UNIVERSITY OF ALBERTA

POLY(D,L-LACTIC-CO-GLYCOLIC ACID) (PLGA) MICROSPHERES AS ANTIGEN DELIVERY SYSTEMS FOR THE INDUCTION OF CELLULAR IMMUNE RESPONSES

By

KIMBERLEY D. NEWMAN

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Date: Sept 20, 2001

To my husband, Gregory Emerson, if there is one thing I have learned in this journey it is that true strength and great accomplishments are made possible through love ...and I am humbled to be blessed with such a love.

"In time the Rockies may crumble Gibraltar may tumble they are only made of clay, but our love is here to stay."

- G. Gershwin and I. Gershwin

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THE UNDERSIGNED CERTIFY THAT THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH FOR ACCEPTANCE, A THESIS ENTITLED **POLY(D,L-LACTIC-CO-GLYCOLIC ACID) (PLGA) MICROSPHERES AS ANTIGEN DELIVERY SYSTEMS FOR THE INDUCTION OF CELLULAR IMMUNE RESPONSES** SUBMITTED BY **KIMBERLEY D. NEWMAN** IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF **DOCTOR OF PHILOSOPHY IN PHARMACEUTICAL SCIENCES.**

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" Such is the constitution of the human mind, that any kind of knowledge, if it be really such, is its own reward."

- John Henry Newman

The Idea of a University

Abstract

The type of immune response generated by an antigen delivery system is paramount to the effectiveness of a vaccine. A study on the adjuvant activity of poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres was performed to evaluate this antigen delivery system for the induction of cellular immune responses. The ability of peptide loaded PLGA microspheres to elicit cell-mediated immune responses specifically T helper type 1 (Th1) responses was investigated using a model peptide antigen, ovalbumin (OVA) peptide and a cancer vaccine candidate, MUC1 mucin peptide. Both the OVA and MUC1 peptide microsphere formulations elicited T cell specific immune responses with antibody and cytokine profiles indicative of Th1 responses. Moreover, an immunomodulator, monophosphoryl lipid A , enhanced Th1 responses when incorporated with the peptide loaded PLGA microspheres.

Intracellular degradation and processing of antigen loaded PLGA microspheres by antigen presenting cells (APCs) was investigated. Both *in vitro* and *in vivo* studies revealed that PLGA microspheres were capable of cytoplasmic delivery - a required step for the induction of cytotoxic T lymphocyte responses. The kinetics of microsphere degradation and cytoplasmic delivery were influenced by polymer molecular weight, where degradation and delivery to the cytosol were more rapid for low molecular weight PLGA microspheres (6000 g/mol) in comparison to the high molecular weight (60,000 g/mol) formulations.

The type of APC taking up antigen *in vivo* has a strong influence on the outcome of the immune response. Following an intraperitoneal immunization the predominant APC taking up PLGA microspheres in the peritoneal cavity was the macrophage whereas an intradermal immunization resulted in uptake of PLGA microspheres by dendritic cells. These results suggest that the profile for cellular uptake varies with the site of injection and more importantly reveal uptake of PLGA microspheres by the most potent APC the dendritic cell.

This research provides insight into the mechanisms of action of antigen loaded PLGA microspheres on the immune system. The results of this study demonstrate the capacity of PLGA microspheres to induce cellular immune responses and reveal their potential as antigen delivery systems for future vaccines.

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ADCC	antibody-dependent cell-mediated cytoxicity
APC	antigen presenting cell
BCA	bicinchoninic acid
BSA	bovine serum albumin
CLSM	confocal laser scanning microscopy
Con A	concanavalin A
CpG	cytosine-phosphate-guanosine
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DIP	N-dodecyl 2-imidazole-proprionate
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DOTAP	1, 2 dioleoyl-3-trimethylammonium-propane
DPM	decays per minute
DTH	delayed type hypersensitivity
EDTA	ethylendiaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FasL	Fas ligand
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
HBSS	Hanks' balanced salt solution
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HSA	human serum albumin
i.d.	intradermal

IFN-γ	interferon-gamma
IL-2	interleukin-2
IL-4	interleukin-4
IL-10	interleukin-10
IL-12	interleukin-12
i.p.	intraperitoneal
ISCOMS	immunostimulating complexes
i.v.	intravenous
KLH	keyhole limpet hemocyanin
LH-RH	luteinizing hormone-releasing hormone
LMP	low-molecular-mass polypeptide
Mϕ	macrophage
mAb	monoclonal antibody
MHC	major histocompatibility complex
mol. wt.	molecular weight
MPLA	monophosphoryl lipid A
NK	natural killer cell
ODN	oligodeoxynucleotides
OVA	ovalbumin
PBS	phosphate buffered saline
PLGA	poly(D,L-lactic-co-glycolic acid)
PLLA	poly(L-lactic acid)
PVA	polyvinyl alcohol
QS-21	Quillaja saponin 21
RER	rough endoplasmic reticulum
s.c.	subcutaneous
SEM	scanning electron microscopy
TAA	tumor-associated antigen

TCR	T cell receptor
Th	T helper
TMB	3, 3′, 5, 5′ tetramethylbenzidine
TMR	tetramethylrhodamine
TNF	tumor necrosis factor
TPBS	0.05% (v/v) Tween 20/phosphate buffered saline
TSA	tumor-specific antigen
UV	ultraviolet
w/o/w	water-in-oil-in-water

Amino acids

A	alanine
D	aspartic acid
E	glutamic acid
G	glycine
H	histidine
I	isoleucine
N	asparagine
P	phenylalanine
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine

Chapter 1

INTRODUCTION

1.1 Introduction

Vaccines are the most cost-effective weapons for disease prevention. Recent advances in the fields of immunology and controlled release technology are now being exploited for the improvement of existing vaccines and the development of new vaccines against diseases such as HIV and cancer. An understanding of the mechanisms of action of novel delivery systems will have a major impact on vaccine strategies. Over the past decade biodegradable biocompatible poly(D,L- lactic-co-glycolic acid) (PLGA) microspheres have been evaluated as vaccine delivery systems (1-3). Early developments in the field of PLGA microspheres focussed on controlling rates of degradation and release with the goal of creating a single-administration vaccine; however more recently emphasis has been placed on the adjuvant activity of PLGA microspheres. This thesis explores and evaluates PLGA microspheres as antigen delivery systems for the induction of cellular immune responses. The following chapter provides the rationale for this research and discusses the mechanisms involved in eliciting and sustaining an effective immune response for protection and immunity.

1.2 Characterization of Immune Responses

1.2.1 Humoral vs. Cellular

An effective vaccine formulation must be capable of activating the humoral and or cellular branches of the immune system. In general, cellular immune responses are required in combating intracellular pathogens (4, 5) and cancer (6), while humoral immune responses are required for the

eradication of extracellular pathogens (7-9). The humoral immune response is characterized by the production of antibodies by B cells. These cells recognize soluble antigen through antibody-specific binding. An antibody response facilitates elimination of pathogens by activating the complement system resulting in lysis of the organism; acting as an opsonin to promote phagocytosis of the pathogen; binding to viruses and preventing their invasion into cells; and binding to Fc receptors on natural killer (NK) cells or macrophages (Mϕ) in antibody-dependent cell-mediated cytotoxicity (ADCC).

Cell-mediated immunity involves antigen specific cells including T helper (Th) cells and cytotoxic T lymphocytes (CTL), and non-specific cells including Mϕs, neutrophils and NK cells (10). Although antibody can be involved, its role is secondary. Cellular immune responses can be divided into two major categories: 1) CTL responses and 2) Th immune responses.

1.2.2 T helper Immune Responses

Activation of Th cells is required for the generation of both humoral and cellular immune responses. The type of immune response is dependent on the subpopulation of Th cells activated by the antigen (11-13). Th cells are CD4+ cells which recognize the antigen complexed with self-MHC class II molecules on the surface of antigen presenting cells (APC), which include B cells, Mϕs and dendritic cells (DC). During the process of activation the Th cell binds to the antigen/MHC class II complex and in the presence of a co-stimulatory signal becomes an effector cell secreting a variety of cytokines.

The co-stimulatory signal may involve interaction of B7 on the APC with CD28 and CTLA-4 expressed on T cells (14).

There are two subsets of Th cells, which differ with respect to their cytokine profiles; however these subsets may not be discrete but rather a continuum of different combinations of cytokine secretion (15). The Th1 subset assists in cellular immune responses and secretes cytokines including interleukin-2 (IL-2) and interferon-gamma (IFN-γ) (16-18), while the Th2 subset is involved in humoral immune responses and secretes cytokines including interleukin-4 (IL-4) and interleukin-10 (IL-10) (16-18). The Th1 and Th2 pathways are mutually inhibitory; IL-10 inhibits the production of cytokines by Th1 cells (19), while IFN-γ inhibits the proliferation of Th2 cells (11). APCs also produce cytokines that regulate the Th response. Mϕs and DCs, in response to microbial antigen will produce interleukin-12 (IL-12) (20, 21)- a cytokine, which induces proliferation of Th1 cells. Furthermore, cytokines produced by the Th1 subset promote production of IgG2a, IgG2b and IgG3 by B cells (22-25). Conversely IgG1 and IgE production is associated with Th2 responses (24, 26).

1.2.2.1 Factors Affecting Th Cell Differentiation

The Th1-Th2 choice may be affected by factors such as antigen dose, co-stimulatory molecules, peptide binding affinity for an MHC molecule and the structure of the T cell receptor (TCR). Early *in vivo* studies on antigen dose indicated that both low and high levels of antigen induced delayed type hypersensitivity (DTH)/cellular immune responses, while intermediate levels

elicited antibody production (27). Later studies, with *Leishmania major* as the infectious model, revealed that Balb/c mice mount an ineffective antibody response when infected with high levels of this protozoan parasite, while very low doses elicit a protective DTH response (28).

Co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86) on APCs have been shown to differentially regulate Th1 and Th2 responses where the former promotes a Th1 response and the latter promotes a Th2 response in the murine system (29, 30). The first reports suggesting that B7.1 and B7.2 molecules were involved in differential signalling of Th cells were based on models of autoimmunity where anti-B7.1 treated mice exhibited Th2 responses and reduced incidence of disease (29). Further studies involving B7.2 transfected cells demonstrated that antigen priming by the B7.2 transfectants preferentially leads to a Th2 response (30). There are however some reports of B7.2 molecules inducing both Th1 and Th2 responses (31) as well as antitumor immune responses (32).

CD28 and CTLA-4, receptors for the B7 ligands, have also been implicated in the Th1/Th2 choice and evidence suggests that CD28 promotes a Th2 response (33, 34) while CTLA-4 inhibits Th2 differentiation (35). Studies have demonstrated that blocking CD28/B7 interactions results in reduced IL-4 production (33) as well as inhibition of Th2 responses using an *in vivo* model of parasitic infection (34). Studies on CTLA-4-deficient mouse strains revealed that CTLA-4 is a potent inhibitor of Th2 differentiation based on the inability of these strains to mount Th1 responses (35).

Peptide binding affinity for MHC molecules has been shown to exert an effect on Th cell differentiation. Studies using peptides from myelin basic protein and human collagen IV protein showed that increasing the affinity of the wild-type peptide for the MHC class II molecule either by mutating the peptide or changing the genotype of the MHC molecule promotes priming of Th1 cells (36-38). Moreover, studies involving peptides from human collagen IV protein (37, 38) or moth cytochrome c proteins (39) demonstrated that decreasing affinity of the wild type peptide for the MHC class II molecule results in enhanced priming of Th2 cells. Hence, the accumulating evidence suggests that high peptide binding affinity for an MHC molecule promotes a Th1 response while low peptide bind affinity promotes a Th2 response. In addition to the peptide/MHC complex, the structure of the TCR also affects Th cell differentiation. A recent study reported that a single amino acid substitution in the TCR at a position that contacts the peptide/MHC class II complex is associated with a shift in Th cell differentiation from Th1 to Th2 (40).

1.2.3 CTL Responses

CTLs are an important component of the cellular immune response and are essential for the eradication of intracellular pathogens and cancer (41-43). CTLs are a population of CD8+ effector cells with lytic capability. They are MHC class I-restricted and hence can recognize antigen/ MHC class I complexes on the surface of target cells. As all nucleated cells express class I MHC molecules, CTLs are capable of recognizing almost any altered cell in

the body. Activation of a CTL requires that the CTL recognize and bind to the antigen/self MHC class I complexes on the surface of the target cells. In the presence of a co-stimulatory signal this precursor CTL then becomes an effector cell. The co-stimulatory signal may be IL-2 (44) or interaction of co-stimulatory molecules B7 on APCs with CD28 or CTLA-4 on T cells (45).

Following activation the effector CTLs mediate target cell destruction by inducing apoptosis through exocytosis of lytic granules, via ligation of Fas or cytokine secretion. In granule-mediated cytotoxicity the CTL releases granules consisting of at least two types of cytotoxins: perforins, pore-forming proteins; and granzymes, members of the enzyme family of serine proteases. In the classical model, perforins generate transmembrane pores through which cytolytic mediators such as granzymes may enter the cell and trigger apoptosis. However, more recent work by R.C. Bleackley suggests that granzyme uptake (i.e. granzyme B) by target cells is mediated by mannose 6-phosphate receptors (46). A number of substrates for granzyme B have been identified including capase 3 which has been linked to DNA fragmentation (47-49). Studies on granule-induced apoptosis revealed that both perforins and granzymes are required for target cell destruction - more specifically, granzymes A and B have been shown to induce target cell apoptosis and cytolysis in a perforin-dependent manner (50-52). In addition to granule-mediated cytotoxicity, CTLs can also induce apoptosis through the use of cell surface receptors on target cells. Receptor-mediated cytotoxicity involves activation of the Fas receptor in the target cell membrane by the Fas ligand

(FasL) expressed on the surface of activated CTLs resulting in transduction of a death signal to the target cell (53). Another mechanism by which CTLs induce apoptosis is through cytokine secretion. More specifically, cross-linking of tumor necrosis factor (TNF) receptors by CTL-derived tumor necrosis factor-beta (TNF-β) has been shown to induce apoptosis in some cell types and most notably in tumor cells (54-56).

1.3 Antigen Processing and Presentation

The manner in which antigen is presented to the immune system can greatly influence the outcome of the immune response. There are two pathways for antigen processing and presentation (57): the MHC class I pathway and the MHC class II pathway. Endogenous antigen is generally processed and presented via the MHC class I pathway- a pathway involved in CTL activation. Conversely, exogenous antigen is processed and presented via the MHC class II pathway- a pathway involved in Th cell activation.

Cellular immune responses generally require that the antigen be internalized and processed by APCs, which include B cells, DCs and Mϕs. The type of APC taking up the antigen *in vivo* is an important factor to consider when designing an antigen delivery system. B cells are poor presenters of soluble antigens due to their inefficient uptake via pinocytosis; however they are very efficient at internalizing antigen via their immunoglobulin receptors (58, 59). Conversely, the Mϕ is a highly phagocytic cell capable of presenting a wide range of antigens, in soluble or particulate form (59-61).

As the antigen presentation function of B cells and Mϕs is well established more recent studies have focused on the DC as the "professional" APC. DCs are the most potent APCs and the most effective stimulators of primary T cell responses (62, 63). In the immature stage DCs are highly efficient at both antigen capture via phagocytosis or macropinocytosis and antigen processing. Following antigen uptake, maturation occurs where the DCs lose their capacity for antigen capture and upregulate their expression of peptide/MHC complexes and costimulatory molecules. The potency of DCs as APCs is based largely on quantitative aspects of expression and regulation. The expression of MHC molecules and MHC /peptide complexes is 10-100 times higher on DCs in comparison to other APCs such as B cells and monocytes (63). Furthermore, unlike B cells and Mϕs, DCs constitutively express high levels of costimulatory molecules such as CD86, making them highly efficient stimulators of T cells.

1.3.1 MHC Class II Presentation

APCs are specialized cells of the immune system, which take up antigen by phagocytosis or endocytosis followed by antigen processing in the endocytic/ MHC class II pathway. This pathway consists of increasingly acidic compartments from the endosome, endolysosome to the lysosome where the antigen is degraded into peptides. These degraded peptides are generally 13-17 residues in length and contain anchor motifs that bind preferentially to different MHC class II allelic products (64, 65). In the rough endoplasmic reticulum (RER) the newly synthesized MHC class II molecules associate with

an invariant chain, which precludes binding with peptides derived from endogenous antigen or other intracellular proteins (66). The class two molecules with the invariant chain are routed from the RER through the Golgi complex to the endocytic pathway. Once inside the endocytic pathway the invariant chain is degraded exposing the peptide binding cleft. The newly generated peptides then associate with MHC class II molecules followed by presentation on the surface of the cell.

1.3.2 MHC Class I Presentation

1.3.2.1 Endogenous Antigen

MHC class I presentation of endogenous antigen occurs in all nucleated cells. In the MHC class I pathway endogenous antigen is degraded into peptides in the cytoplasm by a proteolytic complex, the low-molecular-mass polypeptide (LMP) (67, 68). The resulting peptide is 8-9 residues in length and contains specific anchor residues that are preferentially bound by different class I MHC allelic products. This peptide is transported into the RER by a protein designated TAP, transporter of antigenic proteins. Once in the RER the peptide binds to MHC class I molecule through its anchor motifs followed by presentation on the cell surface.

1.3.2.2 Exogenous Antigen

The classical model of the MHC class I pathway excludes antigen processing and presentation of exogenous antigen; however further investigation revealed the capacity of certain APCs to process exogenous antigen in the MHC class I pathway including B cells (69) DCs (70) and Mϕs

(71, 72). Moreover, both DCs and Mϕs have been shown to process and present particulate antigen in the MHC class I pathway (70, 72). Cytosolic processing of exogenous antigen requires that the antigen cross the endosomal/lysosomal membrane and enter the cytoplasm. Studies have shown that B cells (69) DCs (70) and Mϕs (72) transfer antigen from endosomes/phagosomes to the cytosol where endogenous and exogenous antigens follow a common pathway for MHC class I presentation. The nature of the antigen was found to play an important role in antigen presentation. In both DCs and Mϕs MHC class I presentation of exogenous antigen was enhanced when the antigen was in particulate form and internalized by phagocytosis (70, 71, 73). Furthermore an earlier investigation revealed that IFN-γ, which upregulates MHC class I expression and presentation of endogenous antigen, failed to augment MHC class I presentation of exogenous antigen in Mϕs (71). Hence entry of exogenous antigen into the cytosol may be the rate-limiting step for antigen presentation. In effort to minimize this rate-limiting step pH-sensitive liposomes (74-76) as well as membrane-perturbing agents including N-dodecyl 2-imidazole-proprionate (DIP), a pH-sensitive surfactant (77) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), a cationic lipid (78), have been used to promote cytoplasmic delivery of macromolecules.

1.4 Adjuvants - Modulation of the Immune Response

An adjuvant is defined as a substance that non-specifically enhances or potentiates the immune response to an antigen (79). The use of aluminium

salts as adjuvant for protein antigen was first described in the 1920's. The mechanism of action involves formation of a depot at the injection site as well as uptake of alum-adsorbed particles by APCs (80). Adjuvants based on aluminium salts (ie alum) are currently the only ones accepted for use in humans. Unfortunately, these adjuvants are effective for only a limited number of antigens and more importantly they do not induce cell-mediated immunity (81).

Although the only adjuvant used in humans (i.e. alum) cannot induce cellular immune responses there are other adjuvants currently being studied for their ability to induce cell-mediated immunity-specifically components of bacterial cell walls such as lipid A analogues and CpG motifs. One promising candidate adjuvant is the lipid A analogue monophosphoryl lipid A (MPLA) based on its ability to induce strong cellular immune responses and its established safety in humans (82). The adjuvant activity of MPLA is related in part to its ability to induce the production of cytokines that promote the generation of specific immune responses (83). Research has shown that certain microbial products will stimulate DCs and Mϕs to produce IL-12 (20, 21) - a cytokine that promotes a Th1 response (20, 26). Experiments by Salkowski *et al* demonstrated that MPLA induced IL-12 mRNA expression in mouse macrophages (84). MPLA incorporated in liposomes has been shown to be a safe and effective adjuvant for the generation of humoral immunity (85) and enhancing CTL responses (86). More importantly, the suitability of

liposomal MPLA as an adjuvant for human vaccines has been demonstrated in clinical trials (87).

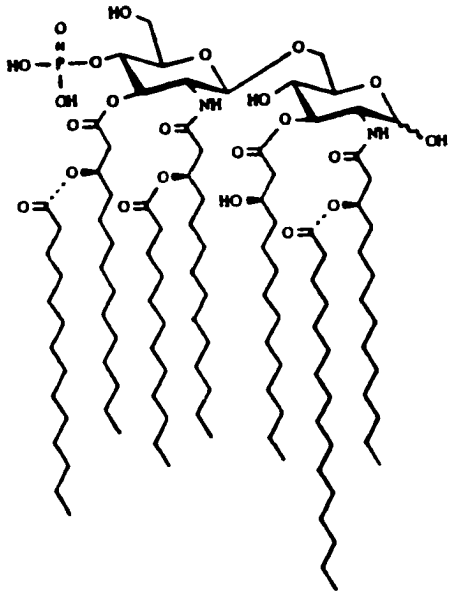

Figure 1-1. Structure of MPLA (adapted from reference (82)).

In addition to lipid A analogues, synthetic oligodeoxynucleotides (ODN) that contain unmethylated CpG motifs are highly effective Th1-inducing adjuvants (88, 89). These unmethylated CpG motifs are common to prokaryotic DNA such as bacterial DNA. Immune effector cells including B cells, DCs and Mϕs are directly stimulated by the microbe-restricted structures of CpG DNA resulting in secretion of cytokines especially Th1-related cytokines (i.e. IL-12), expression of co-stimulatory molecules and an increase in antigen-presenting function (88, 89). CpG ODN has been shown to be an effective adjuvant for a wide variety of vaccines against infectious

agents, cancer antigens and allergens (90-92). A recent study has shown that administration of CpG motifs with peptide/protein immunizations results in enhanced CTL responses and anti-tumor activity (93). Moreover a hepatitis B vaccine using CpG ODNs as adjuvant is in clinical trials (88).

Other novel adjuvants involve controlled release systems where therapeutic molecules are delivered at a predetermined rate for a defined period of time. There exists a wide range of delivery systems including immunostimulating complexes (ISCOMS), vesicles such as liposomes and polymeric microspheres (e.g. PLGA microspheres). The adjuvant activity of these antigen delivery systems is based on their targeted delivery to APCs as well as their "depot" effect where antigen is slowly released into the surrounding medium (94, 95). More specifically, microencapsulation of the antigen results in increased immunogenicity by protecting the antigen from degradation and clearance in a physiological environment. These novel delivery systems have been shown to greatly improve the induction of cellular immune responses over that induced by antigen alone or in combination with standard alum adjuvants (94).

1.5 Cancer Vaccines

1.5.1 Immune Surveillance Theory

Historically, many attempts have been made to produce a vaccine for human cancer. However despite past efforts, there remain numerous obstacles in eliciting an effective immune response for eradication of tumor cells. The rationale for cancer vaccines and immunotherapy is based on the

immune surveillance theory conceptualized by Paul Erlich in the early 1900s (96). He proposed that cancer cells frequently arise in the body but are recognized as foreign and are eradicated by the immune system.

The immune surveillance theory is based on the assumption that cancer cells express unique cell surface markers, which may be antigenic. Tumor antigens can be divided into two categories: Tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs) (97, 98). TSAs are unique to tumor cells and are commonly expressed on physically, chemically and virally induced tumors. Conversely, TAAs are not unique to cancer cells but are also expressed on normal cells. TAAs may be antigens that are expressed on normal cells during fetal development or they may be antigens exhibiting increased expression or altered glycosylation on tumor cells.

1.5.2 MUC1 Mucin

Mucins have attracted attention as potential target antigens in cancer immunotherapy - in particular Mucin 1 (MUC1), which is highly expressed in breast, pancreatic and ovarian carcinomas (99-102). Mucins are large (>200 Kda) complex glycoproteins, which are secreted by and expressed on the surface of both normal and malignant epithelial cells (103, 104). Mucins are composed of large amounts of carbohydrate (≥ 50% by weight) O-linked to a protein core through serine and threonine. The MUC1 mucin or polymorphic epithelial mucin has a core polypeptide consisting of three domains: a cytoplasmic C-terminal domain, a transmembrane region and an extracellular

N-terminal domain containing variable tandem repeats of 20 amino acids (PPAHGVTSAPDTRPAPGSTA) (105).

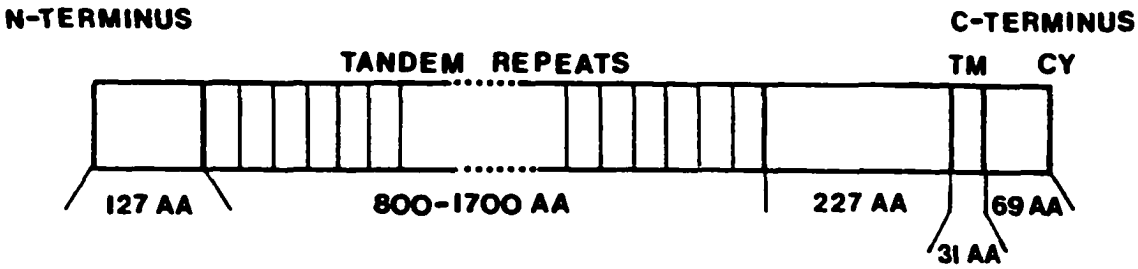

Figure 1-2. Diagram of MUC1 core peptide, consisting of a large extracellular domain with a central tandem repeat region (800-1700 AA) flanked by degenerate regions. The C terminus contains the transmembrane (TM) and the cytoplasmic (CY) regions (adapted from references (102, 105)).

1.5.3 Cancer-associated MUC1 Mucin

1.5.3.1 Altered Expression

The expression of MUC1 mucin is altered in cancer cells (100, 106). Cell surface mucins on normal epithelial cells are located on the luminal surface and therefore are not exposed to the immune system. Conversely, cancer cells exhibit uniform expression of mucin molecules on their surface resulting in exposure of the mucin to the immune system. Furthermore, cancer associated MUC1 mucin frequently exhibits aberrant glycosylation, exposing internal sugar units and normally cryptic peptide sequences (100, 106, 107). These cancer-associated epitopes are exploited as potential targets in cancer immunotherapy for carcinomas expressing MUC1.

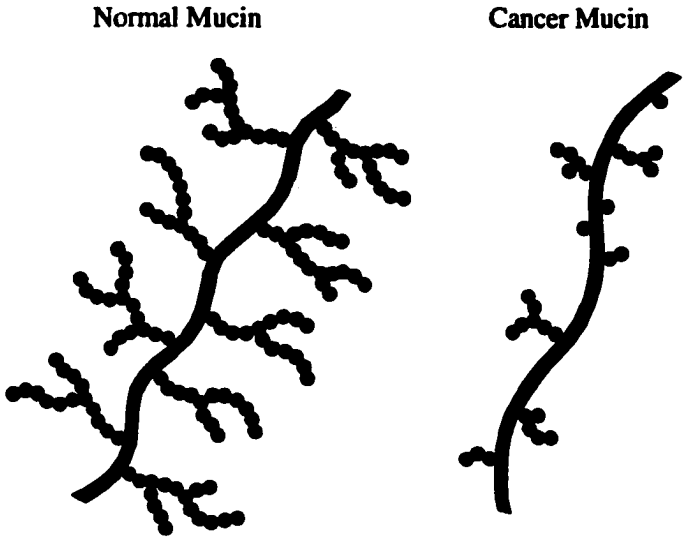

Figure 1-3. The glycosylation pattern of normal vs. cancer-associated MUC1 mucin. The central line represents the core polypeptide, while the side branches represent carbohydrate chains attached to the core peptide. Cancer-associated MUC1 mucins have fewer and shorter carbohydrate chains (adapted from references (100, 102)).

1.5.3.2 Immunosuppression

Tumors expressing mucins are frequently associated with poor prognosis (108). In the case of MUC1 the level of expression is linked with cancer progression in various cancer types (109-112). In addition elevated levels of MUC1 in the serum are associated with poor survival and lower anti-cancer immune responses in metastatic breast, ovarian and colorectal cancer patients following immunotherapy (112, 113). Hence, the accumulating evidence suggests an immunosuppressive role for MUC1 mucin. Efforts are currently being made to elucidate the mechanisms of immunosuppression by

MUC1. Studies have shown that cancer-associated MUC1 mucin inhibits human T cell responses by inducing T cell anergy (114) while other reports implicate apoptosis as the mechanism of inhibition (115). A study by Hiltbold *et al.* reported that the efficiency of MHC class I presentation of MUC1 and the resulting CTL responses are inversely correlated with the degree of glycosylation of the MUC1 antigen (116). Moreover a recent study on antigen processing of MUC1 by DCs revealed that the mechanism of unresponsiveness to serum MUC1 is a block in the intracellular sorting and processing by DCs (117).

1.5.4 MUC1 Mucin Peptides

Cancer-associated MUC1 mucin is significantly underglycosylated resulting in exposure of the peptide core. The exposed peptide sequences have been characterized by MUC1 monclonal antibodies (mAb) such as SM-3 and BCP-8, which are specific for the PDTRP sequence in the 20 amino acid tandem repeat region (118-120). These mAbs bind to the MUC1 mucin on human breast cancer cells but fail to bind to normal epithelial cells. Research has shown that exposed peptide sequences in MUC1 mucin are capable of eliciting immune responses. Antibody responses to the SM-3 epitope of MUC1 mucin have been demonstrated in B cells isolated from the draining lymph nodes of an ovarian carcinoma patient (121). Furthermore, CTL clones specific for MUC1 core peptide have been isolated from pancreatic, breast and ovarian cancer patients (122-125). These clones were able to kill tumor cells expressing MUC1 in an MHC-unrestricted manner but failed to kill breast

epithelial cells or MUC1- negative tumor cells. Hence these studies clearly demonstrate the capacity of cancer-associated MUC1 epitopes to invoke a specific immune response.

MUC1 peptide epitopes have shown promising potential in the field of cancer vaccines and immunotherapy. A MUC1 fusion protein coupled to mannan has been shown to generate MHC class I restricted responses in mice resulting in tumor protection (126). Furthermore, it has been demonstrated that MUC1 peptide conjugated to keyhole limpet hemocyanin (KLH) elicits high antibody titres and delayed type hypersensitivity (DTH) responses resulting in tumor rejection in mouse models (127). In a study by Samuel *et al.* a liposomal formulation of MUC1 peptide and MPLA resulted in significant anti-tumor activity, which correlated with induction of a Th1 response (128). Hence these results indicate that MUC1 peptide-based vaccines are capable of eliciting specific immune responses against an immunosuppressive cancer-associated antigen, MUC1 mucin.

1.6 Current Vaccine Strategies

Traditional vaccines including live attenuated, inactivated vaccines pose many problems with regards to safety and toxicity. Subunit or peptide-based vaccines could overcome many of these difficulties. Unfortunately, protein and peptide antigens are poorly immunogenic and require adjuvants to increase their immunogenicity (129, 130). In general they fail to induce CTL responses and as a consequence of their poor immunogenicity they require multiple immunizations (129).

Proteins and peptides differ significantly with respect to size, structure and immunogenicity. In general, proteins are more immunogenic than peptides due to their enhanced stability *in vivo* and larger variety of epitopes. As a result of these differences, proteins may elicit immune responses, which differ from those of their constituent peptides (131). The use of protein antigen is complicated by two issues: the isolation of protein from host-cell proteins and the expression of the protein in a configuration that evokes the required immune response. Furthermore formulation and delivery of proteins from controlled release systems such as microspheres is complicated by the instability of proteins during microencapsulation, storage, hydration *in vivo* and incubation at 37°C *in vivo* (132, 133). To date the only successful recombinant protein vaccine is the hepatitis B vaccine (134); however this vaccine requires multiple immunizations to achieve a state of immunity.

1.6.1 Synthetic Peptide-based Vaccines

Peptide antigen offers several advantages over protein: the product is chemically defined; enhanced stability; no infectious agent present; the immune response is focused against relevant epitopes; and large-scale production is less expensive (135). In the case of peptide antigen the protein-folding problem is not an issue however poor immunogenicity remains a major obstacle. A common approach has been to increase the immunogenicity of the peptide through conjugation to carrier proteins (e.g. keyhole limpet hemocyanin, diptheria toxoid, tetanus toxoid) (136). Unfortunately, such carrier proteins may induce undesirable immunological

effects: carrier-induced epitopic suppression of peptide-specific responses and predominant anti-carrier immune responses (137). In addition, peptide conjugated to carrier protein has been shown to induce strong humoral responses with minimal capacity for the generation of cell-mediated immunity (138). Furthermore, the conjugation procedure is not without difficulties and may involve modification of peptide epitopes, as well as complications with respect to the characterization and reproducibility of conjugate structures (139).

The poor immunogenicity of peptide antigen is largely a consequence of its rapid degradation in a physiological environment (140, 141). Microencapsulation would protect the peptide from degradation while providing controlled release of the antigen and targeted delivery to APCs. Furthermore, the very nature of peptide antigen with its simple structure and enhanced stability lends itself to encapsulation in controlled release systems where the conditions for encapsulation are frequently harsh and may result in degradation of antigenic structures as seen with protein antigen (132, 133).

1.6.2 Controlled Release Vaccines

Controlled release systems such as microspheres protect the antigen from degradation while providing controlled release of the antigen and targeted delivery to APCs. Polymeric delivery systems in particular offer many advantages including delivery of the antigen in a pulsatile or continuous release profile over a prolonged period of time. Hence, controlled release systems have the potential to improve peptide-based vaccines and possibly

improve compliance in vaccination programs by reducing the number of booster shots to a single administration vaccine.

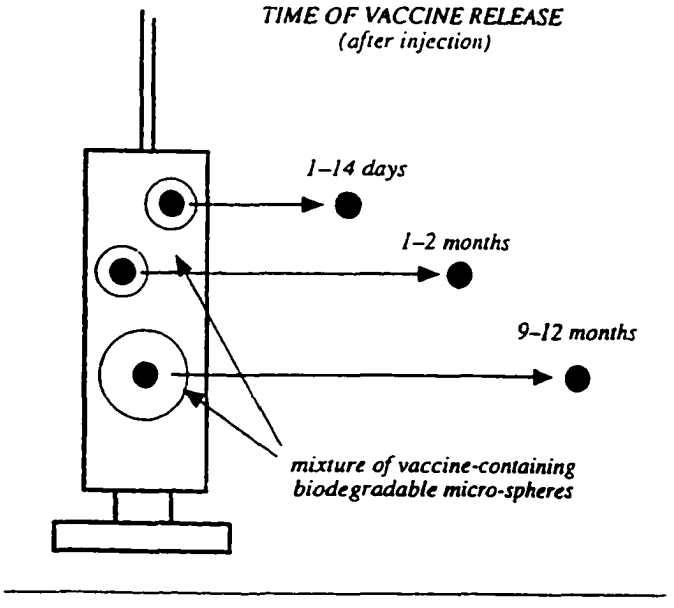

Figure 1-4. Diagram of a pulsatile release profile desired for a single-dose controlled-release tetanus vaccine. WHO/UNDP Programme for Vaccine Development (adapted from references (142, 143)).

When designing a polymeric controlled-release system the polymer should be biodegradable to avoid the need for surgical removal of the polymer remains following immunization. The formulation should be heat stable and the polymer characteristics should allow for manipulation of the release rate and release profile. Several polymers meet these criteria including polyesters, such as PLGA, polyphosphazenes (144) and polyanhydrides (145). PLGA in particular has received much attention in the field of controlled release technology based on its biocompatible nature and its established safety in humans (146, 147).

1.7 PLGA Microspheres as Antigen Delivery Systems

Biodegradable poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres show promising potential as antigen delivery systems. The first application of microencapsulation to vaccine technology in 1979 revealed that polymeric delivery systems were capable of continuous antigen release and stimulation of a humoral immune response (148). The application of PLGA microspheres as antigen delivery systems combines the controlled release properties of a polymeric system with the ease of administration via parenteral or enteral routes.

The use of PLGA microspheres for the delivery of protein/peptide antigen presents some challenges. During the preparation of microspheres, the use of organic solvents, formation of the microspheres and lyophilization are all processes that are capable of denaturing antigen – a major issue for protein antigen (149). Moreover, a reduction in pH in the PLGA microspheres during the degradation process is another potential source of antigen inactivation (149). In comparing liposomes to PLGA microspheres, liposomal formulations can be prepared under mild conditions avoiding exposure to both organic solvents and lyophilization (150, 151). However, the major drawback to liposomes is their physical instability where aggregation and relatively rapid leakage of entrapped substances makes long-term storage difficult (150, 151). Furthermore, upscale production of liposomes is tedious (152). From the point of view of storage and distribution, the use of PLGA microspheres is advantageous over liposomes as the former maintain antigen in a dry state

whereas the latter require aqueous conditions to maintain their carrier characteristics (150). Moreover, the use of dry vaccine formulations, such as PLGA microspheres, will simplify storage and distribution of vaccines and make possible the rapid expansion of immunization programs.

1.7.1 Composition and Release

PLGA microspheres are monolithic particles, consisting of antigen homogeneously dispersed in a matrix of polymer. PLGA is a biodegradable, biocompatible polyester commonly found in resorbable sutures and approved for human use (146, 147).

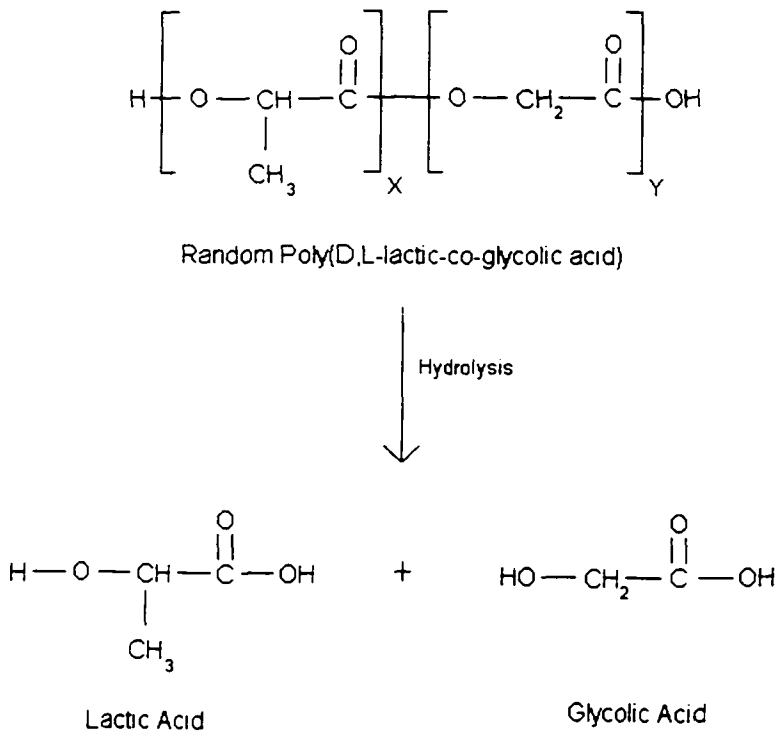

Figure 1-5. Structure and biodegradation products of PLGA.

The polymer is composed of lactic acid and glycolic acid monomers linked together through ester bonds. Extracellular degradation of the polymer *in vivo* involves random nonenzymatic hydrolysis of its backbone ester linkages giving rise to lactic acid and glycolic acid - common metabolic components. The rate of hydrolysis of PLGA and thus the rate of antigen release from PLGA microspheres can be controlled by factors such as lactic acid: glycolic acid ratio and molecular weight of the polymer (153-156). The copolymer ratio affects crystallinity, hydrophobicity and water uptake of the polymer. A polymer with a copolymer ratio of 50:50, lactic acid: glycolic acid, exhibits a higher rate of degradation than those polymers that have a higher proportion of either monomer. For a slower degradation profile lactic acid is usually selected as the predominant species as it is more hydrophobic than glycolic acid. Moreover, D,L-lactic acid is preferred over L-lactic acid as it leads to formation of an amorphous polymer thereby promoting homogeneous dispersion of the antigen or drug within the polymer matrix (150). Degradation of PLGA occurs primarily through bulk erosion and therefore is largely independent of surface area effects. The release profile for PLGA is biphasic with an initial surface release followed by a lag phase and then continuous/zero-order release profile based on bulk erosion of the polymer (157-159).

1.7.2 Cellular Uptake and Processing of PLGA Microspheres

1.7.2.1 Factors Regulating Phagocytosis

Both *in vitro* and *in vivo* studies have demonstrated cellular uptake of PLGA microspheres by macrophages (160-162). Factors affecting phagocytosis and hence microsphere uptake by APCs include size, surface charge and hydrophobicity. The size of the PLGA microspheres has been shown to affect the potentiation of an immune response, with the greatest immune response occurring with microspheres < 10 μm in diameter (163-165) The adjuvant activity of PLGA microspheres < 10 μm in diameter correlates with their ability to undergo phagocytosis and transportation to draining lymph nodes (164). A more recent study demonstrated that macrophages can internalize microspheres < 10 μm in diameter and stimulate a CTL response *in vitro* (166).

In addition to particle size, phagocytosis by Mϕs was shown to be dependent on surface hydrophobicity. A study involving poly(L-lactic acid) (PLLA) and PLGA microspheres revealed that phagocytosis was greater for the more hydrophobic PLLA microspheres than for the more hydrophilic PLGA microspheres (160). Hence phagocytosis of these biodegradable polymer microspheres is not related to bulk properties such as biodegradabililty but to the surface properties. Furthermore, precoating PLGA and PLLA microspheres with protein influences the phagocytic uptake of microspheres. Studies have shown that coating with bovine serum albumin (BSA) decreased phagocytosis due to a reduction in surface hydrophobicity

whereas coating with opsonins such as IgG increased phagocytosis (160, 161).

1.7.2.2 Intracellular Degradation

Following uptake by APCs the PLGA microspheres are degraded and their contents are released. Past research has focused on extracellular degradation of PLGA microspheres involving random hydrolysis of backbone esters (167, 168). Conversely, intracellular degradation is more complex and includes hydrolytic enzymes and acidic compartments of the endosomal/lysosomal pathway (57). Research on intracellular degradation of PLGA microspheres revealed that degradation is dependent on the bulk properties of the polymer and hence can be controlled by modifying properties such as polymer molecular weight (160, 161).

1.7.2.3 Cellular Uptake by DCs

Most studies on cellular uptake of PLGA microspheres have focused on phagocytosis by Mϕs. Based on accumulating evidence of the potency of DCs as APCs, investigation into the cellular uptake of PLGA microspheres by DCs is of growing importance. A recent study revealed uptake of PLGA microspheres by DCs *in vivo* following i.v. and s.c. administration (169); however further studies are required to elucidate the mechanisms involved in cellular uptake and processing of PLGA microspheres by DCs.

1.7.3 Adjuvant Activity of PLGA Microspheres

Microspheres deliver encapsulated antigen to APCs (i.e. Mϕ and DC) via the endocytic/MHC class II pathway or the cytosolic/MHC class I pathway,

where the former supports a Th response and the latter supports a CTL response. A study on MHC class II presentation in B cells revealed that B cells are capable of internalizing and presenting particulate antigen (170). However, the extent to which B cells can phagocytose PLGA microspheres and process microencapsulated antigens is unknown and requires further investigation. In addition to targeted delivery to APCs, microspheres can release antigen into the extracellular medium stimulating immune responses through interaction with circulating antibody or B-cell bound immunoglobulins.

1.7.3.1 Humoral Immune Responses

Early applications of biodegradable microspheres involved the use of protein loaded microspheres for the induction of humoral immune responses (163, 171-175). The induction of systemic and mucosal antibody responses after mucosal administration of PLGA microspheres has been reported for a variety of antigens (172-174). Furthermore, it has been shown that parenteral immunization with protein entrapped in PLGA microparticles induces systemic IgG responses comparable to those elicited by other adjuvants such as complete Freunds adjuvant (CFA) (175) or aluminium hydroxide (163, 171). Moreover, studies have shown that peptide loaded PLGA microspheres are capable of eliciting an antibody response comparable or even superior to that induced by CFA (176).

1.7.3.2 Cellular Immune Responses

The initial focus for PLGA microspheres was on the induction of humoral immune responses; however humoral responses alone may not be

sufficient to provide protection and immunity particularly in the case of intracellular pathogens and cancer. Aside from the ability of PLGA microspheres to elicit antibody responses, there is accumulating evidence of their ability to elicit cell-mediated immune responses.

1.7.3.2.1 Th Responses

Activation of Th cells is required for the induction of both humoral and cellular immune responses. As previously mentioned, there are two subsets of Th cells which differ with respect to their cytokine profiles: Th1, which mediate cellular immune responses and Th2 involved in humoral immune responses. Research has shown that both protein (163, 172, 173, 177) and peptide (176) loaded PLGA microspheres are capable of priming proliferative T cell responses. In most studies the T cell proliferation for antigen loaded PLGA microspheres was comparable or superior to that of the control formulations with adjuvants such as alum (163, 172, 173, 177). Moreover Ertl *et al.* demonstrated that proliferation of lymph node T cells was strongest for a rapid release formulation consisting of low molecular weight PLGA (M.W. 8000) with a particle size of < 10 μm in diameter in comparison to slower release formulations with high molecular weight PLGA (Mol. Wt. ≥ 60,000) and a diameter >10 μm (176). These results correlate with previous research on particle size which reported that microspheres < 10 μm in diameter elicited stronger immune responses than larger microspheres (>10 μm) due to enhanced uptake by phagocytes (164). However the influence of the

antigen release kinetics on the T cell response is uncertain and demands further assessment.

Characterization of the Th response (Th1 or Th2) involves analysis of the cytokine secretion profiles of the proliferating T cells. In a study by Moore *et al.*, immunization with HIV gp120 protein encapsulated in PLGA microspheres elicited a Th1 response where the cytokine secretion profile consisted of high levels of IFN-γ with low or undetectable levels of interleukin-5 (IL-5) (177). In contrast gp120 in alum induced T cells that secreted moderate to high levels of IL-5 and IL-4 and low levels of IFN γ- a cytokine profile consistent with a Th2 response.

1.7.3.2.2 CTL Responses

The induction of CTL responses by microencapsulated antigen requires that the antigen gain access to the cytosol followed by antigen processing in the MHC class I pathway. It was generally assumed that exogenous antigen was excluded from the MHC class I pathway; however, studies have shown that the particulate nature of the antigen may activate certain APCs (i.e. DCs and Mφs) to mediate MHC class I presentation of exogenous antigen thereby promoting a CTL response (70, 71, 73, 166, 178). CTL responses have been reported for protein (173, 177), peptide (179-181) and plasmid DNA (182) loaded PLGA microspheres; however the exact mechanism by which antigen loaded PLGA microspheres induce CTL responses is unknown and the optimal formulation for cytoplasmic delivery has to be defined. Moreover, peptide antigen adsorbed on the surface of

microspheres has been shown to elicit CTL responses (183, 184). This immune response may due to direct binding of the peptide antigen to MHC class I molecules on the surface of APCs or rapid release and cytoplasmic delivery of the antigen following cellular uptake by APCs. Overall the results of these studies require further investigation to elucidate the exact cellular mechanisms involved in MHC class I processing and presentation of exogenous antigen following delivery by PLGA microspheres.

1.8 Research Proposal

The goal of this research was to evaluate PLGA microspheres as antigen delivery systems for the induction of cellular immune responses. Peptide antigen, including a model peptide (i.e. ovalbumin peptide) and a cancer vaccine candidate (i.e. MUC1 peptide) were encapsulated in PLGA microspheres and tested for their ability to elicit specific Th1 responses. Moreover this study explored cellular uptake, processing and presentation of microencapsulated antigen by APCs with the express purpose of optimizing microsphere formulations for inducing cell-mediated immune responses.

1.8.1 Hypotheses

1) Microencapsulation of peptide antigen will accomplish two goals: 1) circumvent the need for traditional adjuvants and carrier proteins and 2) bias the immune response towards a Th1 response.
2) Analogs of bacterial cell wall components, such as MPLA, will enhance Th1 responses when incorporated with peptide loaded PLGA microspheres.

3) PLGA microspheres are capable of cytoplasmic delivery of encapsulated antigen - a required step for the induction of CTL responses.
4) The profile for cellular uptake of PLGA microspheres *in vivo* will vary with the site of injection.

1.8.2 Objectives

1) To induce specific Th1 responses through the use of peptide loaded PLGA microspheres.
2) To investigate the cellular mechanisms involved in MHC class I presentation of exogenous antigen delivered by PLGA microspheres, specifically cytoplasmic delivery.
3) To investigate cellular uptake of PLGA microspheres by APCs *in vivo* at different sites of injection.
4) To demonstrate uptake of PLGA microspheres by DCs *in vivo*.

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Chapter 2

Ovalbumin Peptide Encapsulated in Poly(D,L-lactic-co-glycolic acid) Microspheres is Capable of Inducing a T Helper Type 1 Immune Response

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2.1 Introduction

The effectiveness of an antigen delivery system is determined in part by its ability to activate the humoral and/or cell-mediated branches of the immune system. Humoral immune responses are generally required for the eradication of extracellular pathogens (1-3). Conversely, cellular immune responses are required in combating intracellular pathogens (4-6) and tumor cells (7, 8). The overall immune response is dependent on the subpopulation of Th cells activated by the antigen (9-11); the Th1 subset assists in cell-mediated immune responses while the Th2 subset assists in humoral immune responses.

Peptide-based vaccines have several advantages over traditional live attenuated, inactivated vaccines including enhanced stability and no pathogenic agents present (12). Unfortunately, peptide antigens are poorly immunogenic and require adjuvants to increase their immunogenicity (13). Microspheres offer the advantage of providing controlled release of the peptide antigen while protecting it from degradation and clearance in a physiological environment.

The goal of this study was to determine the specificity and type of Th response elicited by PLGA microspheres containing a model peptide antigen, ovalbumin (OVA) peptide. This OVA peptide, consisting of residues 323-339, contains both Th and B cell epitopes (14) and is capable of eliciting Th1 or Th2 responses. It was hypothesized that microencapsulation of the peptide would accomplish two goals: 1) circumvent the need for traditional adjuvants

and carrier proteins and 2) bias the immune response towards a Th1 response. Furthermore, it was postulated that components of bacterial cell walls, such as lipid A analogues, would enhance a Th1 response when incorporated with peptide loaded PLGA microspheres. In this study monophosphoryl lipid A (MPLA) was used based on its established safety and efficacy in humans, as well as its ability to induce strong cellular immune responses (15).

2.2 Materials and Methods

2.2.1 Preparation of PLGA Microspheres

Poly(D,L-lactic-co-glycolic acid) (PLGA) polymer (composition 50:50, mol.wt. 60,000 g/mol, inherent viscosity 0.6-0.7 dL/g in hexafluroisopropanol, HFIP) was purchased from BPI (Birmingham, AL, USA). Polyvinyl alcohol (PVA) (87-89% hydrolyzed, mol.wt. 31,000-50,000 g/mol) was purchased from Aldrich (Milwaukee, WI, USA). OVA peptide (residues 323-339, sequence: ISQAVHAAHAEINEAGR, mol.wt. 1774.9), synthesized on a solid phase synthesizer, was donated by Biomira Inc. (Edmonton, Alta., Can.). MPLA (Ribi Immunochem Research Inc., Hamilton, MT, U.S.) was provided by Biomira Inc. The ingredients for the phosphate buffered saline (PBS) were purchased from BDH Inc. (Toronto, Ont., Can.) and included 154 mM NaCl, 1.9 mM NaH_2PO_4 and 8.1 mM Na_2HPO_4. Chloroform (HPLC grade) was purchased from Fisher (Nepean, Ont., Can.).

The PLGA microspheres were prepared using a water/oil/water solvent evaporation technique (16). Briefly, 50 μl of a 2.0 mg/ml solution of OVA

peptide in PBS was emulsified with 500 μl of a 20 % w/v solution of PLGA in chloroform for 15 sec using a microtip sonicator (Heat Systems Inc., XL2010, Farmingdale, NY, USA) set at level 4. The resulting 1° emulsion was then combined with 2.0 ml of a 9 % w/v solution of PVA in PBS and sonicated for 20 sec at level 2 to form the 2° emulsion. This 2° emulsion was then added to an 8.0 ml volume of stirring 9 % w/v PVA/PBS and continued to be stirred for 2 hr. The microspheres were then collected via centrifugation using an ultracentrifuge set at 40,000 *g*, 20 °C, for 10 min. The PLGA microspheres were then washed twice with distilled water and centrifuged under the previously stated conditions. The resulting microspheres were then freeze-dried for 2 to 3 days.

For PLGA microspheres containing MPLA, 200 μg of MPLA was dissolved in 100 μl of chloroform:methanol, 4:1. This MPLA solution was then added directly to the PLGA/chloroform solution before the addition of the OVA peptide and formation of the 1° emulsion.

2.2.2 Characterization of PLGA Microspheres

2.2.2.1 Particle Size

Particle size was determined using scanning electron microscopy (SEM). Briefly, 5.0 mg of freeze-dried PLGA microspheres were dispersed in 500 μl of distilled water and air-dried onto metal stubs. The samples were placed in a sputter coater (Edwards, S150B, Sussex, England) for 40 sec to produce a gold coating of approximately 30 nm in thickness. The coated

samples were viewed under a Hitachi S-2500 scanning electron microscope (Hitatchi, Tokyo, Japan) at a magnification of 800X.

2.2.2.2 Peptide Content

The peptide content of the PLGA microspheres was determined using a chloroform/water extraction method. Briefly, 50.0 mg of PLGA microspheres was dissolved in 1.0 ml of chloroform. To this mixture 1.0 ml of water was added followed by stirring for 20 min. The chloroform and water phases were then separated by centrifugation at 1000 *g* for 10 min. The peptide concentration in the aqueous phase was determined by comparing the UV absorbance of the aqueous phase with a standard curve generated under the same conditions. Two hundred μl of the aqueous phase was injected into a Waters HPLC (Waters, Mississauga, Ont., Can.) equipped with a C_{18} reverse phase column (8X10 mm) and a UV detector (Waters, 486) set at 210 nm. The mobile phases employed were A (10 % acetonitrile (Fisher) and 0.1 % triflouroacetic acid (Sigma, St. Louis, MO, USA) in water) and B (70 % acetonitrile (Fisher) and 0.085 % triflouroacetic acid (Sigma) in water). The peptide was eluted using a gradient of 9-61 % B over 18 min. The loading was calculated from the weight of the microspheres and the amount of peptide incorporated.

2.2.3 Immunization of Mice

C57BL/6J female mice (Jackson Laboratories, Bar Harbor, ME, USA), 8 to 12 weeks old, were used during the course of the experiment. There were three experimental groups: i) mice inoculated with OVA peptide loaded

PLGA microspheres, ii) mice inoculated with OVA peptide loaded PLGA microspheres containing MPLA and iii) mice inoculated with a 50 μg/ml solution of OVA peptide in alum (Cedar Lane Laboratories, Hornby, Ont., Can.). There were 5 mice per group. For the alum group, each mouse was immunized via subcutaneous (s.c.) injection with 5 μg of OVA peptide in 100 μl of alum; 50 μl s.c. per flank. In the microsphere groups, each mouse was immunized via s.c. injection with 8.0 mg of microspheres in 200 μl of PBS; 100 μl s.c. per flank. The approximate dosages of OVA peptide and MPLA were 3 μg/mouse and 20 μg/mouse, respectively.

On day 9 following the immunizations, T cells were isolated from the mice and used to set up both the T cell proliferation and cytokine assays. This study was performed 2 times and representative data from 1 study is presented in this chapter.

2.2.4 Immunological Assays

2.2.4.1 T cell Proliferation

The specificity of the immune response elicited by the peptide loaded PLGA microspheres was determined using a T cell proliferation assay, which measured the recall response of the T cells *ex vivo* when challenged with relevant peptide and irrelevant peptides as negative controls.

On day 9 following the immunizations, the mice were sacrificed by CO_2 asphyxiation and cervical dislocation. Their inguinal lymph nodes were removed and the lymph node cells were isolated. These cells were then placed in RMPI 1640 media (Gibco BRL, Burlington, Ont., Can.)

supplemented with 1 mM L-glutamine (Gibco BRL), 55 μm 2-mercaptoethanol (Gibco BRL), 50 U Penicillin (Gibco BRL), 50 μg/ml streptomycin (Gibco BRL), 75 μg/ml gentamicin (Gibco BRL), 10 mM HEPES (Gibco BRL) and 10% v/v fetal bovine serum (Gibco BRL). The T cells were isolated by passing the lymph nodes cells through a nylon wool column, consisting of nylon wool (type 200 L, Robbins Scientific, Sunnyvale, CA, USA) inserted into a leur-lok syringe (BD leur-lok Plastipak, Rutherford, NJ, USA). The T cells were eluted off the column using the supplemented RPMI 1640 media. The purified T cells were then centrifuged at 200 *g* for 8 min and resuspended at $1X10^7$ cells/ml in the supplemented RPMI 1640 media.

Using 96 well microtiter plates (Costar, Cambridge, MA, USA) the T cells were plated at two concentrations: $5X10^5$ T cells/well and $2X10^5$ T cells/well- with the exception of the OVA peptide/alum group, which was plated at only $2X10^5$ T cells/well. At both T cell concentrations each well contained a test antigen at 20 μg/well and antigen presenting cells (APCs) at $1X10^6$ cells/well. The test antigens included the following peptides and proteins: ovalbumin (OVA) (grade V, Sigma); BP1-020 (Biomira Inc.), a mouse MUC1 peptide with the sequence DSTSSPVHSGTSSPATSAPEDSTS; OVA peptide (Biomira Inc.) consisting of residues 323-339. In addition to the wells containing test antigen, there were also negative control wells without antigen. As well, positive control wells at $2X10^5$ T cells/well were set up containing the mitogen, concanavalin A

(ConA) (Sigma), at 150 ng/well in place of the test antigen. All wells were set up in triplicate.

The antigen presenting cells were obtained from the spleen of unimmunized syngeneic mice. After removing the spleen from the syngeneic mice, the spleen cells were collected in the RPMI 1640 media mentioned earlier, and irradiated with 3000 rad using a ^{137}Cs irradiator (Nordion International Inc., Kanata Ont. Can.). These irradiated spleen cells were then collected via centrifugation at 200 *g* for 8 min and resuspended in the supplemented RPMI 1640 media at a concentration of $1X10^7$ cells/well. These spleen cells, serving as APCs, were then plated with the T cells and antigen.

Once the plates were completed they were incubated for 72 h at 37 °C and 5 % CO_2. After the 72 h, ^3H-labeled thymidine (Amersham, Oakville, Ont., Can.) was added to each well at 1 μCi/well and incubation at 37 °C -5% CO_2 was continued for 24 h. After the 24 h, the cells were harvested unto a filtermat (Skatron, Sterling, VA, USA) using a cell harvester (Skatron, Micro 96™ Harvester). Each filter was then added to 2.0 ml of scintillation fluor (Universal liquid scintillation cocktail, Scintiverse® , Fisher, Fair Lawn, NJ, U.S.) and counted in a Wallac 1410 beta counter (Wallac, Turku, Finland). The T cell proliferation was determined by measuring the amount of ^3H-labeled thymidine incorporated into the growing cells in decays per minute (DPM). The stimulation index was defined as the DPM incorporated in culture with antigen/DPM incorporated in culture without antigen.

2.2.4.2 Cytokine Assay

Before purifying the lymphocytes and extracting the T cells for the T cell proliferation assay, lymphocytes were removed from each experimental group to perform the cytokine assay. These cells were centrifuged at 200 *g* for 8 min and resuspended in the supplemented RPMI 1640 media at a concentration of $1X10^7$ cells/well. In a 96 well microtiter plate (Costar) the lymphocyte suspension was added at $1X10^6$ cells/well along with test antigen at 20 μg/well and the supplemented RPMI 1640 media at 150 μl/well. In this case no APCs were added because the APCs were already present in the crude lymphocyte mixture. The test antigens were identical to those used in the T cell proliferation assay and included the mouse MUC1 peptide and OVA peptide. As well, a negative control well was set up without antigen. All of the wells were set up in triplicate with each experimental group plated in triplicate for the 24 h, 48 h and 72 h cytokine collections. During each cytokine collection approximately 270 μl of the supernatant was removed from each well and frozen in 96 well microtiter plates (Costar).

The collected supernatant was then analyzed for the presence of IFN-γ, IL-4 and IL-10 using a sandwich ELISA. Briefly, 96 well microtiter plates (Nunc-immunoplate Maxisorp, Gibco BRL) were coated with a 1° antibody at 50 μl/well: the 1° antibody for IFN-γ, R46.A2 (17) (Biomira Inc.), was added at 75 ng/well; the 1° antibody for IL-4, 11B11 (18) (Biomira Inc.), was added at 25 ng/well; the 1° antibody for IL-10, SXC-2 (19) (Biomira Inc.) was added at

150 ng/well. The plates were then incubated at 37 °C-5 % CO_2 for 30 min and washed once with TPBS- PBS containing 0.05 % v/v Tween 20 (Sigma).

A set of standards was prepared for all three cytokines. For IFN-γ, the standard rIFN-γ (Pharmingen, San Diego, CA, USA) was diluted with the supplemented RPMI 1640 medium to a series of concentrations ranging from 5000 pg/ml to 156 pg/ml. For IL-4, the standard rIL-4 (Pharmingen) was diluted with the RPMI 1640 medium to a series of concentrations ranging from 1666 pg/ml to 52 pg/ml. In the case of IL-10, the standard rIL-10 (Pharmingen) was diluted with the supplemented RPMI 1640 medium to a series of concentrations ranging from 1600 pg/ml to 60 pg/ml. For a given cytokine plate the appropriate series of standards were added in duplicate at 50 μl/well.

Following the addition of the standards to the plates the supernatant of the proliferating T cells was added to the test wells at 50 μl/well. As well, negative control wells were set up containing the supplemented RPMI 1640 medium at 50 μl/well. The plates were then incubated for 45 min at 37 °C. After washing twice with TPBS the 2° antibodies were added to the plates at 50 μl/well: the 2° antibody for IFN-γ, biotinylated XMG1.2 (17) (Pharmingen), was added at 2.5 ng/well; the 2° antibody for IL-4, biotinylated BVD-24G2 (18) (Pharmingen), was added at 10 ng/well; the 2° antibody for IL-10, biotinylated SXC-1 (19) (Pharmingen), was added at 5 ng/well. After a 45 min incubation at 37 °C and 5 % CO_2 the plates were then washed three times with TPBS.

A 23% v/v solution of peroxidase conjugated streptavidin (Jackson Immunoresearch Lab Inc., West Grove, PA, U.S.) in 1% w/v BSA (Sigma) in TPBS was prepared and added to each plate at 50 µl/well, followed by incubation for 30 min at 37 °C and 5 % CO_2. After incubation the plates were washed 4 times in TPBS. Peroxidase solution (KPL, Gaithersburg, MD, U.S.) was then combined in equal parts with 3, 3', 5, 5' tetramethylbenzidine (TMB) peroxidase substrate (KPL) and added to each plate at 100 µl/well. An optical density (O.D.) reading at 2 min was performed at 650 nm using a microplate reader (Molecular Devices, Menlo Park, CA, USA). Once the O.D. reading reached 0.8, 1 M phosphoric acid (BDH Inc.) was added at 100 µl/well, followed by an O.D. reading at 450 nm.

2.2.5 Statistical Analysis

The results are expressed as the mean ± S.D. for each group of mice. An unpaired student's t test was used to compare sample means and assess statistical significance. Results were considered significant if $p < 0.05$.

2.3 Results

The size of the peptide loaded PLGA (50/50) microspheres was 4 ± 2 µm in diameter as determined by SEM. According to the electron micrograph the microspheres were uniform in size with good surface morphology- lacking pores (Fig 2-1). Based on RPHPLC, the encapsulation efficiency was 23% and peptide loading was 0.03% (w/w) of OVA peptide entrapped per dry weight of microspheres.

The specificity of the immune response was determined using a T cell proliferation assay, which measures the recall response of the T cells when challenged *ex vivo* with relevant antigen and irrelevant antigen as negative controls. Figure 2-2A shows the results of the T cell proliferation assay of T cells isolated from mice immunized with OVA peptide loaded PLGA microspheres. The positive control wells containing Con A, at $2X10^5$ T cells/well, exhibited strong proliferation. In the presence of test antigen, wells containing $2X10^5$ and $5X10^5$ T cells/well, exhibited T cell specific immune responses with the greatest proliferative response against the OVA peptide test antigen. The stimulation indexes for the OVA peptide microsphere group at $5X10^5$ and $2X10^5$ T cells/well in the presence of OVA peptide were 31 and 16, respectively. Figure 2-2B shows the results of the T cell proliferation assay of the T cells isolated from mice immunized with OVA peptide PLGA microspheres containing MPLA. The results revealed an antigen-specific T cell response with the greatest proliferative response against the OVA peptide test antigen. The stimulation indices at $5X10^5$ and $2X10^5$ T cells/well in the presence of OVA peptide were 30 and 23, respectively. Figure 2-2C shows the results of the T cell proliferation assay of T cells isolated from mice immunized with OVA peptide in alum. The results indicate the absence of a proliferative T cell response with the exception of the positive control wells containing Con A.

The type of T helper response was determined by analysis of the cytokine secretion profiles of the proliferating T cells. The cytokine assay

tested for the presence of IFN-γ, IL-4 and IL-10. Figure 2-3 shows the cytokine secretion profiles of T cells isolated from mice immunized with OVA peptide loaded microspheres, OVA peptide loaded microspheres containing MPLA and OVA peptide in alum. The cytokine secretion patterns were determined in response to relevant antigen (OVA peptide), and in the absence of antigen. The cytokine profile of the T cells showed the highest level of IFN-γ production occurring 72 h after cell culture (data not shown). IFN-γ production in response to the OVA peptide was significantly greater than the IFN-γ production in the absence of antigen for both the OVA peptide ($p < 0.001$) and OVA peptide + MPLA ($p < 0.001$) microsphere groups (Fig. 2-3A). On the contrary, IFN-γ production for the OVA peptide/alum group was not significantly different from that of the negative control wells without antigen ($p > 0.05$) (Fig. 2-3A). The IL-4 results revealed extremely low levels of IL-4 for both the microsphere groups and OVA peptide/alum group (Fig. 2-3B) with optimal IL-4 production occurring 24 h after cell culture (data not shown). For all three experimental groups IL-4 production in the presence of relevant antigen was not significantly different from that of the negative control wells ($p > 0.05$). In Figure 2-3C the results indicate very low levels of IL-10 with the greatest IL-10 production occurring 48 h after cell culture (data not shown). At 48 h the IL-10 production for the OVA peptide microsphere group, in response to relevant antigen, was found to be significantly lower than that of the negative control wells ($p < 0.005$), while the IL-10 production for the OVA peptide/alum group was not significantly different from that of the

negative control wells ($p > 0.05$) (Fig. 2-3C). Furthermore, for the OVA peptide + MPLA microsphere group, IL-10 production in response to the OVA peptide was significantly greater than the levels detected in the absence of test antigen ($p < 0.025$) (Fig. 2-3C); however these levels were extremely low with a mean value of 16 pg/ml in the presence of relevant antigen (OVA peptide) and 8 pg/ml in the absence of antigen.

In comparing the results between group 1 with OVA peptide microsphere and group 2 with OVA peptide microspheres containing MPLA, the proliferative response for group 2 at $5X10^5$ T cells/well in the presence of OVA peptide was not significantly greater than group 1 ($p > 0.05$). However, at $2X10^5$ T cells/well, the T cell proliferation for group 2 in response to the OVA peptide was significantly greater than that of group 1 ($p < 0.05$). Hence, at $2x10^5$ T cells/well the presence of MPLA in the peptide loaded microspheres appears to have increased the magnitude of the immune response.

The cytokine profiles of the peptide loaded microspheres with and without MPLA both show high levels of IFN-γ with very low levels of IL-4 and IL-10 (Fig. 2-3). Furthermore, at the 72 h collection the level of IFN-γ for the OVA peptide microspheres containing MPLA (12155 pg/ml) was significantly greater than the OVA peptide microspheres without MPLA (6790 pg/ml) ($p < 0.010$) (Fig.2-3A). Hence, the incorporation of MPLA in the peptide loaded microspheres resulted in an increase in IFN-γ production.

2.4 Discussion

Early studies on biodegradable microspheres involved the use of protein loaded microspheres for the induction of humoral immune responses (20-25). Parenteral immunization with protein entrapped in PLGA microparticles has been shown to induce systemic IgG responses comparable to those elicited by other adjuvants such as CFA (25) or aluminum hydroxide (20, 21). Moreover, studies have demonstrated both systemic and mucosal antibody immune responses following mucosal administration of antigen loaded PLGA microspheres (22-24). Furthermore, peptide loaded PLGA microspheres have been shown to elicit antibody responses comparable or even superior to those induced by CFA (26).

Aluminum hydroxide is currently the only widely used adjuvant in humans. Unfortunately, aluminium adjuvants are effective for only a limited number of antigens and they do not induce cell-mediated immunity (27). Aside from the ability of PLGA microspheres to elicit antibody responses, there is accumulating evidence of their ability to elicit cell-mediated immune responses. Previous research has shown that protein loaded PLGA microspheres are capable of priming T cell proliferative responses (20, 22, 23, 28) including Th1 responses (28). Moreover, cytotoxic T lymphocyte (CTL) responses have been reported for both protein (23, 28) peptide (29-31) and plasmid DNA (32) loaded PLGA microspheres.

Studies have shown that human egg-allergic patients produce IgE antibodies specific for OVA peptide (residues 323-339) (33). Hence, the

generation of a Th1 response against OVA peptide is of clinical significance in the treatment of egg allergies. The objective of this investigation was to determine the efficacy of OVA peptide loaded PLGA microspheres in inducing Th1 responses and to study the effects of the immunomodulator, MPLA, on the immune response.

The results of this study clearly show that peptide entrapped in PLGA microspheres is capable of eliciting an immune response without the need for carrier proteins. Moreover, the results indicate that peptide loaded PLGA microspheres are capable of eliciting antigen-specific T cell responses, which is complementary to previous research conducted using protein loaded PLGA microspheres (20, 22, 23, 28). Moreover, the incorporation of MPLA in the peptide loaded PLGA microspheres appears to enhance the T cell proliferative response at $2X10^5$ T cells/well, but not at $5X10^5$ T cells/well. This variation in response with different T cell concentrations may be a reflection of factors limiting cell growth. At $5X10^5$ T cells/well, the proliferative response may plateau earlier due to a larger number of precursor cells in combination with limited nutrients and space. Hence, the T cell proliferation results obtained at lower T cell concentrations (i.e. $2X10^5$ T cells/well) may be a better indicator of differences in the immune responses between experimental groups. In marked contrast to the microsphere groups, the OVA peptide/alum immunization failed to prime for a proliferative T cell specific immune response. The absence of such a response is a direct

indication of the weak immunogenicity of the peptide and its rapid degradation *in vivo*.

The Th1-Th2 choice may be affected by factors such as antigen dose. Early *in vivo* studies indicated that both low and high levels of antigen induced DTH/cellular immune responses, while intermediate levels elicited antibody production (34). Further studies have shown that Balb/c mice mount an ineffective antibody response against *Leishmania major* when infected with high levels of this pathogen, while very low doses elicit a protective DTH response (35). The goal of this study was to induce a Th1/cellular immune response, and based on the current findings it was believed that low peptide loading in the PLGA microspheres would be beneficial. Hence, no further efforts were made to increase peptide loading beyond 0.03% w/w of OVA peptide entrapped per dry weight of microspheres.

In this study, cytokine production for the OVA peptide/alum group was negligible, further confirming the poor immunogenicity of the peptide in the presence of alum. On the contrary, the microsphere groups (i.e. OVA peptide in microspheres, OVA peptide & MPLA in microspheres) exhibited high levels of IFN-γ with low levels of IL-4 and IL-10- a cytokine profile consistent with a Th1/cellular immune response (36, 37). Moreover, it was demonstrated that IFN-γ production can be significantly increased through the use of MPLA.

The adjuvant activity of MPLA is due in part to its ability to induce the production of cytokines, which results in the generation of specific immune responses (38). Studies have shown that both DCs and Mϕs in response to

certain microbial products will produce interleukin-12 (IL-12) (39-41) - a cytokine involved in the Th1 response (39, 42). The exact mechanism by which MPLA enhances a Th1 response elicited by peptide loaded PLGA microspheres is uncertain; however based on these previous studies it is hypothesized that following phagocytosis of the microspheres by APCs (i.e. DC, Mϕ) MPLA stimulates the APC to produce IL-12, which further potentiates a Th1 response. Additional studies must be conducted to determine the validity of this hypothesis.

This report demonstrates that OVA peptide encapsulated in PLGA microspheres is capable of eliciting Th1 responses. The OVA peptide is capable of eliciting both Th1 and Th2 responses, hence the results of this study demonstrate the capacity of PLGA microspheres to bias the immune response towards a Th1 response. Furthermore, the results suggest that lipid A analogues (i.e. MPLA) can enhance Th1 responses when incorporated with peptide loaded PLGA microspheres. Finally, the strong immunogenicity of this vaccine formulation in the absence of traditional adjuvants further confirms that PLGA microspheres may find future applications as antigen delivery systems for peptide-based vaccines.

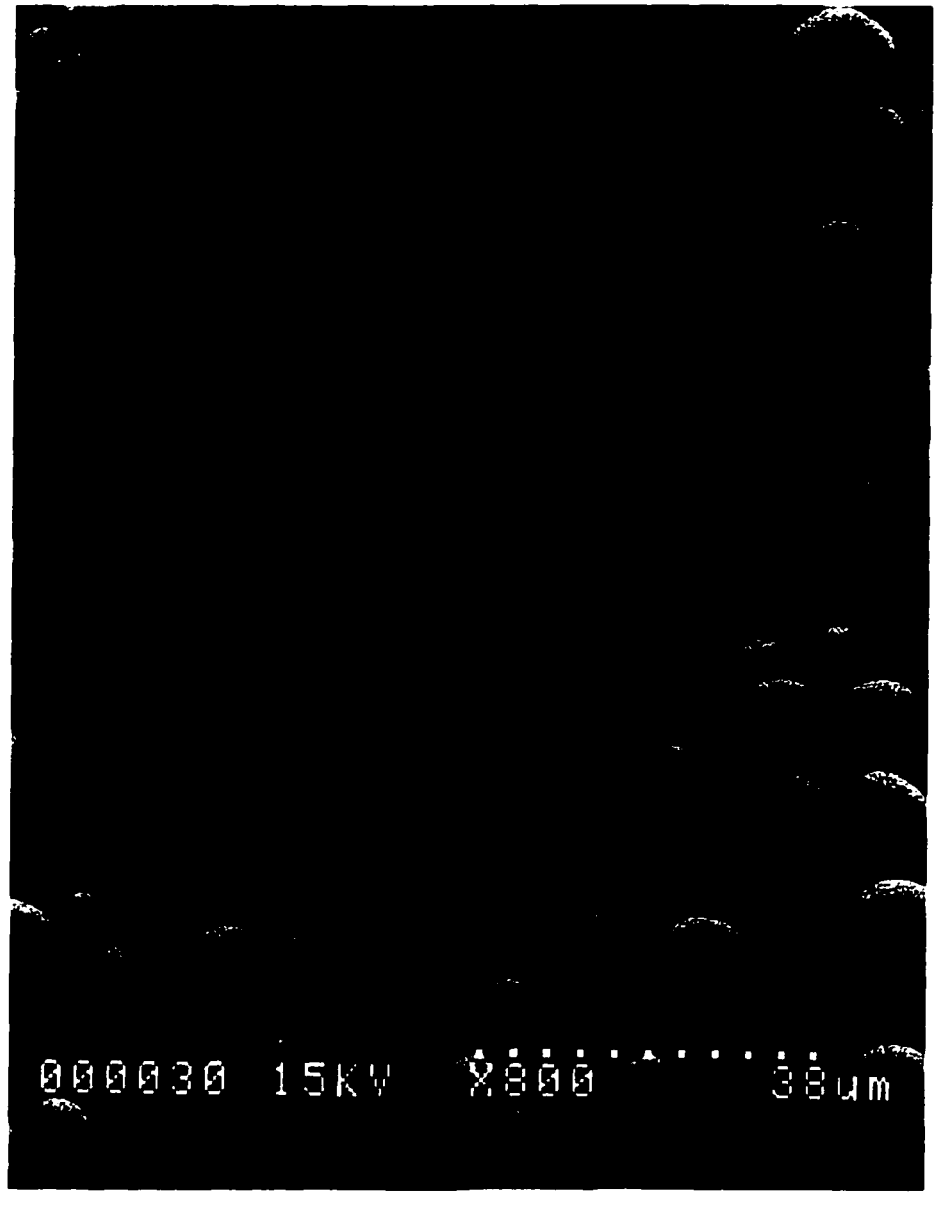

Figure 2-1. Scanning electron micrograph of PLGA microspheres containing OVA peptide. Magnification 800X.

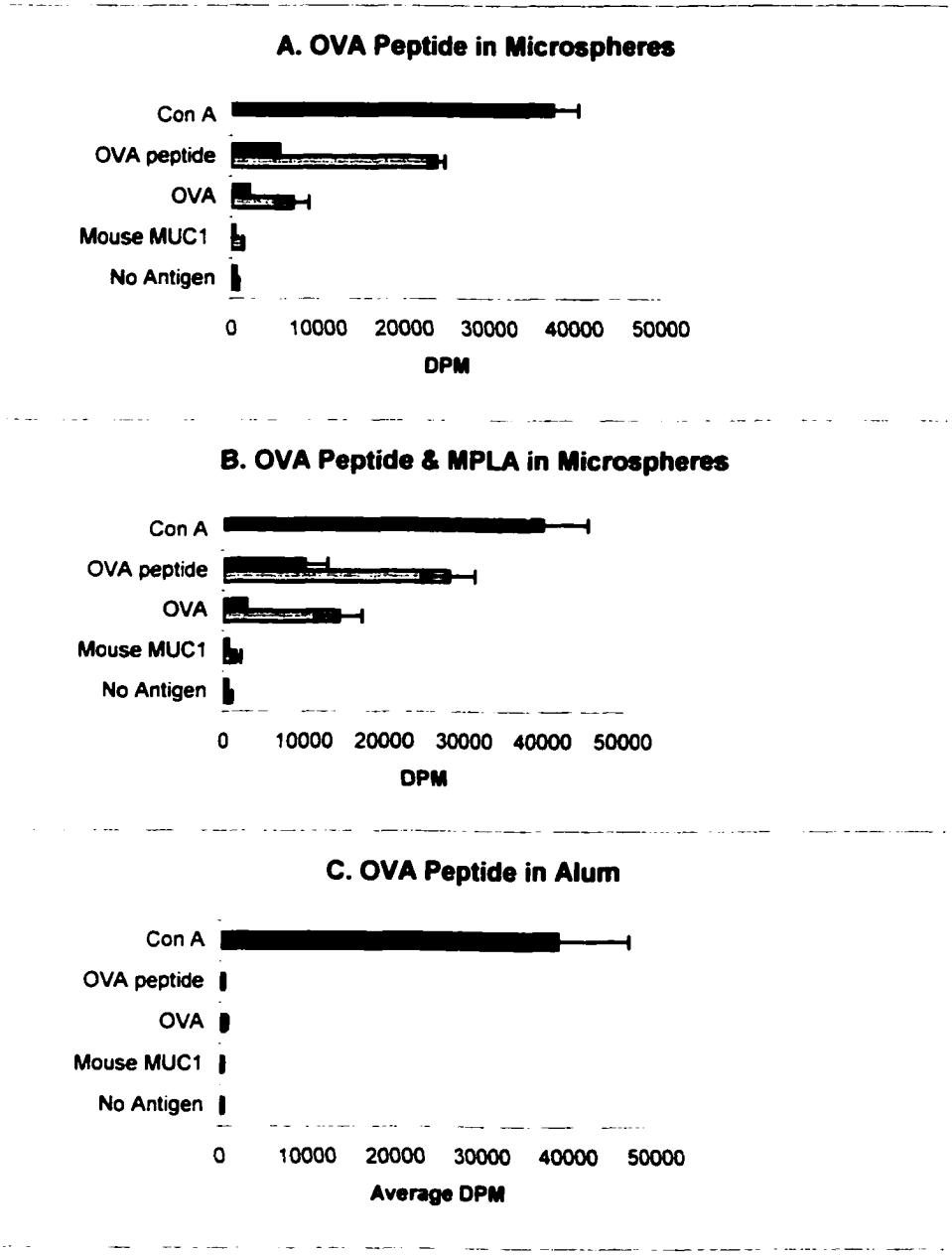

Figure 2-2. Analysis of the T cell proliferation assay of T cells isolated from mice immunized with A) OVA peptide in microspheres, B) OVA peptide & MPLA in microspheres and C) OVA peptide in alum. The proliferative response at $2X10^5$ T cells/well (dark grey column) and $5X10^5$ T cells/well (light grey column) was measured by ^{3}H-thymidine incorporation.

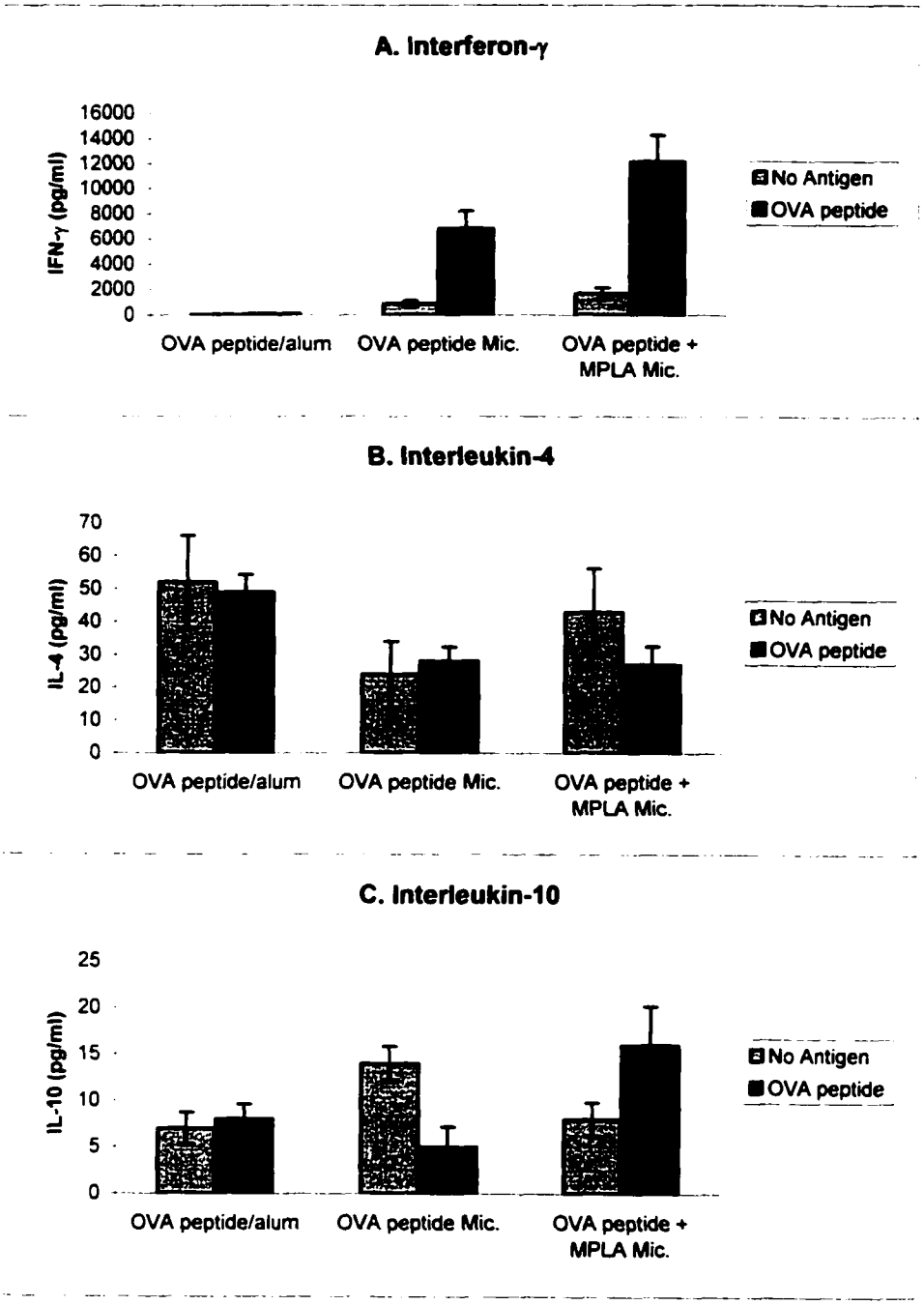

Figure 2-3. Cytokine secretion profile of T cells isolated from mice immunized with OVA peptide in alum, OVA peptide in microspheres, and OVA peptide & MPLA in microspheres. The pattern of cytokine secretion was evaluated in the presence of relevant antigen (i.e. OVA peptide) and in the absence of antigen. The cytokines, measured in pg/ml, included A) IFN-γ, B) IL-4 and C) IL-10.

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Chapter 3

The Delivery of MUC1 Mucin Peptide by Poly(D,L-lactic-co-glycolic acid) Microspheres Induces Type 1 T Helper Immune Responses

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3.1 Introduction

In light of the shortcomings of conventional cancer treatments, immunotherapy and the development of a cancer vaccine represent attractive prospects for cancer research. The immunotherapeutic approach is based on the assumption that cancer cells express unique cell surface markers, which may be antigenic. MUC1 mucin represents a potential target antigen in cancer immunotherapy based on its high expression in many human cancers including breast, pancreatic and ovarian carcinomas (1-4). Human cancer associated MUC1 mucin frequently exhibits aberrant glycosylation, exposing normally cryptic peptide sequences (2, 5). It is these cancer-associated epitopes that provide the basis of vaccine design for carcinomas expressing MUC1.

Studies have shown that cellular immune responses are required for the eradication of cancer (6, 7). Hence, a cancer vaccine formulation should be capable of increasing the immunogenicity of cancer-associated antigens while biasing the immune response towards a Th1/cellular immune response. PLGA microspheres represent attractive antigen delivery systems based their biocompatible, biodegradable nature and their proven safety in humans (8, 9). Studies have shown that protein loaded PLGA microspheres are capable of eliciting cellular immune responses including T cell proliferative responses (10-12) and Th1 responses (12). Furthermore, CTL responses have been demonstrated for both protein (11, 12) and peptide loaded PLGA microspheres. (13). More importantly, the study with ovalbumin peptide

loaded PLGA microspheres (Chapter 2) revealed that this antigen delivery system was capable of biasing the immune response towards a Th1 response.

Based on the results of the OVA peptide study, PLGA microspheres were prepared containing a cancer vaccine candidate, a 24mer human MUC1 mucin peptide. This peptide is derived from the tandem repeat region of MUC1 mucin and contains both T and B cell epitopes (14). It was hypothesized that microencapsulation of MUC1 peptide would prime for antigen-specific Th1 responses without the use of traditional adjuvants or carrier proteins. An immunomodulator, MPLA, was also incorporated into the PLGA microspheres based on its ability to induce strong cellular immune responses and its established safety in humans (15).

3.2 Materials and Methods

3.2.1 Preparation of PLGA Microspheres

PLGA microspheres (polymer composition 50:50; mol.wt. 60,000 g/mol; inherent viscosity 0.6-0.7 dL/g in hexafluroisopropanol) (BPI) containing a MUC1 24mer peptide (Sequence: TAPPAHGVTSAPDTRPAPGSTAPP; mol.wt. 2253.42 g/mol; synthesized on a solid phase synthesizer; donated by Biomira Inc.) were prepared as previously described in section 2.2.1. Briefly, 100 mg of PLGA with 100 μg of MUC1 peptide were used in the preparation of the microspheres with one modification where formation of the 2° emulsion involved sonication at level 4 for 20 s. For PLGA microspheres containing MPLA, 200 μg of MPLA was

dissolved in 100 μl of chloroform:methanol, 4:1. This MPLA solution was then used in the preparation of MPLA loaded PLGA microspheres (see section 2.2.1).

3.2.2 Characterization of PLGA Microspheres

3.2.2.1 Particle Size

3.2.2.1.1 Scanning Electron Microscopy

Particle size was determined using scanning electron microscopy (SEM). The microsphere samples were prepared as previously described in section 2.2.2.1. The gold-coated samples were viewed under a Hitachi S-2500 scanning electron microscope (Hitatchi, Tokyo, Japan) at a magnification of 6000X.

3.2.2.1.2 Dynamic Light Scattering

The diameter of the microspheres was also determined using dynamic light scattering (BI-90 Particle sizer, Brookhaven Instruments Corporation, Holtsville NY, U.S.A.). Two mg of PLGA microspheres were suspended in 2.0 ml of PBS in a plastic cuvette prior to measuring the light scattering.

3.2.2.2 Peptide Content

The peptide content of the PLGA microspheres was determined using a chloroform/water extraction method described in section 2.2.2.2. The peptide concentration in the aqueous phase was determined by comparing the UV absorbance of the peptide in the aqueous phase with a standard curve generated under the same conditions. Two hundred μl of the aqueous phase was injected into a Waters HPLC (Waters, Mississauga, Ont., Can.)

equipped with a C_{18} reverse phase column (8X100 mm) and a UV detector (Waters, 486) set at 210 nm. The mobile phases employed were A (10 % acetonitrile (Fisher) and 0.1 % trifluoroacetic acid (Sigma, St. Louis, MO, USA) in water) and B (70 % acetonitrile (Fisher) and 0.085 % trifluoroacetic acid (Sigma) in water). The peptide was eluted using a gradient of 4-46 % B over 18 min. The loading was calculated from the weight of the microspheres and the amount of peptide incorporated.

3.2.3 Immunization of Mice

C57BL/6J female mice (Jackson Laboratories, Bar Harbor, ME, USA), 8 to 12 weeks old, were used during the course of the experiment. There were three experimental groups: i) mice inoculated with MUC1 peptide loaded PLGA microspheres, ii) mice inoculated with MUC1 peptide loaded PLGA microspheres containing MPLA and iii) mice inoculated with MPLA loaded PLGA microspheres. There were 7 mice per group. Each mouse was immunized via s.c. injection with 8.0 mg of microspheres in 200 μl of PBS; 100 μl s.c. per flank. The approximate dosages of MUC1 peptide and MPLA were 1.6 μg/mouse and 20 μg/mouse, respectively.

On day 9 following the immunizations, T cells were isolated from the mice and used to set up both the T cell proliferation and cytokine assays, while serum was collected followed by analysis using an ELISA. This study was performed 2 times and representative data from one study is presented in this chapter.

3.2.4 Immunological Assays

3.2.4.1 T cell Proliferation

The specificity of the immune responses elicited by the PLGA microspheres was determined using a T cell proliferation assay. In this assay, the recall responses of the T cells isolated from immunized mice were measured *in vitro* against both relevant peptide and irrelevant peptides as negative controls.

On day 9 following the immunizations, the mice were sacrificed by CO_2 asphyxiation and cervical dislocation. Their inguinal lymph nodes were removed and the lymph node cells harvested. The protocol for the T cell proliferation assay is described in section 2.2.4.1. Briefly, in 96 well microtiter plates (Costar, Cambridge, MA, USA) the T cells were plated at two concentrations: $5X10^5$ T cells/well and $2X10^5$ T cells/well. At both T cell concentrations each well contained a test antigen at 20 μg/well and antigen presenting cells (APCs) at $1X10^6$ cells/well. The panel of test antigens included the following peptides: the immunizing MUC1 24 mer peptide (Biomira Inc); a MUC1 30 mer (Biomira Inc.) with the sequence RPAPGSTAPPAHGVTSAPDTRPAPGSTAPP; a mouse MUC1 peptide (Biomira Inc) with the sequence DSTSSPVHSGTSSPATSAPEDSTS; OVA peptide (Biomira Inc.) consisting of residues 323-339 with the sequence ISQAVHAAHAEINEAGR. In addition to the wells containing test antigen, there were also negative control wells without antigen. As well, positive control wells at $2X10^5$ T cells/well were set up containing the mitogen, Con A

(Sigma), at 2 μg/well in place of the test antigen. All wells were set up in quadruplicate.

The plates were incubated for 96 h at 37 °C and 5 % CO_2. ^{3}H-labeled thymidine (Amersham, Oakville, Ont., Can.) was then added to each well at 1 μCi/well and incubation at 37 °C -5% CO_2 was continued for 24 h. After the 24 h, the cells were harvested onto a filtermat (Canaberra Packard, Mississauga Ont. Can.) using a Micromate cell harvester (Canaberra Packard) and each filter was counted in a Matrix 96 Direct Beta counter (Canaberra Packard). T cell proliferation was determined by measuring the amount of ^{3}H-labeled thymidine incorporated into the growing cells in decays per minute (DPM). The stimulation index was defined as the DPM incorporated in culture with antigen / DPM incorporated in culture without antigen.

3.2.4.2 Cytokine Assay

Before pulsing the T cells with ^{3}H-labeled thymidine, 135 μl of supernatant was removed from each well at 96 h to perform the cytokine assay. The collected supernatant was analyzed for the presence of IFN-γ, IL-4 and IL-10 as previously described in section 2.2.4.2

3.2.4.3 ELISA for antibodies

Serum samples obtained from each mouse were analyzed using an ELISA. Briefly, 96 well flat bottom microtiter plates (Immulon 2, Fisher) were coated with a MUC1 16 mer peptide (sequence: GVTSAPDTRPAPGSTA) human serum albumin (HSA) conjugate (Biomira Inc.) at 500 ng/well and

incubated at 4 °C overnight. The coated plates were then washed 4X with PBS and blocked with a 10 % v/v solution of BSA in distilled deionized water (KPL). After a 30 min incubation at 37 °C the plates were washed 3X with TPBS followed by the addition of the test sera. The sera included normal serum from unimmunized C57BL/6J mice, test serum from the 3 experimental groups, and positive serum obtained from C57BL/6J mice immunized with the MUC1 16 mer peptide conjugated to KLH. Serial dilutions of the sera were added to each plate followed by incubation for 1 h at 25 °C. The wells were then washed 5X with TPBS and goat anti-mouse IgM (KPL), IgG (KPL) and anti-IgG isotype (Southern Biotechnology Associates Inc., Birmingham, AL, USA) specific antibodies were added to the appropriate wells. After a 1 h incubation at 25 °C the plates were washed 5X with TPBS and 2, 2'-azino-di(3-ethyl-benzthiazoline sulfonate) (ABTS) substrate (KPL) was then added to the wells. A 15 min O.D. reading at 405 nm was then taken with a microplate reader (Molecular Devices). The antibody titre was defined as the highest reciprocal dilution, which gave an absorbance greater than the mean absorbance ± 2 S.D. of the normal serum diluted from 1/80 to 1/5120.

3.2.5 Statistical Analysis

The results are expressed as the mean ± S.D. for each group of mice. An unpaired student's t test was used to compare sample means and assess statistical significance. Results were considered significant if $p < 0.05$.

3.3 Results

The PLGA microspheres were 700 ± 200 nm in diameter based on both SEM and dynamic light scattering. Hence, there exists good correlation between both methods of size determination. Based on RPHPLC the encapsulation efficiency was 12 % and peptide loading was 0.02% w/w of MUC1 peptide entrapped per dry weight of microspheres.

The mice were immunized via s.c. injection with either 1) MUC1 peptide loaded PLGA microspheres, 2) MUC1 peptide loaded PLGA microspheres containing MPLA or 3) MPLA loaded PLGA microspheres. The specificity of the immune response was determined by measuring T cell proliferation against a variety of antigens including MUC1 24 mer peptide, MUC1 30 mer peptide, OVA peptide, mouse MUC1 peptide, as well as in the absence of antigen (Fig. 3-1). Figure 3-1A shows the results of the T cell proliferation assay of T cells isolated from mice immunized with MUC1 peptide loaded PLGA microspheres. Strong proliferation occurred in the presence of the positive control, Con A. Moreover, in the presence of test antigen, the results revealed T cell specific immune responses with the greatest proliferation occurring against the immunizing antigen MUC1 24 mer peptide and the relevant antigen MUC1 30 mer peptide. The stimulation indexes at $5X10^5$ T cells/well in the presence of MUC1 24 mer peptide and MUC1 30 mer peptide were 22 and 13, respectively. Figure 3-1B reveals the results of the T cell proliferation assay of T cells isolated from mice immunized with MUC1 peptide loaded PLGA microspheres containing MPLA.

Aside from the strong proliferation in the presence of Con A, the results revealed a specific proliferative response against both MUC1 24 mer and MUC1 30 mer peptides. The stimulation indexes at $5X10^5$ T cells/well in the presence of MUC1 24 mer and 30 mer peptides were 29 and 28, respectively. Figure 3-1C shows the results of the T cell proliferation assay of T cells isolated from mice immunized with MPLA loaded PLGA microspheres. The results indicated that these microspheres failed to induce T cell specific immune responses.

In comparing the T cell proliferation of experimental groups a) MUC1 peptide loaded PLGA microsphere and b) MUC1 peptide & MPLA in PLGA microspheres, the results revealed a stronger T cell response for the latter containing MPLA. In the presence of the MUC1 24 mer test antigen, the T cell proliferation of group b) was significantly greater than that of group a) at both $2X10^5$ T cells/well ($p < 0.025$) and $5X10^5$ T cells/well ($p < 0.0005$). Moreover, in the presence of the MUC1 30 mer peptide, the proliferation of group b) was significantly greater than that of group a) at $2X10^5$ T cells/well ($p < 0.005$) and $5X10^5$ T cells /well ($p < 0.001$).

The type of T helper response was determined by analysis of the cytokine secretion profiles of the proliferating T cells. The supernatants of the T cells were tested for the presence of IFN-γ, IL-4 and IL-10. Figure 3-2 reveals the IFN-γ production of T cells isolated from mice immunized with MUC1 peptide loaded PLGA microspheres (Fig. 3-2A), MUC1 peptide loaded PLGA microspheres containing MPLA (Fig. 3-2B) and MPLA loaded PLGA

microspheres (Fig. 3-2C). IFN-γ production for the MUC1 peptide microsphere group was greater in the presence of the MUC1 24 mer peptide than in the presence of irrelevant antigen (OVA 323-339) ($p< 0.05$) and in the absence of antigen ($p < 0.05$) (Fig. 3-2A). For the MUC1 peptide loaded microspheres containing MPLA, IFN-γ levels were significantly greater in the presence of MUC1 24 mer peptide than in the absence of antigen ($p < 0.005$) or the presence of OVA peptide ($p < 0.005$) (Fig. 3-2B). Conversely, in Figure 3-2C IFN-γ production for the MPLA microsphere group in response to relevant antigen was significantly less than that of the negative control wells ($p < 0.05$). For all three experimental groups the levels IL-4 and IL-10 production were below the limit of detection of the cytokine assays (data not shown).

The predominant cytokine for the MUC1 peptide loaded PLGA microspheres with and without MPLA was IFN-γ (Fig. 3-2A and 3-2B) while both IL-4 and IL-10 production was negligible. Furthermore, in comparing the levels of IFN-γ for the 2 groups the results revealed a significant increase in IFN γ production in the presence of MPLA ($p < 0.005$).

Assessment of the antibody response was performed based on the analysis of the mouse sera. IgG and IgM production were negative for both the MPLA loaded PLGA microsphere group and the MUC1 peptide loaded PLGA microsphere group (data not shown). Conversely, the experimental group immunized with MUC1 peptide loaded PLGA microspheres containing MPLA exhibited no IgM production (Fig. 3-3A) with a very small IgG response

(Fig. 3-3B) corresponding to an antibody titre of 160. Further characterization of the IgG response revealed significant levels of IgG2a ($p < 0.010$), IgG2b ($p < 0.001$) and IgG3 ($p < 0.001$) with insignificant levels of IgG1 ($p > 0.05$) (Fig. 3-4). More specifically, the results indicated an absence of IgG1 with the predominant isotype being IgG2b accompanied by very low levels of IgG2a and IgG3 (Fig. 3-4).

3.4 Discussion

Peptides are poorly immunogenic requiring adjuvants (16) and carrier proteins (e.g. tetanus toxoid, diptheria toxoid, keyhole limpet hemocyanin) (17) to increase their immunogenicity. Unfortunately, carrier proteins (18) and the only adjuvant approved for use in humans (i.e. alum) (19), induce predominant humoral immune responses with minimal capacity for cell-mediated immunity. PLGA microspheres have been shown to increase the immunogenicity of peptide antigen based on their ability to induce strong humoral (20) and cellular immune responses (13) (Chapter 2). Moreover, the study in Chapter 2 with OVA peptide loaded PLGA microspheres revealed the capacity of PLGA microspheres to bias the immune response towards a Th1 response.

Plasmid DNA containing MUC1 mucin cDNA (21, 22) and recombinant vaccinia virus MUC1 mucin constructs (23, 24) have been used as cancer vaccines in various models. Moreover, MUC1 peptides have shown promising potential in cancer vaccine formulations (14, 18, 25-27). In particular, MUC1 peptide conjugated to KLH has been shown to induce high antibody titers and

DTH responses, resulting in immune rejection of cancer in mouse models (14). Furthermore it has been shown that a MUC1 fusion protein coupled to mannan generates MHC class I restricted responses resulting in significant tumor protection (25). Moreover, a study by Samuel *et al.* (26), which tested a liposomal formulation containing MPLA and the MUC1 24 mer peptide used in this study, revealed that anti-tumor activity correlated with a Th1 response.

In this study MUC1 24 mer peptide was encapsulated in PLGA microspheres to determine the specificity and type of immune response elicited by the peptide loaded PLGA microspheres. Furthermore, MPLA was incorporated into these PLGA microspheres to determine its ability to enhance a Th1 response. The results, in accordance with the study in Chapter 2, indicate that peptide loaded PLGA microspheres elicit antigen-specific T cell responses, which are enhanced in the presence of MPLA. The lack of a T cell response for the MPLA loaded PLGA microspheres reveals the non-immunogenic nature of the formulation in the absence peptide antigen. Moreover, studies using a similar dose of the MUC1 24 mer peptide in alum failed to induce an immune response (Samuel *et al.*, unpublished results). These results are consistent with the OVA peptide study (Chapter 2) and reveal the importance of microencapsulation in protecting weakly immunogenic peptide antigen.

The type of Th response evoked by the MUC1 peptide loaded PLGA microspheres was determined based on antibody response and cytokine secretion profiles. The negative control, MPLA loaded PLGA microspheres,

had undetectable levels of IgM and IgG. The MUC1 peptide loaded PLGA microspheres failed to elicit IgG or IgM responses, while the MUC1 peptide loaded PLGA microspheres containing MPLA exhibited no IgM production with a very small IgG response. IgG isotyping of the peptide loaded PLGA microspheres with MPLA revealed the presence of IgG2a, IgG2b and IgG3- all of which are associated with Th1 responses (28-31). The gene corresponding to IgG2a in C57BL/6J mice encodes a novel isotype IgG2c (32, 33). This isotype is significantly different from IgG2a expressed in Balb/c mice and consequently commercial reagents for IgG2a may not crossreact substantially with IgG2c. As C57Bl/6J mice were used in this study, both the IgG2b and IgG3 responses serve as better indicators of Th1 responses.

The cytokine profile for the MPLA loaded PLGA microspheres was negative for IFN-γ with undetectable levels of both IL-4 and IL-10. Conversely, the cytokine profile for the MUC1 peptide loaded PLGA microsphere groups with and without MPLA consisted of strong IFN-γ production with undetectable levels of IL-4 and IL-10. Hence, both the antibody and cytokine profiles of the peptide loaded PLGA microsphere groups suggest the presence of Th1 responses. Moreover, MPLA appeared to enhance the Th1 responses based on its ability to increase IFN-γ production. The absence of cytokine and antibody production for the negative control, MPLA loaded PLGA microspheres, further confirms the lack of immunogenicity of this formulation.

Antigen dose has been shown to be a major regulator in the Th1/Th2 choice. Early studies have indicated that both high and low doses of antigen

primed for DTH/cellular responses (34). Such results are also reflected in infectious models where the induction of a healing DTH response occurred in BALB/c mice infected with low numbers of *Leishmania* parasite, while a nonhealing antibody response was induced at higher doses (35). In Chapter 2 with OVA peptide loaded PLGA microspheres, antigen loading was very low and the resultant Th response was Th1. Based on these results, peptide loading in this study was maintained at a low level.

Activation of Th cells requires that the antigen be processed and presented by APCs (i.e. DCs, Mϕs, B cells) (36). The ability of MUC1 peptide loaded PLGA microspheres to bias the immune response towards a Th1 response may be a reflection of the facilitated uptake and presentation of the microencapsulated peptide by phagocytic cells; however, the mechanisms involved require further study. Moreover, the exact mechanism by which MPLA enhances Th1 responses elicited by peptide loaded PLGA microspheres demands further investigation. Antigen covalently linked to microspheres has been shown to induce Th1 responses when co-injected with IL-12 (37) - a cytokine involved in the Th1 response (38). Moreover, DCs and Mϕs have been shown to produce IL-12 in response to certain microbial products (39-41). Hence, as in Chapter 2, it is hypothesized that upon phagocytosis of the microspheres by APCs, MPLA stimulates the APC to produce IL-12, which further potentiates Th1 responses. In conclusion, future studies must be conducted to determine the cofactors and mechanistic

aspects involved in the Th1/Th2 choice for peptide loaded PLGA microspheres.

The results of this study provide useful information on the immunogenicity of MUC1 peptide loaded PLGA microspheres in mice. However, the major limitation of this study is that we are measuring heterologous immune responses against a human MUC1 tumor antigen. Hence, the immune responses against human MUC1 antigen might be greater than expected from the homologous mouse MUC1 sequence. Although the ability of peptide loaded PLGA microspheres to break self-tolerance is unknown, a study by Samuel *et al.* revealed the capacity of a liposomal formulation containing mouse MUC1 peptide, to induce Th1 responses in mice based on IFN-γ producing T cells (unpublished results). Overall, the ideal animal model is a transgenic mouse model, developed by Peat *et al.* (42), which expresses human MUC1 with a distribution similar to that of normal human tissues. Such a model would simulate the self tolerance against epitopes exposed on normal cells and hence would allow for further evaluation of the immunogenicity of MUC1 peptide vaccines.

Research in the area of MUC1 peptide formulations has been characterized by rapid growth and development with several phase I clinical trials demonstrating the safety of MUC1 peptide formulations in cancer patients (43-46). The ultimate goal in cancer immunotherapy is to develop a formulation with cancer-associated epitopes, which is capable of eliciting an appropriate immune response to confer protection against tumorigenesis.

Studies indicate that antibody responses have little influence on tumor rejection (18), while Th1 type responses correlate with immunity and protection against tumor growth and establishment (18, 26). Our study revealed that MUC1 peptide loaded PLGA microspheres are capable of eliciting specific Th1 responses, which may be enhanced through the use of MPLA. The significance of these results resides in the ability of this biodegradable and biocompatible vaccine formulation to harness the protective capacity of the immune system. In summary the results of this study are very encouraging and foretell a promising future for PLGA microspheres as antigen delivery systems in the field of cancer vaccines.

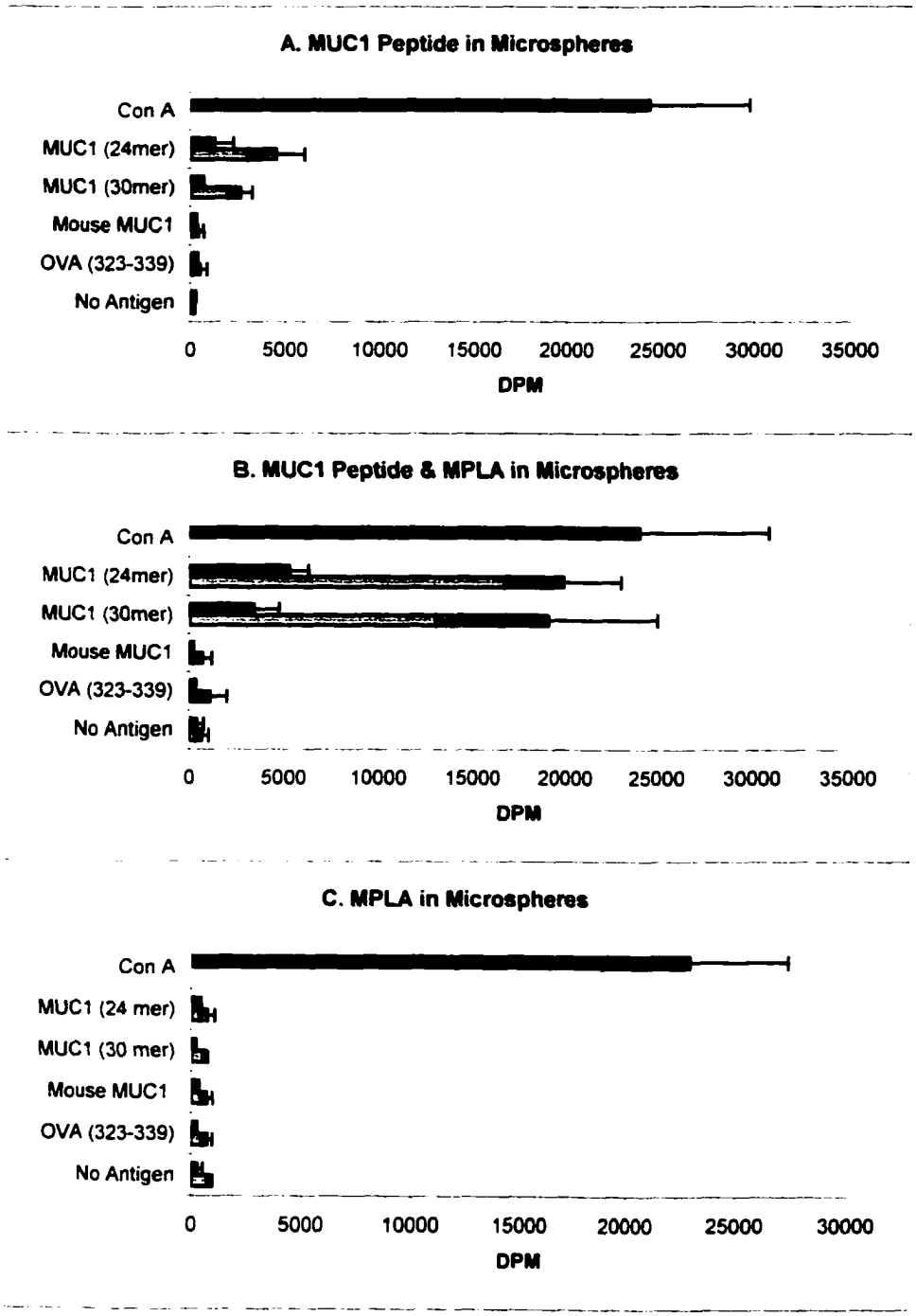

Figure 3-1. Analysis of the T cell proliferation assay of T cells isolated from mice immunized with A) MUC1 peptide in microspheres, B) MUC1 peptide & MPLA in microspheres and C) MPLA in microspheres. The proliferative response at $2X10^5$ T cells/well (dark grey columns) and $5X10^5$ T cells/well (light grey columns) was measured by ^3H- thymidine incorporation.

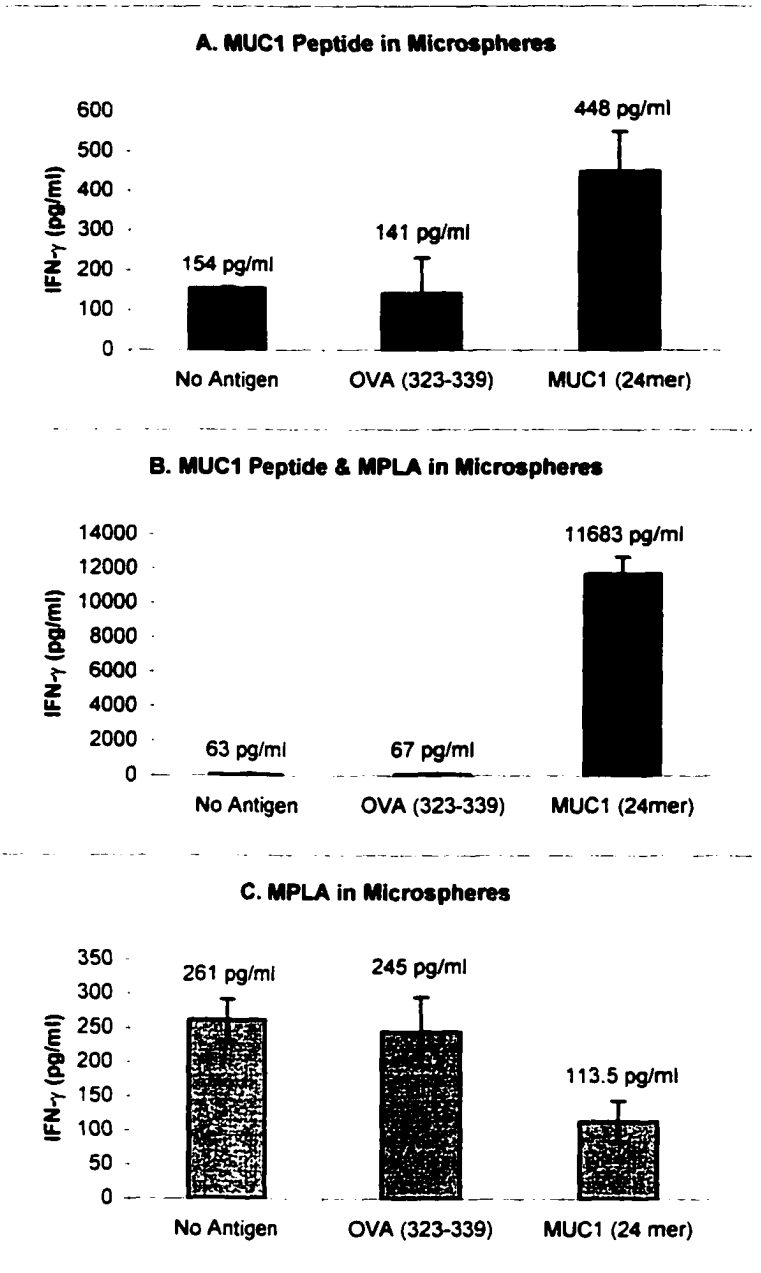

Figure 3-2. IFN-γ production of T cells isolated from mice immunized with A) MUC1 peptide in microspheres, B) MUC1 peptide & MPLA in microspheres and C) MPLA in microspheres. IFN-γ production, measured in pg/ml, was evaluated in the presence of relevant antigen (i.e. MUC1 24 mer peptide) in the presence of irrelevant antigen (i.e. OVA peptide) and in the absence of antigen.

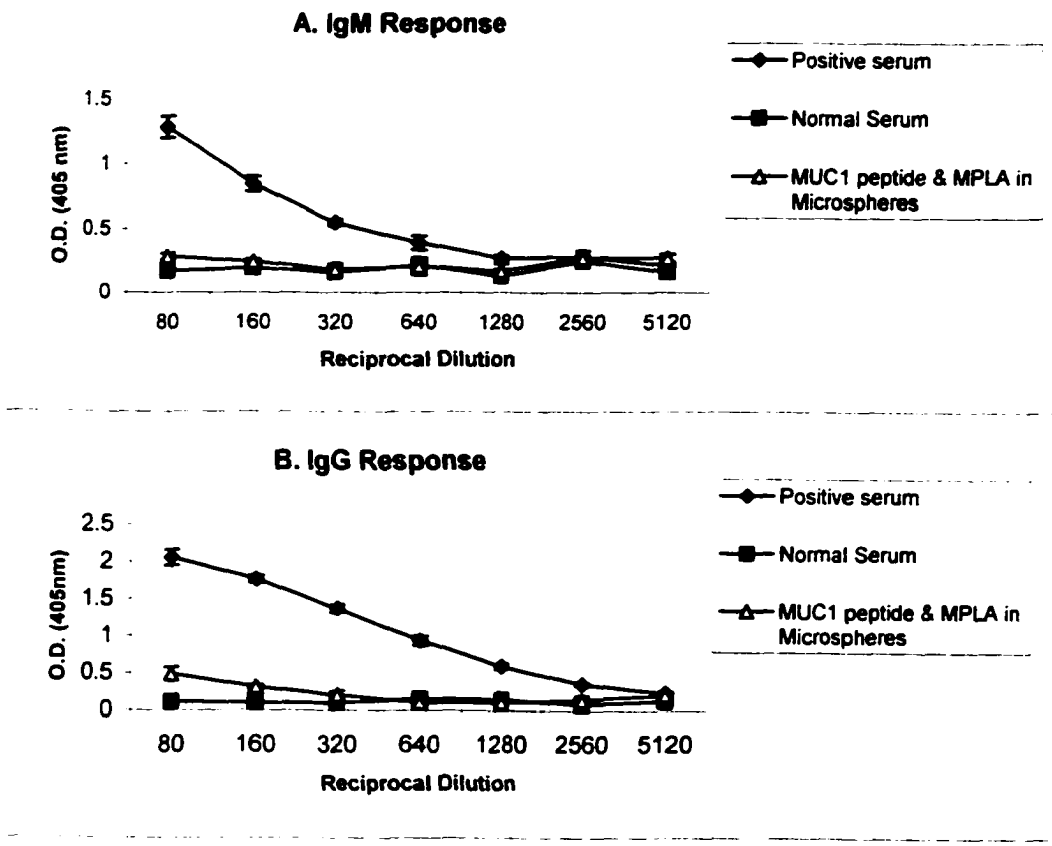

Figure 3-3. A) IgM and B) IgG responses of mice immunized with MUC1 peptide loaded microspheres containing MPLA. Normal serum was obtained from unimmunized mice, while the positive serum was obtained from mice immunized with a MUC1 16 mer peptide (sequence: GVTSAPDTRPAPGSTA) conjugated to KLH. The immunoreactivity of the sera was measured in O.D. readings (background not subtracted).

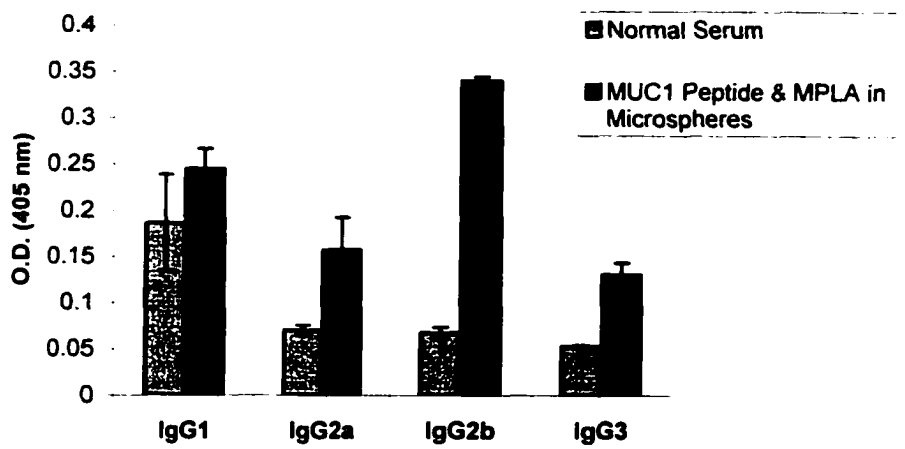

Figure 3-4. IgG isotype profile of serum obtained from mice immunized with MUC1 peptide loaded microspheres containing MPLA. Normal serum was obtained from unimmunized mice. The sera were tested at a 1/80 dilution. The immunoreactivity of the sera was measured in O.D. readings (background not subtracted).

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Chapter 4

Cytoplasmic Delivery of a Macromolecular Fluorescent Probe by Poly(D,L-lactic-co-glycolic acid) Microspheres

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4.1 Introduction

The development of a safe and effective system for the delivery of macromolecules to the cytoplasm of live cells has important applications in the fields of vaccinology and immunotherapy. CTL responses are crucial for the elimination of intracellular pathogens and cancer (1-3). The induction of CTL responses by exogenous antigen requires that the antigen gain access to the cytosol followed by antigen processing and presentation in the MHC class I pathway (4). Although most cells are unable to present exogenous antigen via the MHC class I pathway, both Mϕs (5, 6) and DCs (7) possess this capability. The biocompatible biodegradable nature of PLGA microspheres makes them an attractive system for cytoplasmic delivery and their particulate nature makes them ideal targets for phagocytic cells such as Mϕs and DCs.

Previous research has shown that both protein (8) and peptide (9, 10) loaded PLGA microspheres elicit CTL responses; however, the exact mechanism by which antigen loaded PLGA microspheres induce CTL responses is unknown and the optimal formulation for cytoplasmic delivery is yet to be defined. In our investigation a macromolecular fluorescent probe, Texas red™ labelled dextran, loaded in PLGA microspheres was used as a tool for studying cytoplasmic delivery. It was hypothesized that Texas red™ labelled dextran encapsulated in PLGA microspheres would be delivered to the cytoplasm and thereby provide a means of tracking cytoplasmic delivery. Furthermore, it was postulated that factors such as polymer molecular weight

determine the rate of cytoplasmic delivery. In this study cellular location of the probe was investigated at two different molecular weights of PLGA: 6000 g/mol and 60000 g/mol. Intracellular degradation and processing of Texas red™ dextran loaded PLGA microspheres by mouse peritoneal Mϕs was followed both *in vitro* and *in vivo* over a 7 day period using confocal laser scanning microscopy (CLSM).

4.2 Materials and Methods

4.2.1 Preparation of Microspheres

Texas red™ labelled dextran (mol. wt. 3000 g/mol) (Molecular Probes, Eugene, OR, U.S.A.) was encapsulated in PLGA microspheres (polymer composition 50:50) (BPI) consisting of two different molecular weights of PLGA: 6000 g/mol (inherent viscosity 0.16 dL/g in HFIP) and 60,000 g/mol (inherent viscosity 0.6-0.7 dL/g in HFIP). Briefly, 100 mg of 60,000 g/mol PLGA with 100 μg Texas red dextran and 200 mg of 6000 g/mol PLGA with 200 μg Texas red dextran were used in the preparation of the microsphere formulations as described in section 2.2.1.

4.2.2 Characterization of Microspheres

4.2.2.1 Particle Size

The diameter of the microspheres was determined using dynamic light scattering as described in section 3.2.2.1.2.

4.2.2.2 Loading

The loading of Texas red™ labelled dextran in the PLGA microspheres was determined using a UV spectrophotometer (Ultrospec 3000, Pharmacia

Biotech Ltd., Cambridge, England). Briefly, 15 mg of Texas red™ dextran loaded PLGA microspheres were hydrolyzed in 3 ml of 5 % (w/v) SDS (Sigma, St. Louis, MO) in 0.1M NaOH (BDH Inc.) with overnight stirring. The hydrolyzed sample was then centrifuged and the UV absorbance of the supernatant was measured at 595 nm against a blank consisting of empty PLGA microspheres prepared under the same conditions. The percent loading of Texas red™ dextran was calculated by comparing the UV absorbance of the unknown with a standard curve generated under the same conditions. The percent loading (w/w) is defined as the amount of Texas red™ labelled dextran entrapped per dry weight of microspheres

4.2.2.3 Release

An *in vitro* release experiment was performed to determine the extent of release of Texas red™ dextran from PLGA microspheres over a 24 h period. Briefly, 15 mg of microspheres were placed in a sterile 1.5 ml centrifuge tube. A volume of 1.5 ml of sterile PBS was added to the tube followed by incubation in a shaking water bath at 37 °C, protected from light for 24 hours. At 24 h the supernatant was removed and combined with 1.5 ml of PBS prior to measuring the fluorescence with a spectrofluorometer (Fluoromax™ Spex Industries Inc., Edson, NJ, U.S.A.). The excitation wavelength (λ) was 591 nm and the emission λ was 609 nm. The slit width for excitation and emission was set at 1 mm. A blank consisting of empty microspheres in PBS was prepared in the same manner as the test samples and was subtracted from all the fluorescence readings. The amount of Texas

red™ dextran released was calculated using a standard curve generated under the same conditions.

4.2.3 *In Vitro* Phagocytosis Assay

Female Balb/c mice (Health Sciences Laboratory Animal Services, University of Alberta, Edmonton, Alberta, Canada), 8-12 weeks of age, were used for the *in vitro* phagocytosis assay. Briefly, Balb/c mice were given an intraperitoneal (i.p.) injection of 1 ml of 3 % (w/v) Brewer's thiogylcollate medium (Sigma) in ddH_2O to stimulate the recruitment of Mϕs (11). Seven days after the injection the mice were sacrificed via CO_2 asphyxiation and cervical dislocation. The mouse peritoneal Mϕs were collected by flushing the peritoneal cavity with 5 ml of 5 % (v/v) Hyclone fetal calf serum (FCS) (Gibco BRL, ON, Can.) in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL). The Mϕs were washed once with 5 % (v/v) Hyclone FCS in DMEM followed by centrifugation at 200 g for 7 min. The cells were then resuspended in DMEM supplemented with 1 mM L-glutamine (Gibco BRL), 50 U/ml of penicillin (Gibco BRL), 50 μg/ml streptomycin (Gibco), 75 μg/ml gentamicin (Gibco BRL) and 10% (v/v) Hyclone FCS (Gibco BRL) at a concentration of 4 X 10^5 cells/ml of medium. The cells were placed in 35 mm tissue culture treated Petri dishes (Corning, Corning, NY, U.S.A.) at a concentration of 1 X 10^6 cells/Petri dish.

There were two experimental groups: 1) cells cultured with Texas red™ dextran loaded PLGA microspheres consisting of 6000 g/mol PLGA and 2) cells cultured with Texas red™ dextran loaded PLGA microspheres consisting

of 60,000 g/mol PLGA. Briefly, 1 mg of Texas red™ dextran loaded PLGA microspheres was added to the Mϕ cell culture followed by incubation for 24 h at 37 °C, 5 % CO_2. The Mϕ monolayer was then washed 3X with DMEM followed by the addition of supplemented media to the cell culture and continued incubation at 37 °C, 5 % CO_2. Intracellular degradation of the PLGA microspheres and cellular location of Texas red™ dextran were monitored over a 7 day time course with each time point set up in duplicate. Cell monolayers were fed Texas red™ dextran loaded PLGA microspheres from day –7 to day –1. On day 0 the cells were viewed using confocal laser scanning microscopy (CLSM). Prior to viewing the cells the cytoplasm of the cells was labelled with Cell Tracker™ Green (5-chloromethylflourescein diacetate) (Molecular Probes). The cell cultures were washed once with PBS followed by the addition of 2.5 ml of a 1 μM solution of 5-chloromethylflourescein diacetate in PBS. The cell cultures were incubated for 1 h at room temperature, after which they were washed 3 times with PBS. The cell cultures were then kept in PBS and stored on ice until ready for viewing. This experiment was performed two times and representative data from one experiment is shown.

4.2.4 *In Vivo* Phagocytosis Assay

Female Balb/c mice (Health Sciences Laboratory Animal Services), 8-12 weeks of age, were used during the course of the experiment. Balb/c mice were injected i.p. with 8 mg of Texas red™ dextran loaded PLGA microspheres suspended in 1 ml of PBS. There were two experimental

groups: 1) mice injected i.p. with Texas red™ dextran loaded PLGA microspheres consisting of 6000 g/mol PLGA and 2) mice injected with Texas red™ dextran loaded PLGA microspheres consisting of 60,000 g/mol PLGA. Intracellular degradation of the PLGA microspheres and the cellular location of Texas red™ labelled dextran were monitored over 7 days using CLSM. Mice were injected i.p with the Texas red™ dextran PLGA microspheres from day –7 to day –1. Three mice were injected for each time point in each experimental group. On day 0 the mice were sacrificed via CO_2 asphyxiation and cervical dislocation. The Mϕs were collected by flushing the peritoneal cavity with 5 ml of 5 % (v/v) Hyclone FCS in DMEM. The cells were then washed once with 5 % (v/v) Hyclone FCS in DMEM and resuspended in the supplemented DMEM previously mentioned. The cells were placed in 35 mm tissue culture treated Petri dishes at a concentration of 1 X 10^6 cells/dish. Each time point was set up in duplicate. The cells were incubated for 24 h at 37 °C, 5 % CO_2 to allow for cell attachment. On day +1 the cells were labelled with 5-chloromethylfluorescein diacetate as previously mentioned, followed by viewing using CLSM. This experiment was performed two times and representative data from one experiment is shown.

4.2.5 Confocal Laser Scanning Microscopy

The CLSM images were obtained by simultaneous scanning of the double-labelled specimens using a Leica Confocal Laser Scanning Microscope as previously described (12). The cells were viewed directly in 35 mm Petri dishes, to which a small quantity of mounting medium (50:50, PBS:

glycerol) and a coverslip were added. For uniformity, all physical parameters pertaining to fluorescence illumination and detection were held constant as follows: 63X or 100X oil immersion objective lenses; KP 590 short-pass excitation filter; beam-splitter dual dichroic 488/568; BP535 (fluorescein) barrier filter; LP 590 (Texas red) barrier filter; photomultiplier gain, 800 V; pinhole, 60; offset, -45.

4.3 Results and Discussion

The size of the PLGA microspheres at both molecular weights of PLGA ranged from 2 ± 1 μm in diameter as determined by dynamic light scattering. The loading of the 60,000 g/mol PLGA microspheres was 0.085% w/w of Texas red™ labelled dextran entrapped per dry weight of microspheres, while the loading for the 6000 g/mol PLGA microspheres was 0.050% w/w of Texas red™ labelled dextran entrapped per dry weight of microspheres. The extent of release of Texas red™ labelled dextran from the PLGA microspheres over a 24 h period in PBS at 37°C was 3.6 % for the 60,000 g/mol PLGA microspheres and 4.2 % for the 6000 g/mol PLGA microspheres. Hence, for both formulations the amount of Texas red™ dextran released into the outside medium in 24 h is minimal and should not interfere with the phagocytosis assay.

CLSM was used to monitor cellular location of Texas red™ dextran. The Texas red™ dextran loaded PLGA microspheres appear as punctate red fluorescence in the CLSM images, while the cytoplasm of the macrophages appears green due to labelling with 5-chloromethylflourescein diacetate.

Some of the CLSM images show blebbing of the cells, which may result from cytotoxicity of 5-chloromethylflourescein diacetate. Cytoplasmic delivery was verified by the presence of diffuse yellow fluorescence due to colocalization of fluorescein and Texas red™ in the cytoplasm. The CLSM images for the *in vitro* phagocytosis assay (Fig. 4-1) revealed cytoplasmic delivery on day 1 after phagocytosis for the 6000 g/mol PLGA microspheres (Fig. 4-1A) with minimal cytoplasmic delivery on day 1 for the 60,000 g/mol PLGA microspheres (Fig. 4-1F). On day 2 following phagocytosis the CLSM images revealed cytoplasmic delivery for both the 6000 g/mol (Fig. 4-1B)) and 60,000 g/mol (Fig. 4-1G) PLGA microspheres; however, microsphere degradation appeared to be more rapid for the 6000 g/mol PLGA microspheres as evidenced by the lower number of microspheres present in the cells with concomitant diffuse cytoplasmic labelling. From days 3-7 following phagocytosis of 6000 g/mol PLGA microspheres, the Mϕs exhibited localization of Texas red™ dextran in the cytoplasm (Fig 4-1 C-E, days 6 and 7 not shown). Conversely, on day 3 after phagocytosis of the 60,000 g/mol PLGA microspheres the cells exhibited a small amount of cytoplasmic delivery (Fig. 4-1H) with minimal cytoplasmic delivery on day 4 (Fig. 4-1I) as evidenced by predominant green fluorescence. However, by day 5 following phagocytosis of 60,000 g/mol PLGA microspheres the majority of the cells exhibited strong cytoplasmic delivery of the Texas red™ probe (Fig. 4-1J) with continued delivery on days 6 and 7 (data not shown). Overall cytoplasmic delivery was greater for the cells from the 6000 g/mol PLGA microsphere

group, exhibiting more yellow fluorescence in comparison to the cells from the 60,000 g/mol PLGA microsphere group. A control plate consisting of cells labelled only with 5-chloromethylflourescein diacetate had no red fluorescence (Fig. 4-1K). Furthermore, a plate consisting mouse peritoneal Mϕs cultured in the absence of 5-chloromethylflourescein diacetate and Texas red™ dextran PLGA microspheres exhibited some green autofluorescence with minimal red autofluorescence, typical of unlabelled cells (Fig. 4-1L).

In the *in vitro* phagocytosis assay the Mϕs were in an activated state due to an inflammatory response stimulated by Brewer's thioglycollate medium (13, 14). Consequently, the cellular activity and intracellular degradation processes for these Mϕs may vary significantly from the activity of macrophages under normal *in vivo* conditions. Hence, the second part of the study involved monitoring the intracellular degradation and processing of Texas red™ dextran loaded PLGA microspheres *in vivo* over 7 days using CLSM (Fig. 4-2). On day 1 after phagocytosis of the 6000 g/mol (Fig. 4-2A) and 60,000 g/mol (Fig. 4-2F) PLGA microspheres there was diffuse yellow fluorescence in the cytoplasm. Conversely, on day 2 after phagocytosis both the 6000 g/mol (Fig. 4-2B) and 60,000 g/mol (Fig. 4-2G) PLGA microsphere groups exhibited minimal cytoplasmic delivery. The presence of cytoplasmic delivery on day 1 and a decrease in cytoplasmic delivery on day 2 may be a result of a burst effect involving rapid release of Texas red™ dextran at or near the surface of the PLGA microspheres. In this case the Texas red™

dextran at the surface of the microspheres is rapidly released on day 1; however, by day 2 this probe in the cytoplasm has dissipated and further release of Texas red™ dextran is much slower and dependent on the degradation rate of the polymer. At the polymer molecular weight of 6000 g/mol, cytoplasmic localization of the Texas red™ probe continued from days 3-7 following phagocytosis (Fig. 4-2 C-E, days 6 and 7 not shown); however by day 4 (Fig. 4-2D) all the microspheres had been degraded as evidenced by an absence of punctate red fluorescence. Similar to the 6000 g/mol PLGA microspheres, the macrophages containing 60,000 g/mol PLGA microspheres exhibited cytoplasmic delivery of Texas red™ dextran on days 3-7 following phagocytosis (Fig 4-2 H-J, days 6 and 7 not shown). Overall the 6000 g/mol PLGA microsphere group exhibited stronger yellow fluorescence and hence greater cytoplasmic delivery in comparison to the 60,000 g/mol PLGA microsphere group. Furthermore, the CLSM images also show that the rate of microsphere degradation is more rapid for the 6000 g/mol PLGA microspheres in comparison to the 60,000 g/mol PLGA microspheres. A blank, consisting of mouse peritoneal Mϕs from unimmunized mice labelled with 5-chloromethylflourescein diacetate failed to exhibit any red fluorescence (Fig. 4-2K), while a blank consisting of unlabelled cells isolated from unimmunized mice was devoid of both red and green fluorescence (Fig. 4-2L).

Based on these results it can be concluded that PLGA microspheres are capable of cytoplasmic delivery both *in vitro* and *in vivo*. In comparison to

the *in vitro* phagocytosis assay the *in vivo* assay contains many variables including the dilution of the microspheres *in situ*, the physiological environment such as pH and enzymes and the extent of Mϕ activation elicited by the injection of microspheres. All such factors may affect microsphere degradation, the extent of cytoplasmic delivery and the duration of the fluorescence signal in the cell; however, the goal of our study was to determine cellular localization of the Texas red™ probe and our results clearly show that cytoplasmic delivery of the macromolecular probe is occurring *in vivo*. In comparing the *in vitro* and *in vivo* data the rate of cytoplasmic delivery at a given molecular weight of PLGA is similar under both conditions. More importantly, this study shows that cytoplasmic delivery is affected by the molecular weight of PLGA used in the microsphere formulations. Under both *in vitro* and *in vivo* conditions the kinetics of microsphere degradation and cytoplasmic delivery are more rapid for the 6000 g/mol PLGA microspheres containing Texas red™ dextran than the 60,000 g/mol PLGA microspheres.

Previous studies have focused on the extracellular degradation of PLGA microspheres involving random hydrolysis of backbone esters (15, 16); however, our study focuses on intracellular degradation, which includes exposure to a variety of hydrolytic enzymes and increasingly acidic microenvironments in the endosomal/lysosomal pathway (4). Our results concur with previous research, which indicates that the rate of intracellular degradation of PLGA microspheres is dependent on their bulk properties such as polymer molecular weight (17). To date studies on the intracellular

degradation and processing of particulate antigen and microspheres have been performed *in vitro* (6, 17). This study is the first to look at cytoplasmic delivery of a macromolecular fluorescent probe by PLGA microspheres both *in vitro* and *in vivo*.

An understanding of the processing of PLGA microspheres by macrophages is pertinent for preparing antigen loaded PLGA microspheres optimized for cytoplasmic delivery. In order to elicit a CTL response using antigen loaded PLGA microspheres the antigen must be delivered to the cytoplasm. Previous research has shown that antigen loaded PLGA microspheres are capable of eliciting CTL responses (8-10); however, our study provides physical evidence of cytoplasmic delivery by PLGA microspheres. The results of this study are complimentary to previous studies, which suggest a phagosome-to-cytosol pathway for MHC class I presentation of exogenous particulate antigen (6, 7, 18). Moreover, this study reveals the possibility of modifying formulation parameters for the purpose of manipulating intracellular processing of PLGA microspheres and or optimizing cytoplasmic delivery by PLGA microspheres. Overall, our results clearly demonstrate that PLGA microspheres are capable of cytoplasmic delivery. The development of a tool for studying cytoplasmic delivery and the demonstration of cytoplasmic delivery by PLGA microspheres, has significant implications in the future of this antigen delivery system for the induction of cellular immune responses, specifically CTL responses.

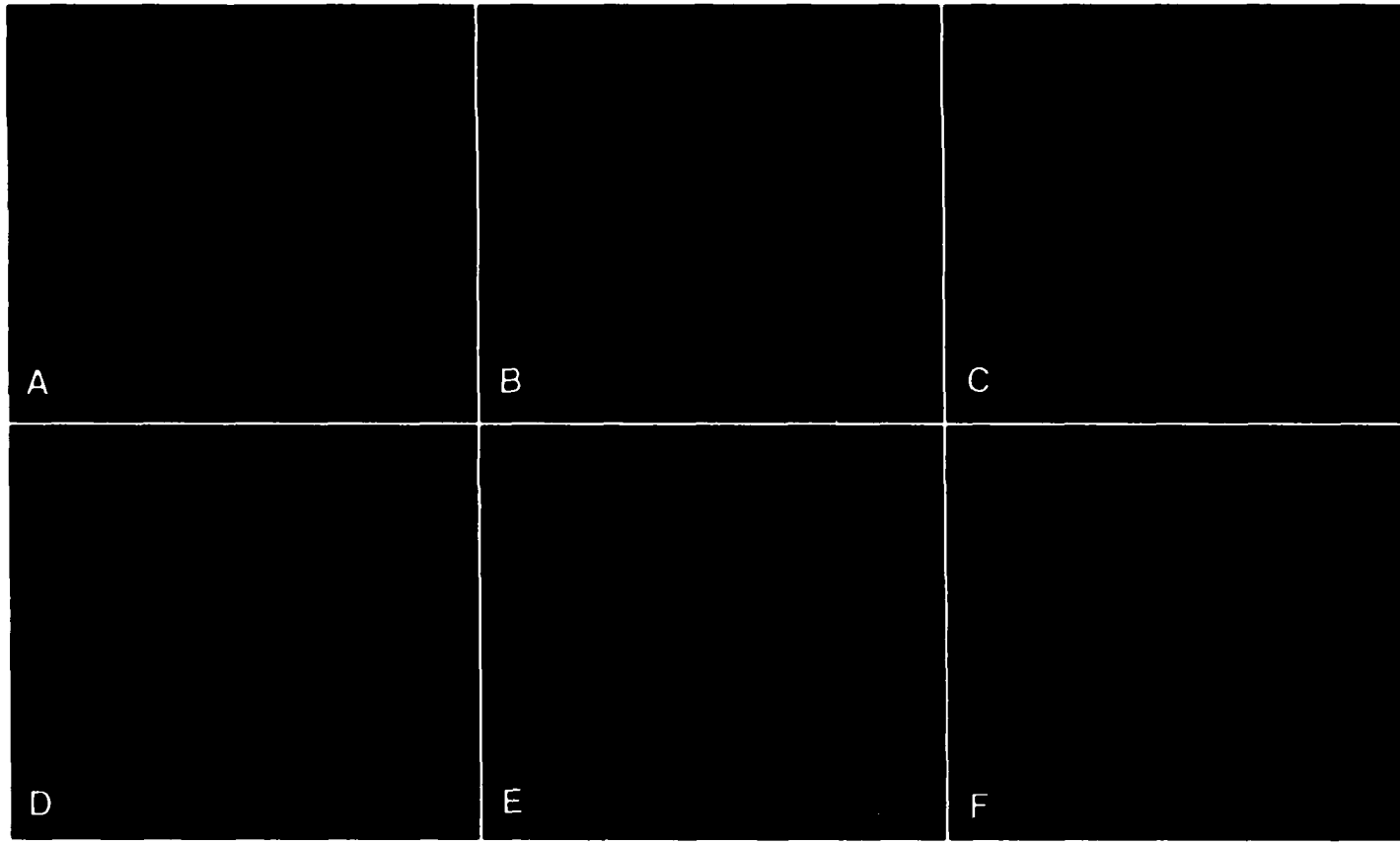

Figure 4-1 (A-F). *In vitro* assay: Cellular localization of Texas red™ dextran on days 1-5 following *in vitro* phagocytosis of Texas red™ dextran loaded PLGA microspheres consisting of 6000 g/mol PLGA (A-E) and 60,000 g/mol PLGA (F-J). The cytoplasm of the cells was labelled with 5-chloromethylfluorescein diacetate. Cytoplasmic delivery of Texas red™ dextran is verified by the presence of diffuse yellow fluorescence. Negative controls consisted of cells cultured in the absence of Texas red™ dextran microspheres both with (K) and without (L) 5-chloromethylfluorescein diacetate. Magnification 63X and 100X.

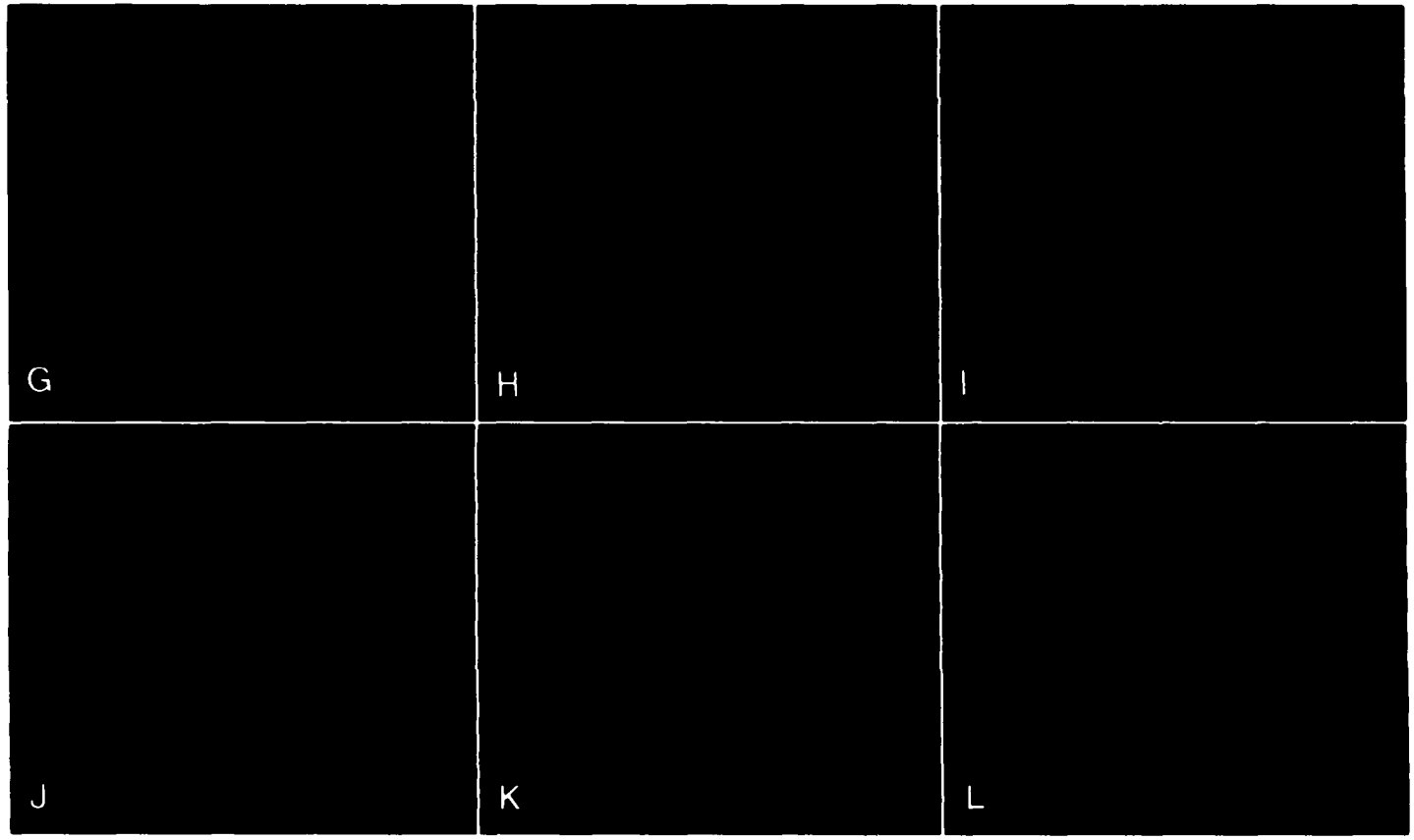

Figure 4-1 (G-L). *In vitro* assay: Cellular localization of Texas red™ dextran on days 1-5 following *in vitro* phagocytosis of Texas red™ dextran loaded PLGA microspheres consisting of 6000 g/mol PLGA (A-E) and 60,000 g/mol PLGA (F-J). The cytoplasm of the cells was labelled with 5-chloromethylfluorescein diacetate. Cytoplasmic delivery of Texas red™ dextran is verified by the presence of diffuse yellow fluorescence. Negative controls consisted of cells cultured in the absence of Texas red™ dextran microspheres both with (K) and without (L) 5-chloromethylfluorescein diacetate. Magnification 63X and 100X.

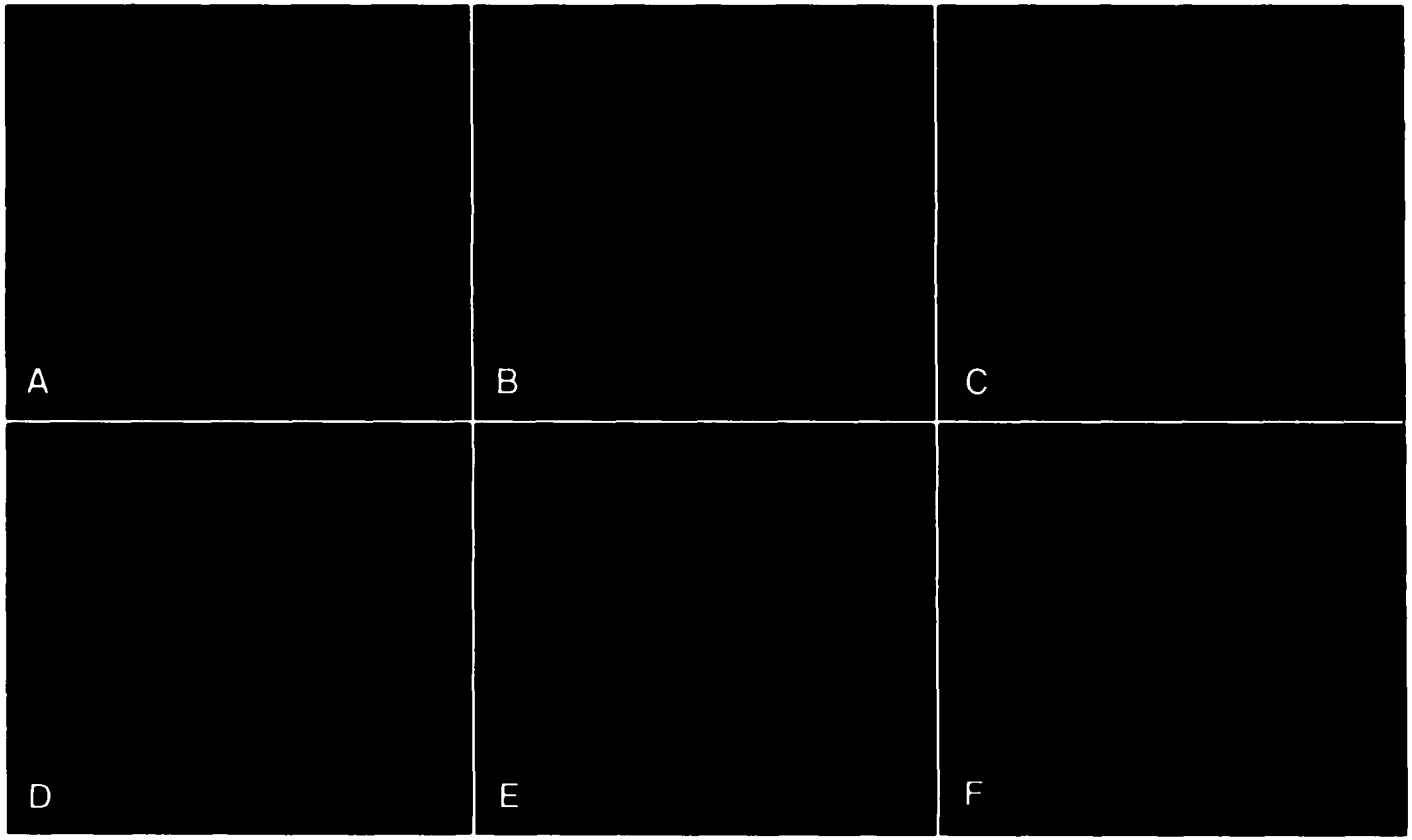

Figure 4-2 (A-F). *In vivo* assay: Cellular localization of Texas red™ dextran on 1-5 days following i.p. immunization with Texas red™ dextran loaded PLGA microspheres consisting of 6000 g/mol PLGA (A-E) and 60,000 g/mol PLGA (F-J). The cytoplasm of the cells was labelled with 5-chloromethylfluorescein diacetate. Cytoplasmic delivery of Texas red™ dextran is determined by the presence of diffuse yellow fluorescence. Negative controls consisted of mouse peritoneal Mϕs isolated from unimmunized mice both with (K) and without (L) 5-chloromethylfluorescein diacetate label. Magnification 100X.

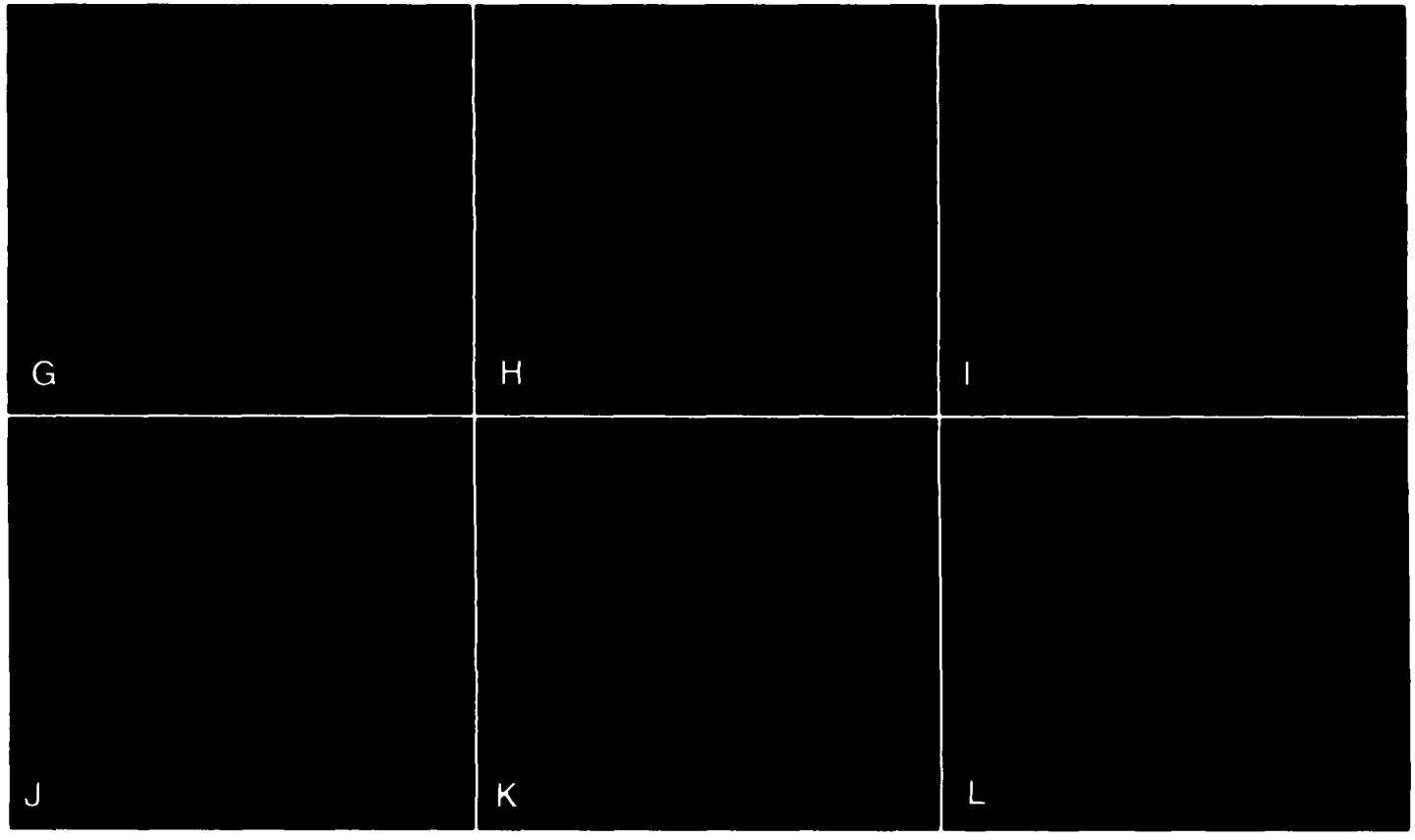

Figure 4-2 (G-L). *In vivo* assay: Cellular localization of Texas red™ dextran on 1-5 days following i.p. immunization with Texas red™ dextran loaded PLGA microspheres consisting of 6000 g/mol PLGA (A-E) and 60,000 g/mol PLGA (F-J). The cytoplasm of the cells was labelled with 5-chloromethylfluorescein diacetate. Cytoplasmic delivery of Texas red™ dextran is determined by the presence of diffuse yellow fluorescence. Negative controls consisted of mouse peritoneal Mϕs isolated from unimmunized mice both with (K) and without (L) 5-chloromethylfluorescein diacetate label. Magnification 100X.

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Chapter 5

Uptake of Poly(D,L-lactic-co-glycolic acid) Microspheres by Antigen Presenting Cells *In Vivo*

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5.1 Introduction

The type of APC taking up the antigen *in vivo* is significant and an important factor to consider when designing an antigen delivery system. Both DCs (1) and Mϕs (2, 3) possess the unique capacity to process and present exogenous antigen including particulate antigen via the MHC class I pathway. DCs are considered the most potent APCs based on their ability to capture and process antigen (4, 5). Furthermore, unlike Mϕ's, DCs constitutively express co-stimulatory molecules such as CD86 making them key professional APCs and the most effective stimulators of primary T cell responses (4, 5).

Past research has demonstrated the potential of PLGA microspheres as antigen delivery systems including studies, which involved microencapsulation of peptide antigen (6-8) (Chapter 2, Chapter 3), immunomodulators (Chapter 2, Chapter 3) and more recently plasmid DNA (9, 10). PLGA microspheres are biocompatible, biodegradable antigen delivery systems and their microparticulate nature make them ideal targets for uptake by APCs. The purpose of this study was to investigate the type(s) of APC taking up the PLGA microspheres *in vivo* and to determine if this cell profile varies with the site of injection or location of the microspheres. A fluorescent probe, tetramethylrhodamine (TMR) labelled dextran was loaded into PLGA microspheres and cellular uptake of the PLGA microspheres was investigated at two injection sites: intraperitoneal (i.p.) and intradermal (i.d.). It was hypothesized that TMR labelled dextran loaded in PLGA microspheres

would be taken up by APCs and thereby provide a means of studying cellular uptake of PLGA microspheres *in vivo*. The cell phenotype of the APCs was established through double-color immunofluorescence analysis using flow cytometry and confocal laser scanning microscopy (CLSM).

5.2 Materials and Methods

5.2.1 Preparation of Microspheres

Tetramethylrhodamine (TMR) labelled dextran (mol. wt. 40000 g/mol) (Molecular Probes) was encapsulated in PLGA microspheres (polymer composition 50:50; mol. wt. 60,000 g/mol; inherent viscosity of 0.6-0.7 dL/g in HFIP) (BPI) using a w/o/w solvent evaporation technique. Briefly, 100 mg of 60000 g/mol PLGA and 1 mg of TMR labelled dextran were used in the preparation of the microsphere formulations as described in section 2.2.1.

5.2.2 Characterization of Microspheres

5.2.2.1 Particle Size

The diameter of the microspheres was determined using dynamic light scattering as described in section 3.2.2.1.2.

5.2.2.2 Loading

The loading of TMR labelled dextran in the PLGA microspheres was determined based on a method previously described in section 4.2.2.2. The UV absorbance of the samples was measured at 555 nm. The percent loading of TMR dextran was calculated by comparing the UV absorbance of the unknown with a standard curve generated under the same conditions.

The percent loading (w/w) is defined as the amount of TMR labelled dextran entrapped per dry weight of microspheres

5.2.3 Intraperitoneal Immunization

Female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), 8 to 12 weeks old, were used during the course of the experiment. Briefly, C57BL/6J mice were injected i.p. with 8 mg of TMR dextran loaded PLGA microspheres suspended in 1 ml of PBS. Twenty-four hours after the injection, the mice were sacrificed via CO_2 asphyxiation and cervical dislocation. The mouse peritoneal cells were collected by performing a peritoneal lavage with 5 ml of 5% (v/v) Hyclone FCS (Gibco BRL, ON, Canada) in Dulbecco's modified eagle's medium (DMEM) (Gibco BRL). The cells were resuspended at a concentration of 2.5×10^5 cells/ml in FACS buffer consisting of 1 % (v/v) FCS and 0.09 % (w/v) NaN_3 (Fisher) in PBS. The cells were then stained for cell surface markers followed by flow cytometric analysis. The data shown are from a representative experiment of 2 independent experiments.

5.2.4 Intradermal Immunization

Female Balb/c mice (Health Sciences Laboratories Animal Services, University of Alberta, Edmonton, Alberta, Canada), 8 to 12 weeks old, were used for the i.d. immunizations. Briefly, the mice were anesthetized with metofane (Janssen Ortho Inc., North York, ON, Canada) and their abdomens were shaved in preparation for the injections. The mice were then given four i.d. injections on the lower abdomen each consisting of 2 mg of TMR dextran

loaded PLGA microspheres suspended in 50 μl of PBS. Twenty-four hours following the injections the mice were sacrificed via CO_2 asphyxiation/cervical dislocation and the inguinal lymph nodes were collected.

DCs were isolated from the draining lymph nodes based on a method described by van Wilsem *et al.* (11). Briefly, the inguinal lymph nodes were broken down into small fragments followed by digestion with collagenase IV (Sigma: 0.5 mg/ml) and DNAse I (Gibco BRL: activity 40 U/ml) in 7.5 ml of RPMI-1640 (Gibco BRL) supplemented with 10 mM Hepes (Gibco BRL) and 2 % FCS (Gibco BRL). Digestion was continued for 30 min at 37 °C with continuous agitation in a shaking water bath. A 0.1M solution of ethylenediaminetetraacetic acid (EDTA) (BDH Inc.) was then added to the digestion mixture at 10% (v/v). The fragments were then resuspended and incubation was continued for 5 min at 37 °C. The resulting cell suspension obtained after enzyme treatment was spun down through 2 ml of FCS/ EDTA (10 ml FCS + 1 ml 0.1M EDTA) at 500 g for 7 min. The pellet was then resuspended in 10 ml of Hank's balanced salt solution (HBSS) (Gibco BRL) / FCS/EDTA, where HBSS (w/o Ca^{2+} Mg^{2+} and phenol red) was supplemented with 25 mM Hepes, 0.2 mg/ml EDTA and 10 % (v/v) FCS/ EDTA (10 ml FCS + 1 ml 0.1M EDTA). The resulting cell suspension was then counted with trypan blue and the cells were resuspended in the HBSS/FCS/EDTA at a concentration of 5 x 10^6 cell/ml. Five ml aliquots of the cell suspension were layered onto 2 ml aliquots of Nycodenz solution (Accurate Chemical and Scientific Corporation, Westbury, NY) (14.5 g Nycodenz/ 100 ml

HBSS/FCS/EDTA) and centrifuged for 20 min at 600 g. Following centrifugation, 1 ml aliquots were removed sequentially from each gradient. A total of seven 1 ml aliquots were removed from each tube. These aliquots were then pelleted and resuspended in 400 μl of FACS buffer. The aliquots were then analyzed for cells containing TMR dextran loaded PLGA microspheres using flow cytometry. DC isolated from the inguinal lymph nodes of unimmunized Balb/c mice were used to set up the gates for flow cytometric analysis. Those aliquots containing cells positive for TMR loaded PLGA microspheres were pooled together and used in the preparation of cytospins. Representative experiments that have been repeated once are shown.

5.2.5 Flow cytometry

Single cell suspensions consisting of 2.5 x 10^5 cells/ml were prepared in FACS buffer. Cells were stained for CD14 (Mϕ marker), CD86 (B7.2; costimulatory molecule) and DEC-205 (NLDC 145; DC marker) (12) cell surface markers. The JAWS II mouse DC line (American Type Culture Collection (ATCC), Manassas, VA) served as a positive control cell line for the DEC-205 cell surface marker.

Prior to incubating with the 1° antibody, cells were preblocked with Fc block^R (Pharmingen) at 0.25 μg/10^6 cells followed by incubation on ice for 3-5 min. Cells that were stained for CD14 were preblocked with mouse IgG (Sigma) at 2.5 μg/10^6 cells followed by incubation on ice for 15 min. The antibody for CD14 was FITC-conjugated rat anti-mouse CD14 IgG1

monoclonal antibody (mAb)(Pharmingen) used at a concentration of 0.25 $\mu g/10^6$ cells. The 1° antibody for CD86 was rat anti-mouse CD86 IgG2a mAb (Pharmingen) used at 0.06 $\mu g/10^6$ cells, while the 1° antibody for DEC-205 was rat anti-mouse DEC-205 IgG2a mAb (a gift from Dr. Beatrice Leveugle, Faculty of Pharmacy, University of Alberta, Edmonton, Alberta, Canada) used at 1 $\mu g/10^6$ cells. The isotype controls for CD86 and DEC-205 (rat IgG2a (anti-KLH) (Pharmingen)) and CD14 (rat IgG1 (Pharmingen)), were prepared at the same concentration as the antibody of interest. Following the addition of the 1° antibody the cells were incubated on ice for 30 min, protected from light. The cells were then washed three times with FACS buffer followed by the addition of a 2° antibody when necessary. The 2° antibody for both CD86 and DEC-205 was fluorescein isothiocyanate (FITC)- conjugated mouse anti-rat IgG2a mAb (Pharmingen) used at a concentration of 0.06 $\mu g/10^6$ cells. Following the addition of the 2° antibody the cells were protected from light and incubated on ice for 30 min. The cells were then washed three times with FACS buffer followed by flow cytometric analysis.

Analytical flow cytometry was performed using a FACScan^R flow cytometer (Becton Dickinson, San Jose, CA) and the data was processed using Cellquest software (Becton Dickinson). FITC (530/30nm) and phycoerythyrin (585/42 nm) band pass filters were used for immunofluorescence analysis of the cell suspensions. The gates for FACS analysis were set to include large cells of high granularity- those cells, which have taken up microspheres.

5.2.6 Cytospin preparations

Cytospins, (consisting of 10000-15000 cells/slide,) were prepared using a Cytospin 3 cytocentrifuge (Shandon Inc., Pittsburgh, PA). Briefly, 1-1.5 x 10^4 cells in 200 µl of PBS were centrifuged onto each microscope slide and allowed to air-dry overnight at 37 °C protected from light. The cytospins were then viewed using bright-field microscopy followed by immunofluorescent staining for DEC-205 and CD86 cell surface markers.

The antibodies for immunofluorescent staining of the cytospins were the same as those used for flow cytometry. When staining for CD86 the slides were blocked with 1 % BSA (Sigma)/PBS and Fc block^R (Pharmingen). Briefly, 20 µl of 1 % BSA/PBS was added to each cytospin followed by a 10 min incubation at room temperature. The blocking buffer was then removed by careful blotting with a piece of fiber-free paper and the Fc block^R was then added at 5 µg/ml, 20 µl/cytospin, followed by incubation at room temperature for 15 min. This blocking agent was then removed by careful blotting with a piece of fiber-free paper followed by the addition of the 1° antibody. All antibody dilutions for CD86 and the corresponding isotype control were prepared using 1 % BSA/PBS. Conversely, when staining for DEC-205 no blocking step was performed and all antibody dilutions were prepared in PBS. The 1° antibody for CD86, rat anti-mouse CD86 IgG2a mAb, was used at 10 µg/ml, while the 1° antibody for DEC-205, rat anti-mouse DEC-205 IgG2a mAb, was used at a concentration of 5 µg/ml. The isotype controls for CD86 and DEC-205 consisted of rat IgG2a (anti-KLH) prepared at the same

concentration as the antibody of interest. Following the addition of the 1° antibody at 20 μl/cytospin, the slides were incubated at 37 °C for 1 h in a humidified container, protected from light. The slides were then washed three times in sterile PBS- 20 min /wash. The 2° antibody for both CD86 and DEC-205, FITC-conjugated mouse anti-rat IgG2a mAb, was used at 60 μg/ml and 30 μg/ml, respectively. A 20 μl volume of the 2° antibody was added to each cytospin followed by a 1 h incubation at 37 °C in a humidified container protected from light. The slides were then washed three times in PBS- 20 min/wash. A small quantity of mounting medium (50:50, PBS: glycerol) and a coverslip were added to each cytospin preparation prior to viewing the samples with CLSM.

Cytospins of irrelevant cells were set up as negative controls. More specifically, the J774A.1 murine Mϕ cell line (ATCC) was used as a negative control when staining for the DEC-205 marker, while the EL4 T lymphocyte cell line (ATCC) was the negative control when staining for the CD86 cell surface marker.

5.2.7 Confocal Laser Scanning Microscopy

The CLSM images were obtained by simultaneous scanning of the double-labelled specimens using a Zeiss 510 NLO Confocal Laser Scanning Microscope (Carl Zeiss Microscope Systems, Jena, Germany). All scans were performed in a multi-tracking mode using a 25 mW Argon laser (488 nm) and a 1 mW Helium Neon (HeNe) laser (543 nm). For uniformity, all physical parameters pertaining to fluorescence illumination and detection were held

constant as follows: 40X oil immersion objective lens with a zoom of 2X; beam-splitter dual dichroic 488/543; LP 505 (fluorescein) barrier filter; LP 560 (Rhodamine) barrier filter; pinhole diameter, 2.00 Airy units.

5.3 Results

The microspheres used in the i.p. immunizations were 5 ± 3 μm in diameter with a loading of 1.427 % w/w of TMR labelled dextran encapsulated per dry weight of microspheres. Following the i.p. immunization, cells collected from the peritoneal cavity were analyzed for microsphere uptake and cell phenotype using flow cytometry. The cell phenotype was established by immunofluorescent staining for cell surface markers including DEC-205 (DC marker) CD86 (costimulatory molecule) and CD14 (Mϕ marker). The JAWS II mouse DC line served as a positive control cell line for DEC-205 (data not shown). Flow cytometric analysis of the peritoneal cells revealed low expression of CD86 with 7 % of the gated population double positive for TMR loaded PLGA microspheres and the CD86 cell surface marker (Fig. 5-1: A1). The isotype control for CD86 showed minimal staining with 2 % of the gated population containing TMR loaded PLGA microspheres and staining for the isotype control (Fig.5-1: A2). FACS analysis further revealed strong expression of CD14 with 46 % of the gated population double positive for TMR loaded PLGA microspheres and the CD14 cell surface marker (Fig.5-1: B1). Conversely, the isotype control for CD14 exhibited minimal nonspecific staining with 3 % of the gated population containing microspheres and staining for the isotype control (Fig.5-1: B2). The expression of DEC-205 on

cells positive for TMR loaded microspheres was negligible where the level of staining was less than that of the isotype control (Fig.5-1: C1, C2). Hence the results reveal that cells taking up PLGA microspheres in the peritoneal cavity are negative for DEC-205 with strong expression of CD14 and low expression of CD86.

The PLGA microspheres used for the i.d. immunizations were 800 ± 200 nm in diameter with a loading of 0.978 % w/w of TMR labelled dextran entrapped per dry weight of microspheres. The PLGA microspheres used for the i.d. immunizations were made slightly smaller than those used for the i.p. immunizations based on both the route of injection and studies by Samuel *et al.*, which revealed that smaller microspheres elicited stronger cellular immune responses than microspheres above 1 μm (unpublished data). Following the i.d. immunization, the draining lymph nodes were collected and the DCs were isolated. Cell counts revealed that the isolated DCs constituted 1 % of the total number of cells in the lymph node cell suspension. Flow cytometric analysis of the aliquots of isolated cells revealed cells positive for TMR dextran loaded PLGA microspheres (Fig 5-2). A2 and B2 in Figure 5-2 represent peak fractions of cells positive for TMR dextran loaded PLGA microspheres, while A1 and B1 represent the corresponding fractions of cells isolated from unimmunized mice. The markers were set based on cells isolated from unimmunized mice (Fig 5-2: A1, B1). Flow cytometric analysis revealed that 11 % of the cells in fraction A were positive for TMR dextran loaded PLGA microspheres (Fig 5-2: A2), with a background fluorescence of

2 % (Fig 5-2: A1). In fraction B 7 % of the gated cells were positive for TMR dextran loaded PLGA microspheres with a background fluorescence of 2 % (Fig 5-2: B1).

The aliquots containing cells positive for TMR dextran loaded PLGA microspheres were used in the preparation of cytospins followed by double-color immunofluorescence analysis using CLSM. Cytospin preparations were stained for DEC-205 and CD86 cell surface markers. The cytospins contained cells exhibiting typical dendritic cell morphology with veils /dendrites as determined by bright-field microscopy (data not shown) and CLSM (Fig. 5-3A). The TMR dextran loaded PLGA microspheres appeared as punctate red fluorescence while the labels for the cell surface markers appeared green. The CLSM images clearly showed that the cells containing TMR loaded PLGA microspheres were positive for DEC-205 (Fig 5-3A). The DEC-205 isotype control revealed some weak non-specific labeling of the cytospins (Fig 5-3B); however, the fluorescence intensity was less than that of the DEC-205 labelled cytospins. Immunofluorescence analysis further demonstrated that the microsphere-positive cells exhibited expression of CD86 (Fig 5-3C). There was weak labeling with the CD86 isotype control (Fig 5-3D); however, the fluorescence intensity was less than that for the CD86 labelled cytospins. Images were also taken of unlabelled cells both with (Fig 5-3E) and without (Fig 5-3F) TMR dextran loaded PLGA microspheres. The unlabelled cells exhibited some red autofluorescence with minimal green autofluorescence. Furthermore, cytospins of irrelevant cells, J774A.1 murine

Mϕs and EL4 murine T lymphocytes, were negative for DEC-205 and CD86, respectively (data not shown).

5.4 Discussion

The type of APC taking up antigen *in vivo* has a significant effect on the outcome of the immune response. DCs are the most potent APCs and the most effective at eliciting primary T cell responses (4, 5). The goal of this study was to investigate the type(s) of APC taking up PLGA microspheres *in vivo* at different injection sites: i.p. and i.d.. For the i.p. immunization, flow cytometric analysis of the peritoneal cells revealed cellular uptake by cells negative for DEC-205 with weak expression of CD86 and strong expression of CD14 - a cell phenotype indicative of Mϕs. Hence, based on this study it was concluded that the predominant cell taking up TMR dextran loaded PLGA microspheres in the peritoneal cavity was the Mϕ.

Based on the large population of DCs (i.e. Langerhans cells) in the skin, the second injection site that was explored was the intradermal route. It was postulated that an i.d. immunization would promote cellular uptake of PLGA microspheres by DCs. Following the i.d. immunization, DCs isolated from draining lymph nodes were analyzed for cellular uptake of TMR dextran loaded PLGA microspheres using flow cytometry. Cell phenotype and morphology of the cells positive for TMR dextran loaded PLGA microspheres were determined by double-color immunofluorescence analysis using CLSM. This study revealed that cells containing microspheres exhibited typical DC morphology (i.e. veils) and were clearly positive for both DEC-205 and CD86 -

a cell phenotype indicative of DCs. Furthermore, the level of expression of cell surface markers provides information on the activation and maturation state of the cells. More specifically DEC-205 is expressed on immature DCs with increased expression on mature DCs (13), whereas CD86 is more strongly expressed on mature activated DCs (13, 14). The focus of this study was to establish the cell type taking up PLGA microspheres *in vivo* and further study is required to determine the extent of activation and maturation state of the cells following uptake of PLGA microspheres.

In comparing the results of the i.d. and i.p. immunizations, the i.d. immunization resulted in uptake of PLGA microspheres by DCs while the i.p. injection resulted in the ingestion of PLGA microspheres by Mϕs. Hence, these results suggest that the profile for cellular uptake varies with the site of injection and that targeted delivery to specific APCs (i.e. DCs) may be achieved based on the route of administration. It has long been demonstrated that PLGA microspheres can be phagocytosed by Mϕs both *in vitro* and *in vivo* (15, 16) (Chapter 4). Based on the potency of DCs as APCs one of the goals of our study was delivery of PLGA microspheres to DCs and the results of the i.d. immunization clearly demonstrate uptake of PLGA microspheres by DCs *in vivo*. Moreover, this investigation strongly suggests that DCs have migrated from the skin to draining lymph nodes following uptake of PLGA microspheres. Alternatively, it is possible that free microspheres could pass through the lymphatic system to draining lymph nodes where they are taken

up by DCs. Hence, further study is required to determine the location of cellular uptake of microspheres following an i.d. immunization.

This study on cellular uptake following i.d. immunization concurs with previous research by Lunsford *et al.* (17), which demonstrated uptake of PLGA microspheres by DCs *in vivo* following intravenous (i.v.) and subcutaneous (s.c.) administration. In the Lunsford study the DCs were analyzed for fluorescent PLGA microspheres using FACS. Conversely, in this study cellular uptake of PLGA microspheres by DCs was determined using flow cytometry followed by a more detailed investigation on cell morphology and phenotype of the microsphere-positive cells through double-color immunofluorescence analysis using CLSM. Both the FACS data and the CLSM images revealed uptake of PLGA microspheres by DCs following i.d. immunization. Furthermore, this study established the cell phenotype of the microsphere-positive DCs based on cell staining for CD86 and DEC-205 cell surface markers, both of which are relevant to the maturation and activation state of DCs (13, 14). Hence this work is complementary to the Lunsford study (17); however, it also provides crucial information with regards to cell phenotype. More importantly, this study provides conclusive evidence of uptake of PLGA microspheres by DCs *in vivo*.

The unique ability of DCs to take up antigen and activate naïve T cells makes them an attractive target for vaccines. In the field of cancer immunotherapy studies have demonstrated that DCs, including dermal DCs and Langerhans cells, when pulsed with tumor-associated antigen (TAA)

generate tumor-specific immune responses and anti-tumor activity (18, 19). Furthermore, clinical trials involving *in vivo* immunization of TAA-pulsed DCs have shown promising results in human cancers (18, 20). These studies demonstrate the potency of DCs in antigen presentation and suggest an important role for DCs in combating diseases such as cancer.

Based on the strong influence of the APC on the immune response we investigated cellular uptake of PLGA microspheres *in vivo*. In this study uptake of PLGA microspheres by APCs occurred at both i.p. and i.d. injection sites without the use of adjuvants or immunomodulators thereby demonstrating the adjuvant activity of this antigen delivery system. More importantly, this study provides direct and conclusive evidence of uptake of PLGA microspheres by DCs *in vivo*. Hence, PLGA microspheres represent a safe biodegradable, biocompatible formulation that may be used for targeted delivery to APCs and more importantly DCs.

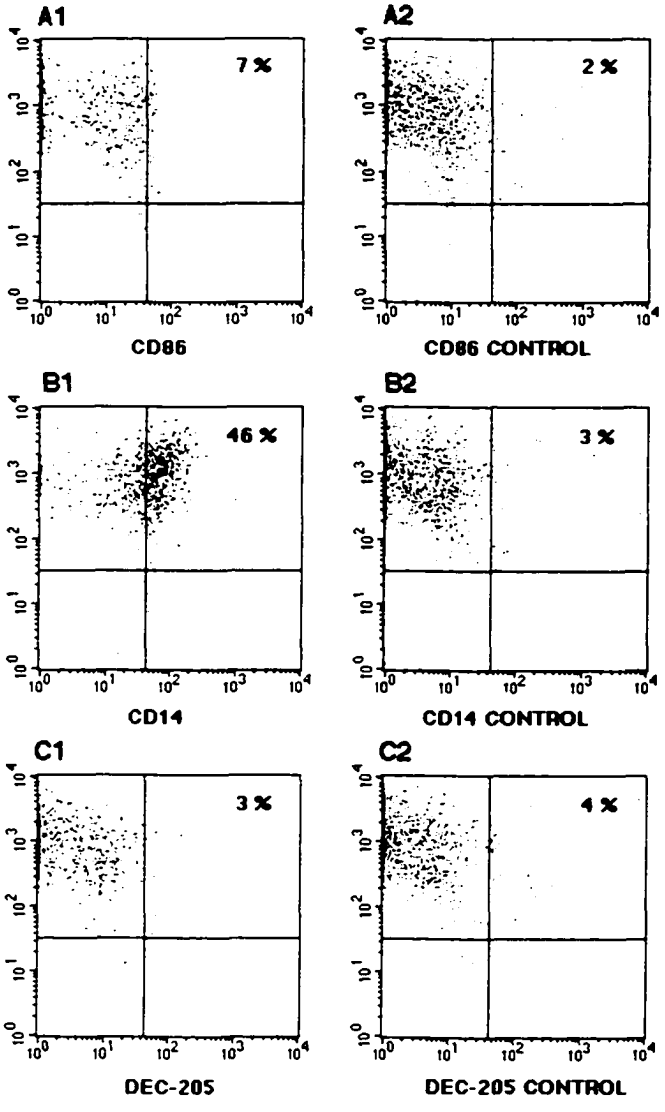

Figure 5-1. Cell phenotype of microsphere–positive cells isolated from the peritoneal cavity of mice following i.p. immunization with TMR dextran loaded PLGA microspheres. The phenotype was established through double-color immunofluorescence analysis using flow cytometry. The cells were stained for cell surface markers: A1) CD86, A2) CD86 isotype control, B1) CD14, B2) CD14 isotype control, C1) DEC-205 and C2) DEC-205 isotype control. Profiles were gated on microsphere-positive cells. Numbers within the dot plots represent percentages within the quadrants.

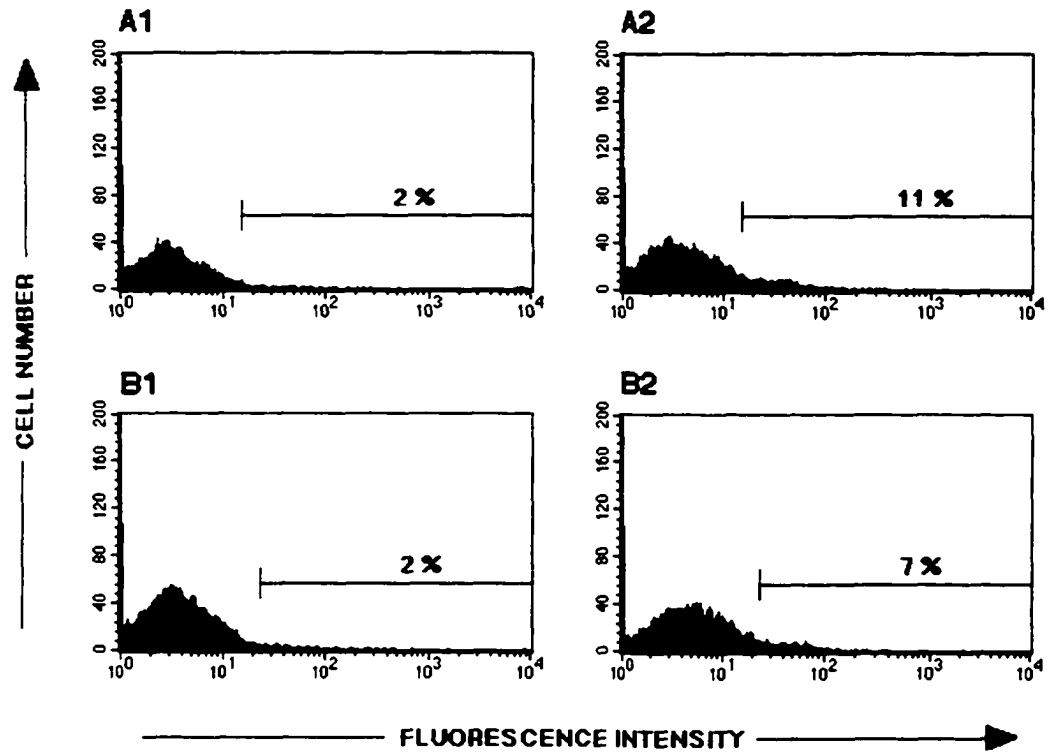

Figure 5-2: Single-color immunofluorescence analysis of DCs isolated from the draining lymph nodes of mice following i.d. immunization with TMR dextran loaded PLGA microspheres. A2 and B2 represent peak fractions of microsphere-positive cells isolated from immunized mice, while A1 and B1 are the corresponding fractions of unlabelled DCs isolated from unimmunized mice. Fractions A2 and B2 were analyzed for cellular uptake of TMR dextran loaded PLGA microspheres. The profiles were gated on large cells of high granularity. Markers were set based on DCs isolated from lymph nodes of unimmunized mice (A1 and B1). The frequency of fluorescent cells and the fluorescence intensity of the cells were evaluated by flow cytometry. The percentage of fluorescent cells in each fraction is indicated above the marker.

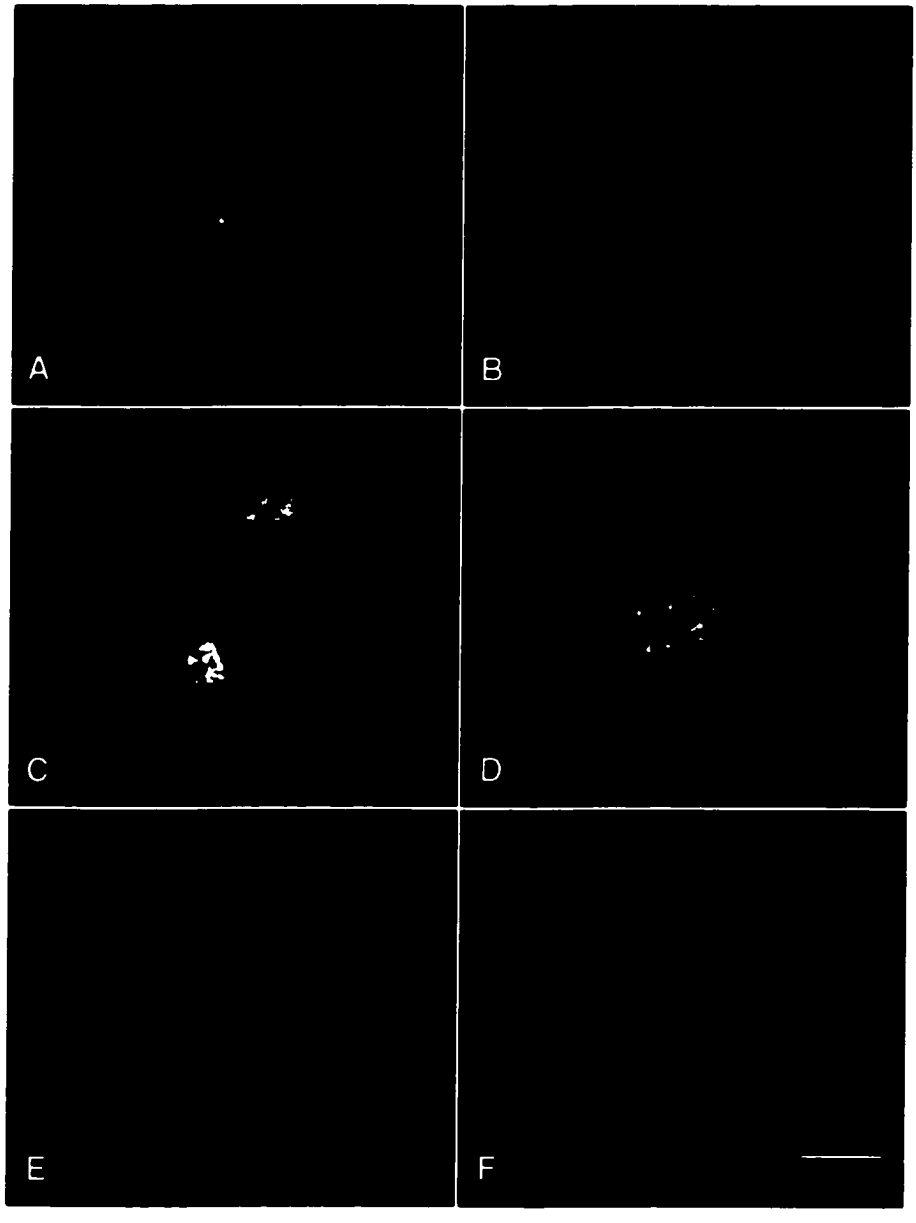

Figure 5-3. Cellular uptake of PLGA microspheres by DCs *in vivo*. The cytospins were stained for cell surface markers: A) DEC-205, B) DEC-205 isotype control, C) CD86 and D) CD86 isotype control. In the CLSM images the microspheres appear as punctuate red fluorescence while the cell surface markers appear as green fluorescence. Negative controls consisted of unlabelled cells both with E) and without F) TMR dextran loaded PLGA microspheres. Magnification 40X, zoom 2X.

5.5 References

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Chapter 6

General Discussion and Conclusions

6.1 Historical Perspective

Early developments in the field of PLGA microspheres focused on the biocompatible nature and controlled release properties of the polymer PLGA (1-5). One of the primary interests was in formulating a single administration vaccine by manipulating the bulk properties of PLGA (i.e. molecular weight and copolymer ratio) to tailor rates of bioerosion and release. More specifically, a single-injection vaccine for neonatal tetanus in Third World countries was and continues to be a primary focus for PLGA microsphere-based vaccines (6, 7). However, this vaccine aside, the full potential of PLGA microspheres as antigen delivery systems is only beginning to be realized with vast applications in synthetic peptide (8-11) and recombinant subunit vaccines (12, 13) as well as DNA vaccines (14, 15).

More recent interest in the adjuvant activity of PLGA microspheres has prompted many studies on the immunogenicity of antigen loaded PLGA microspheres. Earlier studies evaluated antibody responses elicited by PLGA microsphere formulations (8, 16-21); however, current research has focussed on cell-mediated immune responses including Th (12, 16, 18, 20, 22) and CTL responses (9, 10, 12, 14, 20, 23), both of which are necessary for combating intracellular pathogens and cancer. As the type of immune response generated by an antigen delivery system will determine host immunity to infection and disease, exploration and study of PLGA microspheres as antigen delivery systems is necessary to understanding their

mechanisms of action and engineering PLGA microspheres capable of eliciting the desired immune responses.

6.2 General Discussion

Early studies on PLGA microspheres investigated antibody responses elicited by microencapsulated protein antigen including tetanus toxoid and model antigens such as OVA and BSA (13). However, advances in immunology and biochemistry allowed for mapping of immunodominant epitopes and synthesis of the constituent peptides. Such peptide antigen has several advantages over protein antigen including enhanced stability based on its simple structure and focussing the immune response against relevant epitopes (24). Unfortunately peptide antigen due to its rapid degradation in vivo is poorly immunogenic (25, 26), requiring adjuvants or microencapsulation to improve its immunogenicity. A study by Ulunma Imo tested peptide stability in human serum comparing the half-life for soluble OVA peptide, residues 257-264, to OVA peptide (257-264) encapsulated in PLGA microspheres (27). The results revealed over a 100-fold increase in peptide half-life for the microencapsulated OVA peptide (257-264). This increase in half-life can be attributed to the adjuvant activity of the PLGA microspheres, where the peptide is protected from enzymatic degradation under physiological conditions.

Previous studies on peptide antigen loaded in PLGA microspheres revealed the capacity of microencapsulated peptide to elicit antibody responses (8), CTL responses (9, 10, 23) as well as T cell proliferative

responses (8). In Chapter 2 a model peptide antigen, OVA peptide, was encapsulated in PLGA microspheres to study the specificity and type of Th response elicited by peptide loaded PLGA microspheres. This OVA peptide contains both T and B cell epitopes (28) and is capable of eliciting both Th1 and Th2 responses. It was hypothesized that microencapsulation would avoid the need for traditional adjuvants and bias the immune response towards a Th1 response - a response required for the eradication of intracellular pathogens and cancer (29-31). Furthermore, an immunomodulator, MPLA, was incorporated into the peptide loaded PLGA microspheres to determine its efficacy in enhancing Th1 responses. The results revealed that OVA peptide loaded PLGA microspheres elicited antigen-specific T cell responses with a cytokine profile suggestive of a Th1 response. Moreover, MPLA appeared to enhance the Th1 response based on an increase in both the T cell proliferative responses and IFN-γ production. Hence, it was concluded that peptide loaded PLGA microspheres are capable of biasing the immune response towards a Th1 response.

The ability of peptide loaded PLGA microspheres to elicit Th1 responses makes it an attractive system for immunotherapy in the treatment and prevention of cancer. The immunotherapeutic approach is based on eliciting immune responses against antigens expressed by cancer cells. Human MUC1 mucin is a cancer-associated antigen, which is highly expressed on breast, pancreatic, ovarian and colon carcinomas (32-36). In cancer cells altered glycosylation of MUC1 mucin results in the exposure of

core peptide sequences that may be used as cancer-associated epitopes (34, 37). In Chapter 3 a cancer vaccine candidate, MUC1 mucin peptide was incorporated into PLGA microspheres to evaluate the specificity and type of Th response elicited by the microsphere formulation. This MUC1 peptide consists of 24 amino acids corresponding to the variable tandem repeat region of MUC1 mucin, and contains both T and B cell epitopes (38). The results revealed a T cell specific immune response with cytokine and antibody profiles indicative of a Th1 response. Moreover, the incorporation of MPLA into the MUC1 peptide loaded PLGA microspheres increased the magnitude of the Th1 response. Studies using the same MUC1 peptide in a liposomal formulation demonstrated tumor protection with a Th1 response (39). Based on the ability of MUC1 peptide loaded PLGA microspheres to elicit Th1 responses further studies should involve testing this formulation in murine tumor models - more specifically MUC1 transgenic mice (40).

A liposomal formulation of MUC1 peptide is now in clinical trials for the treatment of breast cancer; however, several MUC1 peptide formulations have shown promising results with phase I clinical trials demonstrating the safety of these formulations in cancer patients (31, 41-43). Recently published clinical trials include a MUC1-KLH conjugate plus QS-21 (44). This formulation was immunogenic and well tolerated in cancer patients; however the immune response was primarily an antibody response with no evidence of T cell activation. As cellular immune responses are generally required for eradication of tumor cells (31), a formulation capable of biasing the immune

response towards a Th1 response would be beneficial in the treatment and prevention of cancer. The results in Chapter 3 demonstrate the capacity of MUC1 peptide loaded in PLGA microspheres to elicit Th1 responses. Such results are promising and warrant further study of PLGA microspheres as antigen delivery systems in cancer vaccines and immunotherapy.

In addition to Th responses, CTL responses are an integral component of cell-mediated immunity and are crucial for combating intracellular pathogens and cancer (45-47). The induction of CTL responses by exogenous antigen requires that the antigen gain access to the cytosol followed by antigen processing and presentation in the MHC class I pathway (48). Studies have demonstrated that antigen loaded PLGA microspheres are capable of eliciting CTL responses (9, 10, 20, 23); however, the exact cellular mechanisms involved in this process remain unknown. The study in Chapter 4 tracked cellular location of a macromolecular fluorescent probe encapsulated in PLGA microspheres following phagocytosis by murine peritoneal Mϕs. This study demonstrated that PLGA microspheres are capable of cytoplasmic delivery and that delivery to the cytosol can be controlled by modifying polymer molecular weight. Moreover, this investigation unveiled one of the key steps for MHC class I presentation of exogenous antigen (i.e. cytoplasmic delivery) and is complementary to earlier studies on the cross presentation of exogenous antigen in the MHC class I pathway (49-52). Such studies on processing of PLGA microspheres are crucial for understanding the mechanisms of action of this delivery system

and furthermore, are necessary in optimizing PLGA microspheres for eliciting CTL responses.

Cellular immune responses generally require that the antigen be processed and presented by APCs, which include B cells, DCs and Mϕs (48). There is accumulating evidence suggesting that the type of APC taking up and processing antigen has a strong influence on the immune response. Research has shown that both Mϕs and DCs are capable of processing and presenting exogenous antigen, including particulate antigen in the MHC class I pathway (49-51, 53). Moreover, MHC class I presentation of exogenous antigen is enhanced when the antigen is in particulate form and internalized via phagocytosis (49, 50, 53). The APC that is currently receiving much attention is the DC. Unlike Mϕs, DCs constitutively express high levels of costimulatory molecules making them highly potent APCs and the most effective stimulators of primary T cell responses (54, 55).

Based on the strong influence of the APC on the immune response, a study was undertaken to investigate cellular uptake of PLGA microspheres by APCs *in vivo* (Chapter 5). The objective of the study was to determine the type(s) of APC taking up PLGA microspheres at different injection sites: i.p. and i.d. Following an i.p. immunization the predominant cell taking up microspheres in the peritoneal cavity was the Mϕ while an i.d. immunization resulted in uptake of PLGA microspheres by DCs. The exact influence of the injection site on the cellular uptake requires further investigation beyond the scope of this project; however, these results strongly suggest that the profile

for cellular uptake varies with the site of injection and that delivery to specific APCs may accomplished based on the route of administration. Based on this study it was concluded that PLGA microspheres can be used for targeted delivery to APCs without the use of adjuvants or immunomodulators. More importantly, this is the first study to provide direct and conclusive evidence of uptake of PLGA microspheres by the most potent APC, the DC.

The role of DCs in priming immune responses has lead to many studies on the use of DCs in cancer immunotherapy (55-59). Moreover, clinical trials using antigen loaded DCs administered as a therapeutic vaccines to cancer patients have shown promising results (56, 57). The clinical data reveals the influence of DCs on the immune response and suggests an important role for DCs in combating diseases such as cancer. Hence, targeted delivery of antigen to DCs may represent a key factor in the design of an effective vaccine. The study in Chapter 5 clearly demonstrates delivery of PLGA microspheres to DCs. The biocompatible biodegradable nature of PLGA microspheres in addition to its versatility makes it an attractive system for antigen delivery to DCs and represents a viable option to pursue in the design of future vaccines.

PLGA microspheres as antigen delivery systems show a strong capacity for eliciting cellular immune responses. With further study and exploration of this system it is expected that PLGA microspheres will have a significant impact on the development of vaccines against diseases which would benefit from cellular immune responses including HIV and cancer. The

full potential of PLGA microspheres has yet to be exploited; however with continuing interdisciplinary studies based on pharmaceutics and immunology, PLGA microspheres as vaccine delivery systems may one day become a reality.

6.3 Conclusions

1. Peptide loaded PLGA microspheres are capable of biasing the immune response towards a Th1 response. Both the OVA and MUC1 peptide studies revealed that peptide loaded PLGA microspheres are capable of eliciting antigen-specific T cell responses. The cytokine and antibody profiles for the peptide loaded PLGA microspheres strongly suggest the presence of Th1 responses. Moreover, OVA peptide in alum failed to prime a T cell response, thereby demonstrating the weak immunogenicity of the peptide and its rapid degradation *in vivo*.
2. MPLA enhances Th1 responses when incorporated with peptide loaded PLGA microspheres. Incorporation of MPLA in the peptide loaded PLGA microspheres enhanced the T cell proliferative response and resulted in increased IFN-γ production; however MPLA alone in microspheres was nonimmunogenic failing to elicit any T cell response.
3. MUC1 peptide derived from human MUC1 mucin, an immunosuppressive cancer-associated antigen, elicits antigen-specific Th1 responses when encapsulated in PLGA microspheres.

4. Texas red™ labelled dextran encapsulated in PLGA microspheres can be used as a model for studying cytoplasmic delivery of antigen under both *in vitro* and *in vivo* conditions.
5. PLGA microspheres are capable of cytoplasmic delivery. Moreover, delivery to the cytoplasm can be controlled by modifying formulation parameters, specifically polymer molecular weight. Both *in vitro* and *in vivo* studies revealed that the kinetics of microsphere degradation and cytoplasmic delivery were more rapid for the 6000 g/mol PLGA microspheres than the 60,000 g/mol PLGA microspheres.
6. PLGA microspheres can be delivered to the most potent APC, the DC, *in vivo*.
7. PLGA microspheres can be used for targeted delivery to APCs without the use of adjuvants or immunomodulators. The study on cellular uptake of PLGA microspheres *in vivo* demonstrated uptake of microspheres by Mϕs and DCs following i.p. and i.d. administration, respectively.

6.4 Future Perspectives

Over the past decade PLGA microspheres have been studied extensively for their applications in drug delivery and vaccine technology. Such research has produced several PLGA-based sustained release formulations of peptides (i.e. luteinizing hormone-releasing hormone (LH-RH) analogs) currently on the market including Lupron Depot®, Zoladex® and Decapeptyl SR® (60). These LH-RH formulations have found applications in the treatment of endometriosis, uterine fibroids as well as prostate and breast

cancers (61). Despite this success the development of a PLGA-based vaccine continues to be a much slower process. The World Health Organization (WHO) Special Programme for Vaccine Development launched in the late 1980s had a major impact on the research and development of PLGA microsphere-based vaccines- specifically a single administration vaccine for neonatal tetanus in the Third World (6). However in over 10 years of research in this field, published clinical trails on microsphere-based vaccines remain few (62).

With the recent focus on the adjuvant activity of PLGA microspheres, this delivery system is now being considered relevant for conventional, recombinant and synthetic subunit vaccines as well as DNA vaccines. PLGA microspheres have proven to be suitable for a large variety of protein and peptide antigens from various sources and having different structures (8-13, 16-22, 63, 64). This feature opens up prospects for incorporating two or more antigens into a multivalent microsphere preparation. Although such research is in its infancy, previous studies on diptheria toxoid and tetanus toxoid multivalent PLGA microsphere vaccines show promising results (65, 66). Future possibilities could include the development of a multivalent single-administration PLGA microsphere vaccine. Furthermore, the formulation of a non-invasive PLGA-based microsphere vaccine specifically a nasal or oral vaccine is under intense exploration (11, 67-69). The development of such vaccines would have enormous socio-economic implications with improved

patient compliance, reduced costs of administration as well as reduced morbidity and mortality.

The emergence of plasmid DNA vaccines in the early 1990s had a major impact on the field of vaccinology (70). Plasmid DNA offers many advantages over protein and peptide antigen. Based on its ability to mediate production of endogenous antigen, it is expected that plasmid DNA will induce superior CTL responses in comparison to peptide or protein antigen. However as research continues to expose the limitations of naked DNA injections (71, 72) and the need to improve efficiency such vaccines, microspheres are now gaining attention as potential delivery systems for DNA vaccines. A study by D. Wang *et al.* demonstrates that PLGA microspheres can deliver intact functional plasmid DNA at controlled rates and that these microspheres may be used jointly to deliver genes and immunomodulators to APCs (15). Moreover plasmid DNA encapsulated in PLGA microspheres has been shown to elicit CTL responses (14). Such results are promising and demonstrate a strong future for PLGA microspheres in the rapidly expanding field of DNA vaccines.

The effectiveness of a vaccine is determined in part by its ability to elicit the appropriate immune response - an antibody-mediated immune response for the eradication of extracellular pathogens (73-75) and a cell-mediated immune response for the elimination of intracellular pathogens and cancer (29-31, 76, 77). PLGA microspheres have been studied extensively for their ability to elicit humoral immune responses (8, 16-21); however with the

challenges presented by diseases such as HIV and cancer, current research has shifted focus towards the development of PLGA microsphere formulations for the induction of cellular immune responses (i.e. Th1 and CTL responses). There are many studies demonstrating the capacity antigen loaded PLGA microspheres to elicit cellular immune responses including CTL responses as well as Th1 responses (9-12, 14) (see chapters 2 and 3). The ability of PLGA microspheres to induce cellular immune responses makes it an attractive system for cancer vaccines. The study in chapter 3 clearly demonstrates the capacity of MUC1 loaded PLGA microspheres to elicit Th1 responses and serves as a solid foundation for further study of this formulation in the treatment and prevention of cancer. In addition to cancer-associated antigens, coencapsulation of antigen with Th1-related cytokines to enhance the cellular immune response remains another area of intensive exploration for cancer vaccines. Previous attempts to encapsulate IFN-γ and IL-2 in PLGA microspheres have been successful (78, 79). Moreover, the delivery of Th1-related cytokines by biodegradable microspheres has been shown to elicit anti-tumor activity (80-83).

Studies on both cellular uptake and antigen processing and presentation of antigen loaded PLGA microspheres are crucial for engineering microspheres capable of eliciting cellular immune responses. In order for the antigen to be processed the antigen must first be taken up by APCs. The study in chapter 5 demonstrated that targeted delivery of PLGA microspheres to APCs, most notably DCs. Based on the potency of DCs and

the clinical observations (56, 57) to date, the targeted delivery of antigen loaded PLGA microspheres to DCs using additives such as targeting moieties demands further exploration (84). Other areas requiring further investigation include MHC class I and II processing of antigen loaded PLGA microspheres. The study in Chapter 4 demonstrated that PLGA microspheres are capable of cytoplasmic delivery - a required step for the induction of CTL responses. A study by Kovacsovics-Bankowski *et al.* proposed that the rate limiting step in MHC class I presentation of exogenous particulate antigen may be delivery of antigen to the cytosol (50). Hence, incorporating membrane-perturbing agents into the PLGA microsphere formulations may be a viable route for enhancing cytoplasmic delivery and inducing CTL responses (85, 86). Moreover additives located on the surface or encapsulated to manipulate intracellular processing, trafficking and presentation of antigen may further enlarge the potential of PLGA microspheres as vaccine delivery systems.

In summary, future prospects for PLGA microsphere-based vaccines remain numerous including microencapsulation of multiple antigens and adjuvants as well as DNA. The past decade of research has uncovered the many facets of PLGA microspheres and with continued progress in this field it is expected that PLGA microspheres will play a key role in the development and design of future vaccines.

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The individual chapters in this thesis have been published in international scientific journals.

Research Articles

1. Newman, K.D., Elamanchili, P., Kwon, G.S., Samuel, J., Uptake of poly(D,L-lactic-co-glycolic acid) microspheres by antigen presenting cells *in vivo*, *J. Biomed. Mat. Res.* (accepted for publication, 2001).

2. Newman, K.D., Kwon, G.S., Miller, G.G., Chlumecky, V., Samuel, J., Cytoplasmic delivery of a macromolecular fluorescent probe by poly(*d,l* lactic-co-glycolic acid) microspheres, *J. Biomed. Mat. Res.* 50: 591-597 (2000).

3. Newman, K.D., Sosnowski, D.L., Kwon, G.S., Samuel, J., Delivery of MUC1 peptide by poly(*d,l* lactic-co-glycolic acid) microspheres induces type I T helper immune responses, *J. Pharm. Sci.* 87(11): 1421-1427 (1998).

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Abstracts

1. Newman, K.D., Samuel, J. and Kwon, G. Biodegradable poly(*d,l* lactic-co-glycolic acid) microspheres as antigen delivery systems for the selective induction of cellular immune responses against synthetic peptides, *Pharm. Res.* September Supplement 13(9): S-77 (1996).

2. Newman, K.D., Kwon, G., Miller, G., Chlumecky, V., Samuel, J., Cytoplasmic delivery of a fluorescent probe by poly(*d,l* lactic-co-glycolic acid) microspheres, *AAPS PharmSci* Supplement 1(4) (1999).