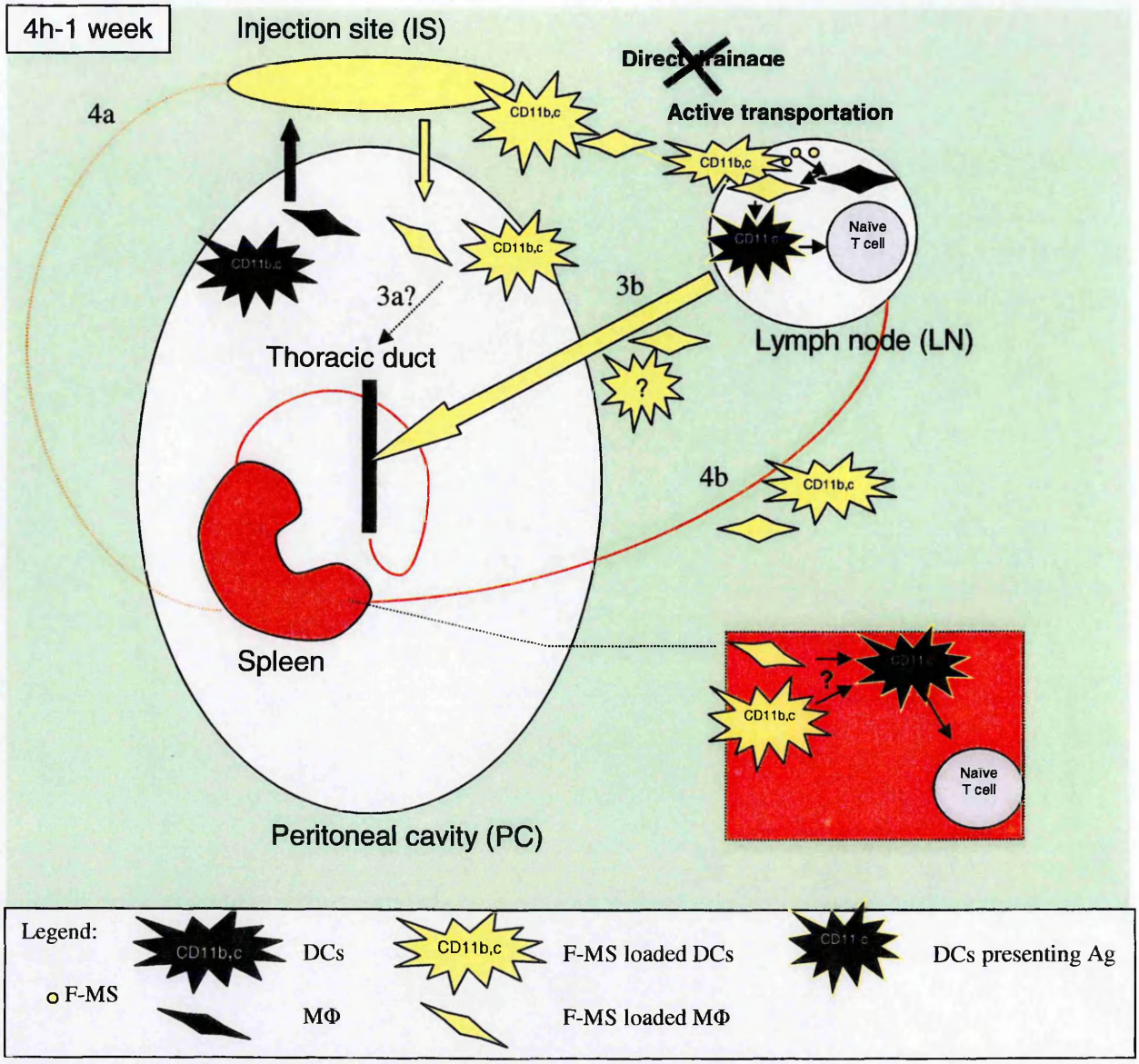
1a: recruitment of inflammatory cells from PC to IS including macrophages and dendritic cells

1b: direct drainage from IS to LN through lymphatic canals

2a: active transportation of F-MS via MΦ and DCs from IS to PC

2b: active transportation of F-MS via MΦ and DCs from IS to LN

The antigen is presented in the LN to naïve T-cells via cross presentation by MΦ to DCs and/or directly by DCs.



3a: F-MS loaded MΦ and DCs from the PC might reach the spleen via the thoracic duct

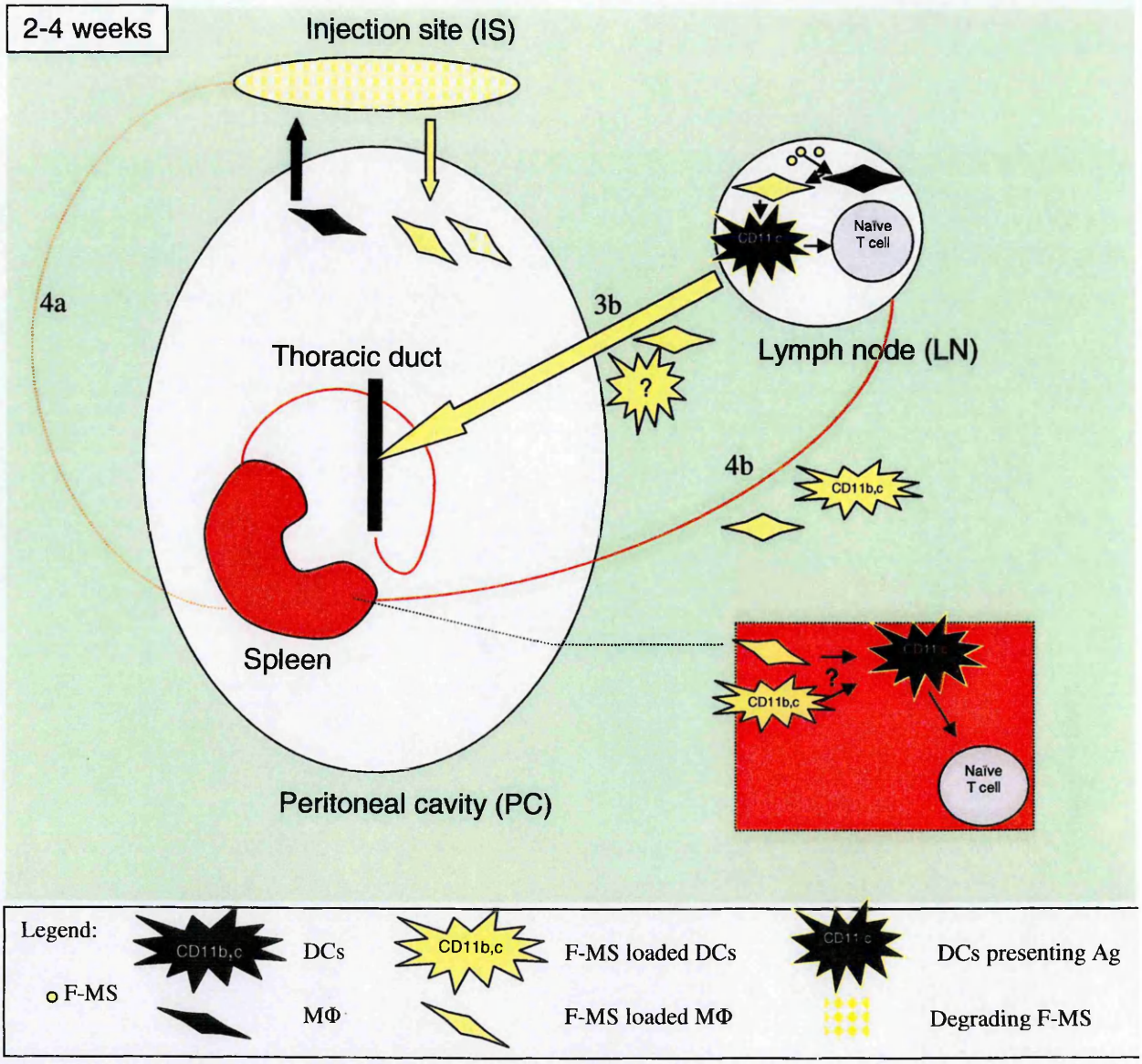
3b: F-MS loaded MΦ and maybe DCs migrate to the spleen via the thoracic duct

4a: F-MS loaded MΦ and DCs might migrate through the blood circulation from the IS after few da blood vessels appear at the IS during formation of a nodule or granuloma

4b: F-MS loaded MΦ and DCs might migrate through the blood circulation from the LN

After 1 or 2 days, there is no more direct drainage between the IS and the LN

The antigen is presented in the spleen to naïve T-cells via cross presentation by MΦ to DCs and/or directly by DCs.

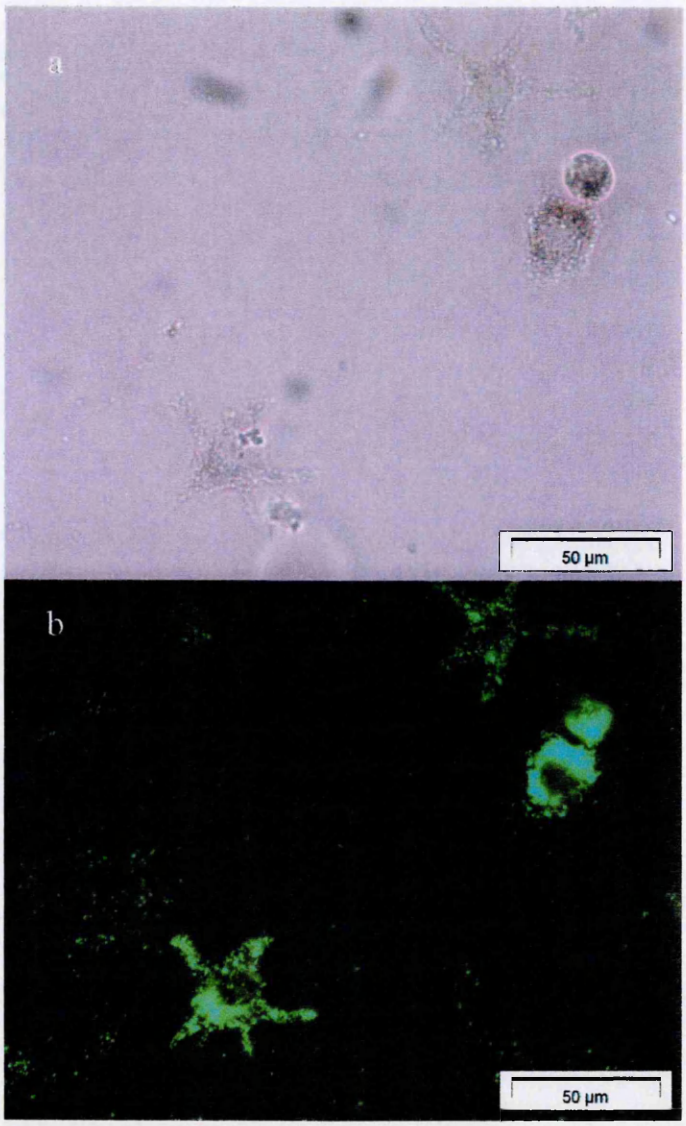


No more transportation from the IS to the LN.

No presence of DCs in the PC.

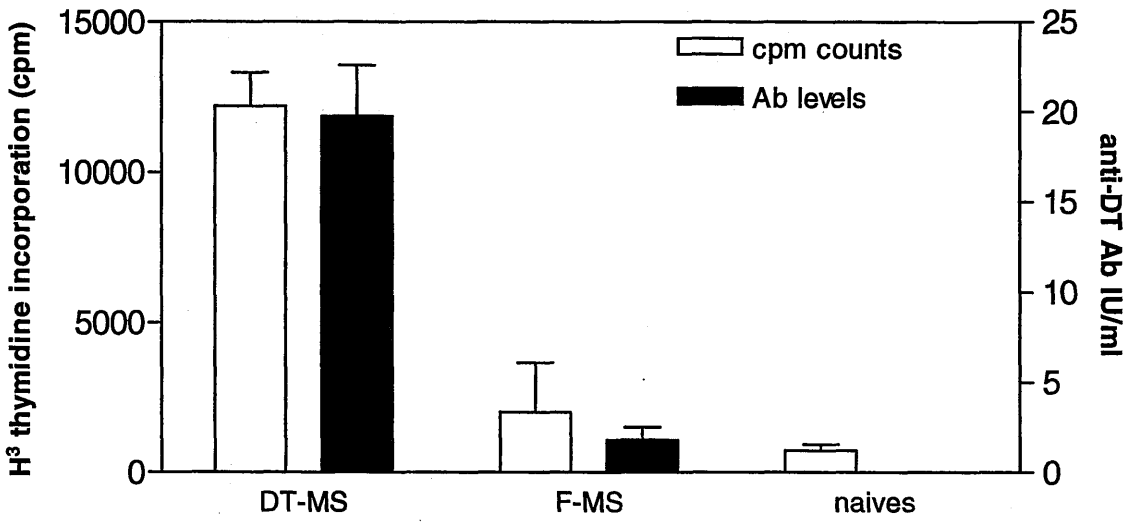
The F-MS are starting to degrade at the IS and inside the cells.

APPENDIX 3: *In vitro* uptake of PS beads by macrophage cell line



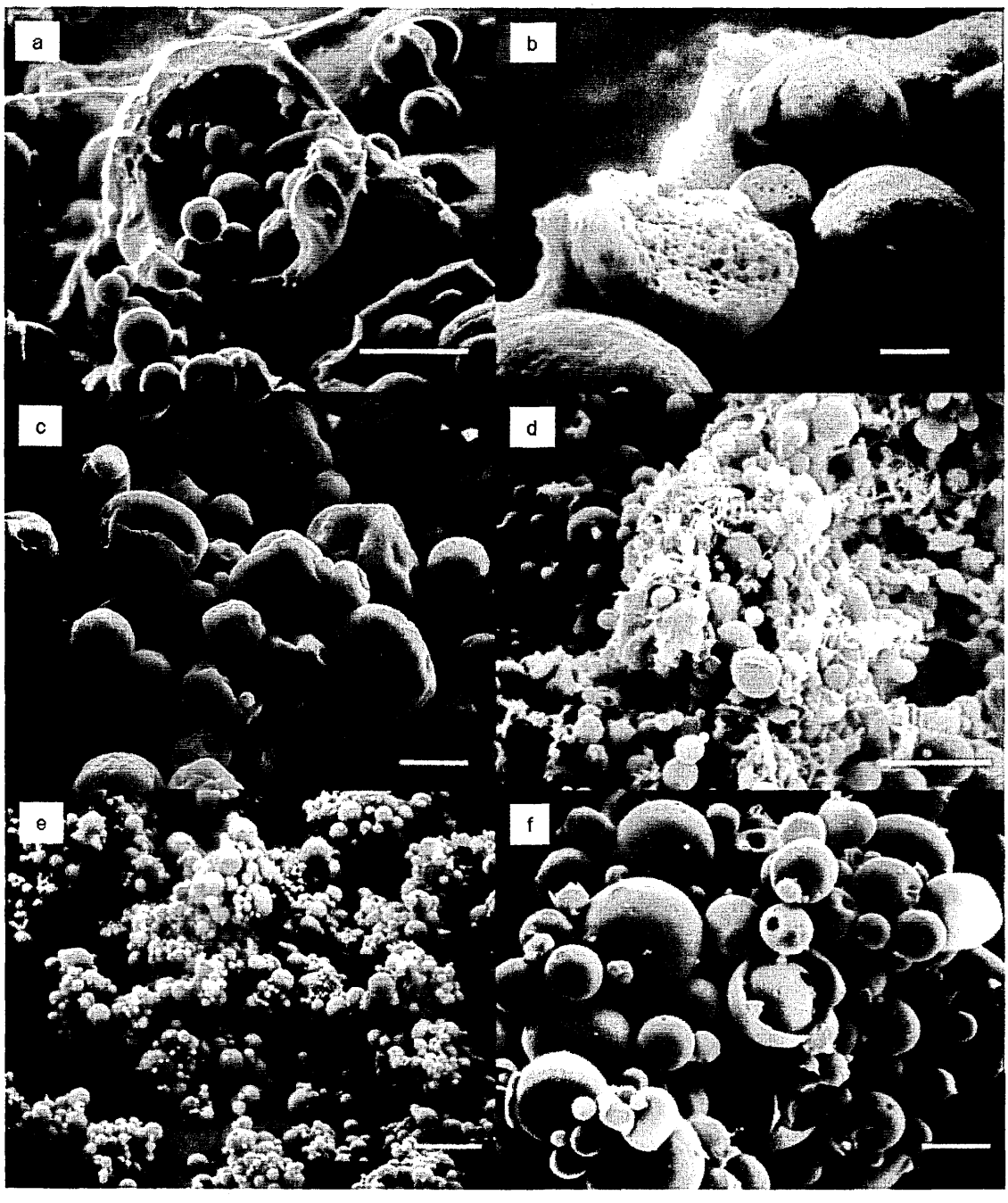
Epifluorescence micrographs (20 x) of *in vitro* cultivated macrophages (J774 cell line) that have ingested PS beads (0.5-1 µm diameter). (a) bright field image; (b) fluorescein emission at 488nm. The PS beads were highly uptaken by macrophages *in vitro*.

APPENDIX 4: Influence of fluorescent dyes on the immunological performances of the MS-DT formulation.



Cellular (cpm counts) and humoral (Ab titers) responses at 4 weeks post-immunisation (s.c) of mice with DT-MS or fluorescent DT-MS (F-MS). The results are expressed as geometric means ± SEM from 3 individual animals. The immune response appears to decrease when fluorescent compounds are present in the formulation

APPENDIX 5: SEM photographs of small- or large-sized PLGA and PLA microspheres



Microsphere types made from PLGA (b, c, d, e) and PLA (a, f); collapsed particles (c) and coalesced particles (d). The MS were produced either by spray-drying to yield small-sized MS (a, d, e) or by coacervation to yield large-sized MS (b, c, f).Scale bars = 10μm