Title
Antigen delivery by liposomes in vitro

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Publication Date
1989

Peer reviewed|Thesis/dissertation

ANTIGEN DELIVERY BY LIPOSOMES IN VITRO

by

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B.S. 1976

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACEUTICAL CHEMISTRY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

to my wife

"Be active, be energetic, be
enthusiastic and faithful,
and you will accomplish your
object"

- Ralph Waldo Emerson

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Frank C. Szoka for the opportunity to work under his guidance on this project. I am grateful for his encouragement, optimism, patience, and financial support. Our numerous discussions have benefited me scientifically as well as personally.

I would like to thank the members of my thesis committee, Dr. Joel Goodman and Dr. Wolfgang Sadee for their helpful comments and suggestions during the course of this work.

I am grateful to Dr. Philippa Marrack from the National Jewish Hospital, Denver, CO., Dr. Ronald Schwartz from the National Institutes of Health, Bethesda, MD.,and Dr. Daniel Stites for generously providing the cell lines employed in this work.

I must thank the graduate students, post-docs, and others who have kindly offered me their friendship and support. I thank the people of the Department of Pharmaceutical Chemistry and the University of California.

Finally, I am grateful to the City of San Francisco and Bay Area for giving me the pleasure of two Super Bowls, a World Series, becoming a Democrat, and the Pacific Ocean.

TITLE: ANTIGEN DELIVERY BY LIPOSOMES IN VITRO

ABSTRACT

An in vitro antigen processing and presentation system was used to investigate the effect of association of protein antigens with liposomes on immune responses. Antigen processing and presentation of non-encapsulated and liposome associated antigens by two different antigen presenting cell (APC) types, B cells and macrophages, was studied in culture. Interleukin-2 (IL-2) secretion by antigen-specific T cells in a major histocompatibility complex-restricted fashion was the indicator of antigen processing and presentation.

The macrophage line, P388D1, presented chicken ovalbumin (cOVA) to cOVA-specific T cells. Presentation was a function of the amount of antigen, level of Class II expression, and ratio of APC to T cell used. The IL-2 response was antigen specific and genetically restricted. Encapsulation of cOVA in liposomes resulted in a 5-10 fold enhancement in efficiency of presentation over unencapsulated cOVA.

Encapsulation of pigeon cytochrome-c (PCC) modestly increased the presentation when the peritoneal cells were the APC but totally eliminated presentation when a B cell was the APC. The increased presentation by the macrophage was correlated with an increase in uptake and rate of processing of the liposomal antigen. The time course and extent of uptake and presentation was influenced by the lipid composition of the liposome. The absence of presentation by the B cell was because this cell type could not internalize and degrade the encapsulated PCC. These results support the concept that the macrophage is the primary cell type involved in the initial

stages of an immune response to a liposome encapsulated protein antigen in vivo.

Pigeon cytochrome-c covalently coupled to the liposomal surface was a 30-40 fold more potent stimulus for IL-2 secretion than the free protein when presented by peritoneal cells. In the case of the B cells, the liposome form was less efficiently presented than unmodified PCC. A 24 amino acid peptide representing the T cell epitope of PCC was a potent stimulus of IL-2 secretion with both antigen presenting cell types. Covalent attachment of the peptide to the liposome surface resulted in a less efficient stimulus of IL-2 secretion than the unmodified peptide.

These studies indicate that the nature of the association of the antigen with the liposome (encapsulated versus covalently attached) can influence which cell types process and present the antigen to T lymphocytes.

BY: Paul Dal Monte

Thesis Chairman: Francis C. Szoka, Ph.D.

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APC	Antigen presenting cell
BCGF	B cell growth factor
BCDF	B cell differentiation factor
BCG	Bacillus Calmette Guerin
BGG	Bovine gamma globulin
BSA	Bovine serum albumin
CH	Cholesterol
CGN	Carrageenan
CON A	Concanavalin A
cOVA	Chicken ovalbumin
CP	*Corynebacterium parvum*
CTL	Cytotoxic T lymphocyte
DCMDP	Dichloromethylene diphosphonate
DCP	Dicetylphosphate
DLPC	D-L-α-dilaurylphosphatidylcholine
DMPC	D-L-α-dimyristoylphosphatidylcholine
DMPE	L-α-dimyristoylphosphatidylethanolamine
DMPG	L-α-dimyristoylphosphatidylglycerol
DOPC	D-L-α-dioleylphosphatidylcholine
DPPC	D-L-α-dipalmitoylphosphatidylcholine
DPPE	L-α-dipalmitoylphosphatidylethanolamine
DPPG	L-α-dipalmitoylphosphatidylglycerol
DRV	Dehydration-rehydration vesicle
DSPC	D-L-α-distearoylphosphatidylcholine

ABBREVIATIONS (continued)

DT	Diphtheria toxoid
EPC	Egg yolk-phosphatidylcholine
EPG	Egg yolk-phosphatidylglycerol
FBS	Fetal bovine serum
FACS	Fluorescence activated cell sorter
FCA	Freund's complete adjuvant
HBsAg	Hepatitis B surface antigen
HRP	Horse radish peroxidase
HSA	Human serum albumin
id.	intradermal
IFA	Incomplete Freund's adjuvant
IFN	interferon
IL-2	Interleukin 2
IL-1	Interleukin 1
im.	intramuscular
ip.	intraperitoneal
ISCOM	Immuno-stimulating complex
iv.	intravenous
LPS	Lipopolysaccharide
LUV	Large unilamellar vesicle
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
MLV	Multilamellar vesicle
MØ	Macrophage
NBD-PE	4-nitrobenzo-2-oxa-1, 3 diazole-phosphatidylethanolamine

ABBREVIATIONS (continued)

PA	Phosphatidic acid
PCC	Pigeon cytochrome-c
PE	Phosphatidylethanolamine
PEC	Peritoneal exudate cells
PI	Phosphatidylinositol
PS	Phosphatidylserine
RCS	Rat Con A stimulated spleen cell supernatants
REV	Reverse-phase evaporation method
RPMI-CM	RPMI Complete medium with FBS
SA	Stearylamine
SAC	Spleen adherent cells
sc.	subcutaneous
SM	Sphingomyelin
SUV	Small unilamellar vesicles
Tc	Transition temperature
TDA	Thymus dependent areas

ANTIGEN DELIVERY BY LIPOSOMES IN VITRO

BY: PAUL DAL MONTE

FEBRUARY, 1989

Liposomes have been used as carriers of a wide variety of therapeutic agents. Their use as a carrier of antigens is one of the more promising clinical applications. In 1974, Allison and Gregoriadis first demonstrated the adjuvant effect of liposomes on the immune response to diphtheria toxoid in vivo. A multitude of studies have confirmed this observation and have raised the question as to the role of the liposome in enhancing the immune response.

OBJECTIVE OF RESEARCH

It is the objective of this work, to establish an in vitro antigen presentation system to answer basic questions about the liposome adjuvant effect. These results will help to understand the mechanism(s) of the effect at the level of the antigen presenting cell and should aid in the optimization of liposome vaccine formulations.

HYPOTHESIS OF RESEARCH

It is proposed that the immune-enhancing effect observed with liposomes in vivo is due to the rapid and efficient uptake of liposome-associated antigen by the macrophage which in turn augments the efficiency of these cells to stimulate T-lymphocytes. At least two contributing factors in the adjuvant effect of liposomes could be the extent to which

liposomes are taken up by macrophages and the rate by which antigen is released from the liposome and processed once inside the cell. To test this hypothesis, an in vitro model of an immune response is used. This model makes use of antigen presenting cells (APC) and antigen-specific T-cell hybridomas. The immune response is quantitated by measuring interleukin-2 (IL-2) secretion by the T-cell after the antigen is processed and presented by the APC.

CHAPTER I: INTRODUCTION: THE USE OF LIPOSOMES AS ADJUVANTS IN IMMUNE RESPONSES

The focus of this chapter will be to review the literature on the use of liposomes as adjuvants for antibody responses to protein antigens. To direct the reader, this chapter consists of five sections. The first two sections will provide general information necessary to aid in the understanding of the liposome adjuvant effect. Section 1 includes a brief description of relevant liposome characteristics and section 2 will focus on the immunology of antibody responses to protein antigens. The review of the liposome adjuvant literature is in section 3 and is followed by a section on mechanisms of liposome adjuvanticity proposed by the various authors. Section 5 and 6 will provide the reader with the rationale and scope of this thesis.

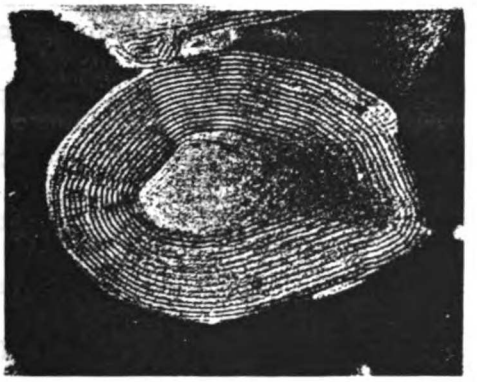
SECTION I.1: DESCRIPTION OF LIPOSOMES

I.1.1 DEFINITION OF LIPOSOMES

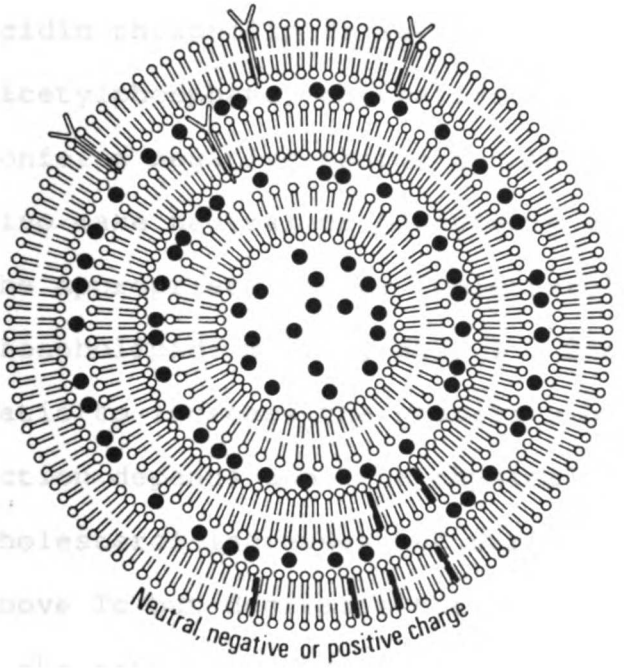
Phospholipid vesicles (liposomes) are microscopic structures consisting of one or more phospholipid bilayers stacked upon one another and enclosing an aqueous space. The structures are formed spontaneously upon hydration of phospholipids as originally described by Bangham (Bangham et.al., 1965). The amphiphilic nature of the phospholipid molecules makes these structures possible. Essentially, the hydrophilic parts of the phospholipid molecules are oriented on either side of the bilayer in contact with the aqueous phase whereas the hydrophobic acyl chains form the core of the bilayer. An electron micrograph of a multilamellar liposome (MLV) is shown in Figure I.1.a. Figure I.1.b demonstrates the capability of the liposome to entrap substances as determined by physico-chemical parameters.

I.1.2 TYPES OF LIPOSOME PREPARATIONS

Methods of preparation of liposomes have been extensively reviewed (Szoka and Papahadjopoulos, 1980; Deamer and Uster 1983; Gregoriadis, 1984). Liposomes may be prepared from a variety of lipids of natural or synthetic origin (Machy and Leserman, 1987). Liposomes composed of a single component and maintained at a temperature below the phase transition temperature (Tc) of that lipid are considered to be solid or in the gel state. Liposomes held at



(a)



(b)

Figure I.1: a) An electron micrograph of an MLV showing electron dense spaces between lipid bilayers. b) A diagramatic representation of an MLV with bilayer stacks separated by aqueous spaces. Molecules, such as protein antigens, will distribute in the aqueous or lipid phase depending on their physico-chemical properties. Figure obtained from: Gregoriadis, 1979. Permission requested.

a temperature higher than the Tc are designated to be fluid or liquid crystalline. Solid liposomes have rigid, ordered acyl chains whereas fluid liposomes have their acyl chains in a disordered state free to move within the core of the bilayer.

The surface charge of liposomes is determined by the lipid composition. Specific phospholipids, for example, the acidic phospholipids such as phosphatidylserine, dicetylphosphate, phospatidic acid, phosphatidylglycerol will confer a negative charge on the liposome surface. One can also make neutral or positively charged liposomes by choosing the appropriate lipid. Cholesterol is often added to the phospholipid mixture at a 1 : 2 cholesterol : phospholipid ratio to increase the stability of the liposomes. Cholesterol action depends upon the physical state of the phospholipid. Cholesterol increases the surface density of the acyl chains above Tc but decreases the surface density below Tc. At a 1 : 2 cholesterol : phospholipid ratio it eliminates the phase transition (Machy and Leserman, 1987). Addition of cholesterol to liposomal preparations decreases the ability of serum proteins such as high density lipoprotein to intercalate into the bilayer thereby increasing the liposomal stability in vivo (Machy and Leserman, 1987).

Depending on the method of preparation, liposomes of various size ranges and number of lamellae can be obtained. The method of liposome preparation is commonly determined by the quantity of agent which one needs to entrap or attach on

the surface. Entrapment signifies either the incorporation of a lipophilic substance into the lipid bilayer or the passive encapsulation of a water soluble substance in the aqueous compartment of the system. In the case of macromolecules, with both hydrophobic and hydrophilic regions, the method of preparation will influence the distribution between the lipid and aqueous phase. The charge distribution of the macromolecule and the charge of the liposome will also influence liposome-macromolecule interactions.

Some of the more commonly employed techniques for preparing liposomes are compiled in Table I.1.

I.1.3 IN VIVO DISPOSITION OF LIPOSOMES

Numerous studies have shown that the majority of liposomes injected intravenously localize primarily in the liver, spleen, and bone marrow.

Liposome retention in these organs is due primarily to their uptake by mononuclear phagocytes (macrophages) which line the vascular sinusoids. This has been demonstrated by tissue fractionation and ultrastructural studies(Poste et.al., 1982). In addition to uptake by fixed phagocytic cells, liposomes can also be phagocytosed by circulating blood monocytes (ibid). The characteristics of liposomes which influence their rate of clearance from the circulation are extensively described (Poste et.al., 1983; Senior, 1986; Hwang, 1987). The major parameters which dictate liposome clearance are: vesicle size, dose, and liposome surface

Table I.1: Description of different liposome types resulting from commonly used preparation methods.

Liposome type	Lamellae	Method of preparation	Diameter (nm)	Entrapped volume (µl/µmole lipid)*	Reference of origin
MLV	oligo	vigorous vortexing	400-3500	1-5	Bangham,et al, 1965
DRV	oligo.	lyophilized SUV then rehydration	?	?	Kirby & Gregoriadis, 1984
SUV	uni.	sonication of MLV	20-50	0.5	Papahadjopoulos & Miller,1967
SUV	uni.	detergent dialysis	40-180		Kagawa & Racker 1971
LUV	uni.	serial extrusion	100-200	15	Szoka, et.al. 1980
REV	uni.	reverse phase evaporation	100-1000	8-17	Szoka & Papahadjopoulos 1978

* based on encapsulation of a polar marker (Szoka and Papahadjopoulos, 1978)

characteristics such as charge, hydrophobicity, and additional ligands attached to the surface. The clearance of liposomes is analogous to the behaviour of other inert particulate materials injected intravenously such as colloidal carbon, latex particles, nanoparticles, microspherees, immune complexes, and erythrocytes (Poste et. al., 1983).

Early studies on the effect of vesicle size in the rate of clearance of liposomes demonstrated that the rate of clearance was biphasic in nature. Juliano and Stamp (1975) reasoned that the biphasic pattern was due to the fact that the liposome preparations yielded heterogeneous size populations of liposomes. Furthermore, these studies were dependent on the leakiness of the entrapped markers which were used to assess the liposomal clearance. The circulating half-lives after intravenous (iv.) administration were 7.5 hours for SUV, 1.5 hours for REV (0.2 μm filtered), and 0.2 hours for REV (0.4 μm filtered). Vesicle size clearly influences rate of clearance from the circulation (Senior, 1986).

The lung becomes one of the primary organs for the uptake of liposomes in the order of 5-10 μm in diameter (Poste and Kirsh, 1983). This uptake probably occurs by the trapping of liposomes in the capillary beds. These liposomes are slowly cleared either by disintegration of the bilayers or uptake by alveolar macrophages. The other major sites of liposome accumulation after iv. injection are in the

mononuclear phagocytes of the spleen and the bone marrow. Liposome accumulation in the spleen is enhanced under conditions where liver uptake is reduced or eliminated. After multiple injections of liposomes, uptake by liver, spleen, and bone marrow macrophages can become saturated resulting in toxicity due to impaired reticuloendothelial function (Senior, 1986).

The presence of a specific ligand on the surface may influence the disposition of liposomes in vivo. In cases where targeting the liposomes is desired, liposomes carrying surface attached immunoglobulin will result in more rapid clearance of the liposomes (Hwang, 1987). Another example of ligand modification of liposomal clearance is demonstrated by the addition of carbohydrate ligands which confer specificity for uptake by receptors on parenchymal cells of the liver. This has been nicely demonstrated for SUV liposomes of less than 0.1 μm diameter. These small liposomes are transported through continuous capillaries of the liver and are subsequently taken up via endocytosis by liver parenchymal cells (Hwang, 1987).

The rate of clearance of liposomes can also be influenced by the route of administration. Several routes other than iv. administration have been studied. Liposomes administered intraperitoneally (ip.) are absorbed intact into the blood circulation with an initial lag phase. Once in the circulation, the liposomes are taken up by phagocytic cells in a manner similar to iv. administration. The transport of

intact liposomes from the peritoneal cavity to the circulation is via lymphatic drainage. It has been suggested that the lag phase in appearance of liposomes into the circulation depends on the surface charge, lipid composition, size, or amount of liposome dose. Subcutaneous (sc.) injection of liposomes results in the accumulation and retention of a large percent of the administered dose at the site of injection. A portion of the dose however, has been demonstrated to be taken up by cells of the regional lymph nodes and is dependent on liposome size with small liposomes showing higher lymph node uptake than larger liposomes. Liposomes may also be administered via intramuscular (im.), topical, intrathecal, intra-articular, subconjunctival, and oral routes although these routes are less commomnly studied (Hwang, 1987).

Section I.2. DESCRIPTION OF THE IMMUNE RESPONSE

I.2.1 CELLS OF THE IMMUNE SYSTEM

The immune system of vertebrates consists of a number of organs and several different cell types which have evolved to recognize non-self antigens and to eliminate them. All the cells of the immune response arise from pluripotent stem cells through two main lines of differentiation: 1) the lymphoid lineage, producing lymphocytes and 2) the myeloid lineage, producing phagocytes and other cells (Figure I.2).

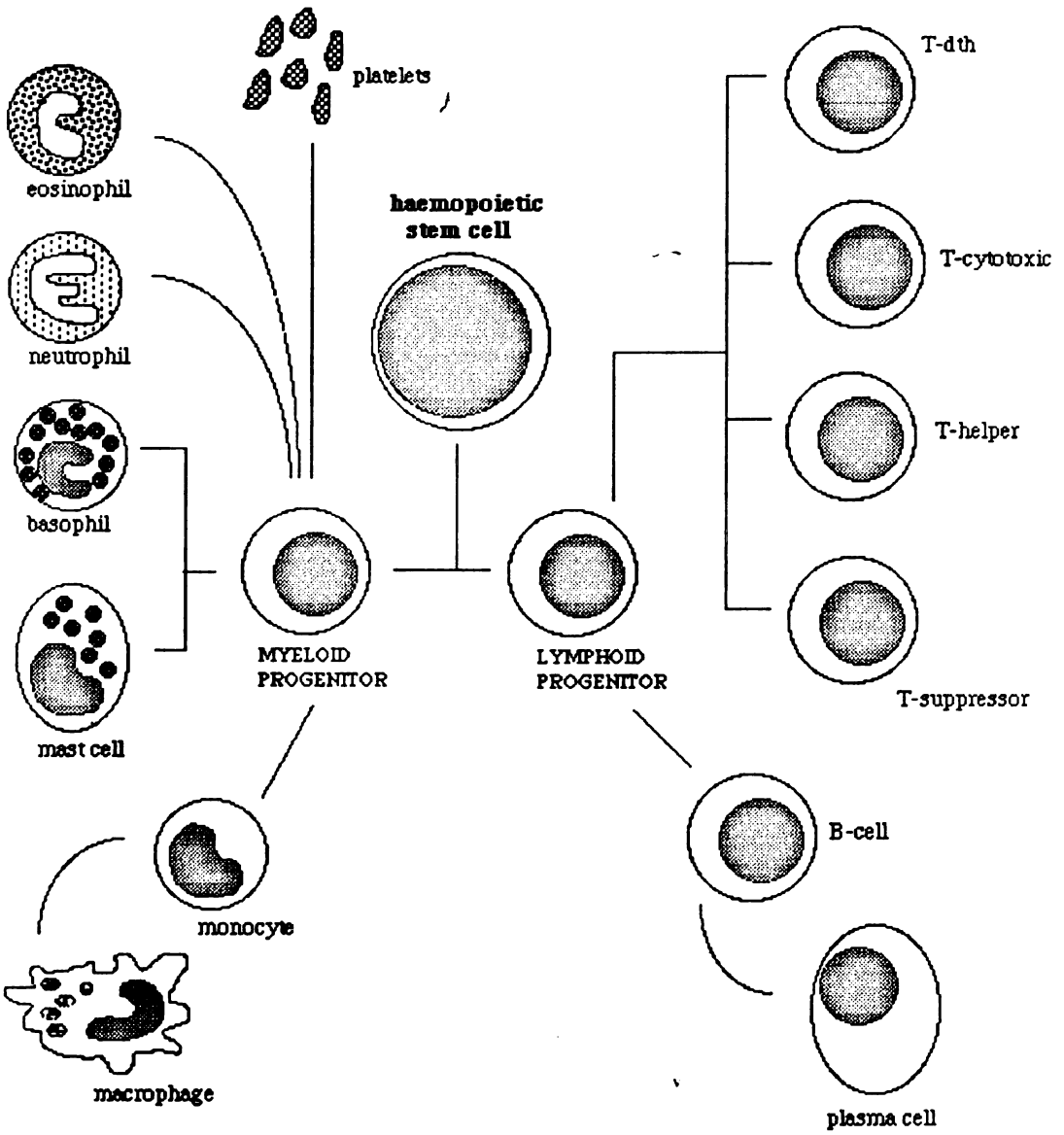


Figure I.2: Cells involved in the immune response and their origin. Modified from: Roitt et al, 1985.

There are two different types of lymphocytes which serve different functions, T lymphocytes and B lymphocytes. T cells differentiate initially in the thymus while B cells differentiate in the adult bone marrow and in the fetal liver and spleen. During their development, both T and B cells acquire specific receptors for antigen which commit them to a single antigenic specificity for the rest of their life-span.

B cells represent about 5-15% of the circulating lymphoid pool and are classically defined by the presence of immunoglobulin IgM and IgD on their surface. Activated B cells become terminally differentiated plasma cells and are responsible for the production of antibodies specific for determinants on an antigen. Antibodies produced by a single plasma cell are of one specificity and immunoglobulin class. On the basis of many investigations carried out since the 1950's, it is now generally accepted that antibody producing plasma cells can first be detected in the spleen following iv. immunization and the spleen is the primary site for antibody synthesis (Richter et.al., 1986)

In the case of T cells, there are three basic responses, each carried out by a specific subpopulation of T cells. Cytotoxic T cells specifically kill foreign or virus-infected cells. Helper T cells help specific T or B lymphocytes respond to antigen and can activate cells such as macrophages. Supressor T cells supress the response of T or B lymphocytres.

Bone marrow derived myeloid progenitors give rise to cells of the mononuclear phagocyte system which has two main functions: 1) to remove particulate antigens by phagocytic macrophages and 2) to present antigen to antigen specific lymphocytes thereby aiding in the antibody response (discussed in section I.2.6). The bone marrow precursors give rise to a circulating pool of blood monocytes which migrate into various organs and tissues to become tissue macrophages.

I.2.2 TYPES OF IMMUNE RESPONSES

There are two classes of immune responses: humoral and cellular. Humoral immunity involves the production of antibodies by activated B lymphocytes whereas T lymphocytes are responsible for cellular immunity.

Following primary antigenic challenge, whether humoral or cellular, the immune response will appear after an initial lag period of several days followed by a rapid logarithmic rise to a plateau which eventually declines (Figure I.3). This is known as the primary immune response. The secondary immune response has a shorter lag phase, an extended plateau, and a more gradual decline. The plateau levels are much greater in the secondary response than in the primary response. In the case of a humoral antibody response, for example, differences in the production of antibody classes occur during the primary and secondary responses. Antibodies of the IgM type form a major proportion of the primary

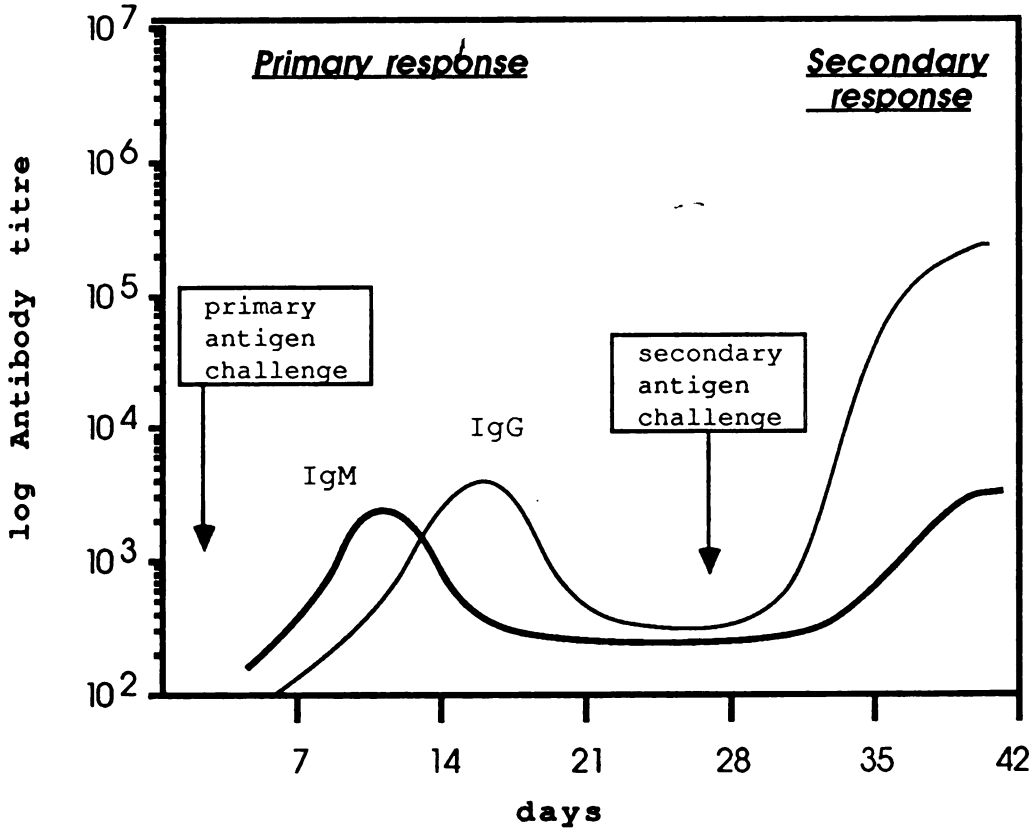


Figure I.3: The antibody level following primary and secondary antigenic challenge. Modified from: Roitt et al, 1985.

response whereas the secondary response consits of almost entirely IgG.

I.2.3 T-DEPENDENT AND T-INDEPENDENT ANTIGENS

Antigens which require both B and T cells to be recognized as foreign are called T-dependent antigens. Animals which have been surgically thymectomized or are genetically deficient of thymus function cannot mount an immune response to T-dependent antigens. There are some antigens, however, which can activate B lymphocytes without T-cell help and are therefore referred to as T-independent antigens. The T-independent antigens share a number of common properties some of which include: 1) large polymeric molecules with repeating antigenic determinants, 2) the ability at high concentrations to activate B cell clones other than those specific for that antigen, 3) many T-independent antigens are resistant to degradation. Examples of T-independent antigens include: lipopolysaccharide (LPS), ficoll, and dextran. Primary antibody responses to T-independent antigens in vitro are generally weaker than the responses to T-dependent antigens. The secondary response to T-independent antigens is very similar to the primary response in that it is weak and almost entirely confined to IgM production.

I.2.4 CLONAL EXPANSION OF THE IMMUNE RESPONSE

During the primary response virgin T and B cells are stimulated to differentiate into pools of effector cells and memory cells. Effector cells are immediately available to produce an immune response whereas memory cells are readily induced to become effector cells upon secondary challenge of antigen. This forms the basis of the clonal selection theory. Ada and Nassal recently published an excellent review on this topic (1987).

I.2.5 CELL INTERACTIONS IN THE ANTIBODY RESPONSE

The fundamental basis of the recognition of foreign protein antigens by B cells and T cells stems from a large number of studies from as early as 1959 (Gell and Benacerraf, 1959) employing diverse protein antigens. B cells recognize the antigen via surface Ig receptor (mIg) which is specific for conformational determinants on the antigen. The antibodies produced by the B cell are also specific for conformational determinants on the native molecule.

T cells do not recognize the antigen in its native form but instead recognize determinants found on the unfolded molecules or in fragments of it. T cell help in the production of antibody can be delivered in at least three ways: 1) via direct interaction of the helper T cell and the responding B cell, 2) through the production of soluble, nonspecific, helper factors known as lymphokines, and 3) by the localized release of lymphokines at the site where helper

T cell-B cell interaction occurs. Among the lymphokines are IL-2, B cell growth factors, and B cell differentiation factors which help in the proliferation and differentiation of antigen specific B cells into antibody secreting plasma cells.

I.2.6 ANTIGEN PRESENTATION

The process by which helper T cells recognize protein antigens is known as antigen presentation. Extensive reviews have been written on this topic (Unanue, 1984; Schwartz, 1985; Unanue and Allen, 1987). Helper T cells do not recognize antigen in its native form but instead recognize a complex of the modified antigen in conjunction with a gene product of the major histocompatibility complex (MHC) known as the Class II or Ia glycoprotein on the surface of an antigen presenting cell (APC). Class II glycoproteins, heterodimers made of 34 and 28 kD chains, are found mainly on the surfaces of macrophages, B-cells, Langerhans cells in the skin, and dendritic cells. The exact amino acid sequence of the MHC Class II and the T cell repertoire are important factors in selecting the antigenic determinant that is recognized. The epitopes of the protein which are recognized vary depending upon the MHC haplotype involved in the presentation to helper T cells and the T cell repertoire itself.

The molecular events involved in the interaction between the APC and the T helper cell were discovered using

macrophages as the APC. In general, for effective presentation of protein antigens, the APC must have the capacity to: 1) take up the antigen, internalize it, and "process" it, 2) express MHC Class II molecules on its surface, and 3) secrete molecules such as IL-1 which are required to stimulate T helper cells. The induction of IL-1 in antigen presentation will not be discussed any further in this review. For more detail, see Unanue (1988). It has been suggested that IL-1 may not be required for stimulation of activated T cells (Beller, 1984) or T helper hybridomas (Watts and McConnell, 1988). This is relevant to our studies since we employ T cell hybridomas.

Antigen processing can be defined as the conversion of an antigen by an APC, which expresses Ia, from a native to a non-native form. The necessity for antigen processing by APC before T cells could recognize the antigen was first shown by Ziegler and Unanue (1981; 1982) who discovered two major features of the antigen processing event: 1) APC fixed with paraformaldehyde after a processing period presented antigen to T cells whereas APC fixed prior to processing were inactive. 2) Treatment of the APC with lysosomotropic agents such as chloroquine and ammonium chloride abrogated the presenting capacity of the treated APC. From these studies and those of others, it was concluded that antigen processing involved proteolytic degradation of foreign antigen. This was demonstrated for antigens such as lysozyme (Allen and Unanue, 1984), ovalbumin (Shimonkevitz et.al., 1983), myoglobin

(Streicher et.al., 1984), cytochrome-c (Kovac and Schwartz, 1985), insulin (Falo et.al., 1985), and ribonuclease (Lorenz and Allen, 1988).

More recent studies indicate that processing can involve more than simple proteolytic cleavage. For example, the unfolding of hen egg lysozyme was enough for APC to present the antigen to specific T cell hybridomas (Allen and Unanue, 1984). Similar results were shown for myoglobin (Streicher et.al., 1984).

Depending on the antigen and the T cells being studied, Allen proposed that there exist at least three levels of antigen processing: 1) none, 2) unfolding, and 3) cleavage. The immunogenic sequence in each case possesses two distinct features: 1) it must be able to make contact with the T cell receptor and 2) it must bind to an Ia molecule on the surface of the APC (Allen, 1987).

Although the studies that led to the present understanding of processing and presentation were mostly done with macrophages, it now has become clear that antigen presentation is not exclusive to macrophages. Other APC include Langerhans cells, dendritic cells which carry high levels of Ia molecules, B lymphocytes and even L cells which have been transfected with the genes encoding Class II glycoprotein expression (Unanue and Allen, 1987). Although all Ia-bearing cells are APC, the in vivo relationship between the various APC has not been determined. Figure I.4

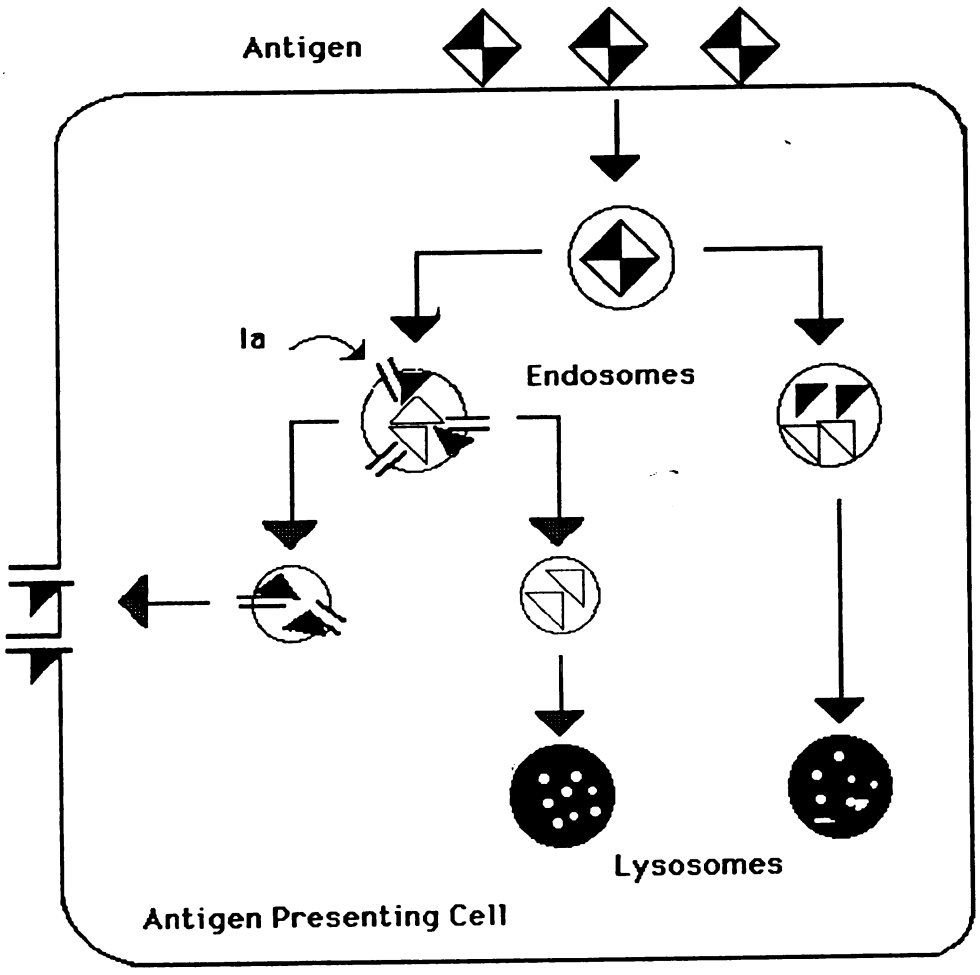


Figure I.4: The cellular events that result in the expression of an immunogenic fragment of a protein antigen on the surface of an antigen presenting cell. The protein is endocytosed into an acidic endosome and degraded. If the endosome has Ia molecules (left side), those peptide fragments which have affinity for Ia will be transported to the surface. Those fragments which have no affinity for Ia will be transported to lysosomes for complete degradation. Those endosomes which have no Ia molecules will transport the exogenous protein into lysosomes for complete digestion. Modified from: Unanue and Allen, 1987.

depicts a model which describes the proposed cellular events that result in presentation of immunogenic fragments on the surface of APC (Unanue and Allen, 1987).

I.2.7 ANTIGEN PRESENTATION BY B CELLS

Mitchison and coworkers showed that in the induction of a specific antibody response, antigen specific B cells interact with antigen specific helper T cells and that the interaction is MHC restricted (Mitchison, 1971). As mentioned, B cells bind antigen in their native conformation via mIg whereas T cells recognize processed antigen associated with Class II on the surface of an APC. The question was raised as to how the antigen receptor on T cells which recognizes processed antigen in conjunction with Class II detects the native antigen bound on the mIg receptor on the surface of B cells. The paradox of T-B cell co-recognition of antigens was resolved by assuming that specific B cells would use their mIg to capture antigen and would subsequently process it and present it to T cells in an MHC-restricted fashion as do phagocytic APC (Kakiuchi et.al., 1983).

Furthermore, Lanzavecchia showed that B cells can use their antigen specific mIg to capture antigen at concentrations 1000 to 10000 times lower than that required by nonspecific APC such as macrophages (Lanzavecchia, 1987). A similar difference in the efficiency of presentation

between specific and non-specific B cells was also reported in different experimental sytems (Kakiuchi et.al., 1983).

I.2.8 Overview of the Antibody Response

The typical immune response to a protein antigen is summarized schematically in Figure I.5. Briefly, the primary response to antigen can be initiated in essentially two ways: 1) The antigen may encounter an antigen specific B cell. The probability of this occurring is low. However, if specific B cells are encountered, the recognition would be specific resulting in efficient uptake. 2) The antigen may make contact with a non-specific APC such as a macrophage, B cell, monocyte, or dendritic cell with a high probability, no specific recognition, and low efficiency of uptake. The next step in the response is the presentation to helper T cells with subsequent antibody production.

In the naive animal, the frequency of antigen specific B cells and/or T cells is low, so that in a primary response the antigen must be provided in high doses and in a physical form that can interact efficiently with other APC such as macrophages, thereby enabling antigen specific T cells to easily encounter APC bearing processed and presented antigen. This results in the proliferation and clonal expansion of antigen specific T cells which will in turn increase the probability that an antigen specific B cell will encounter an antigen specific T cell. The outcome of these events is the primary response.

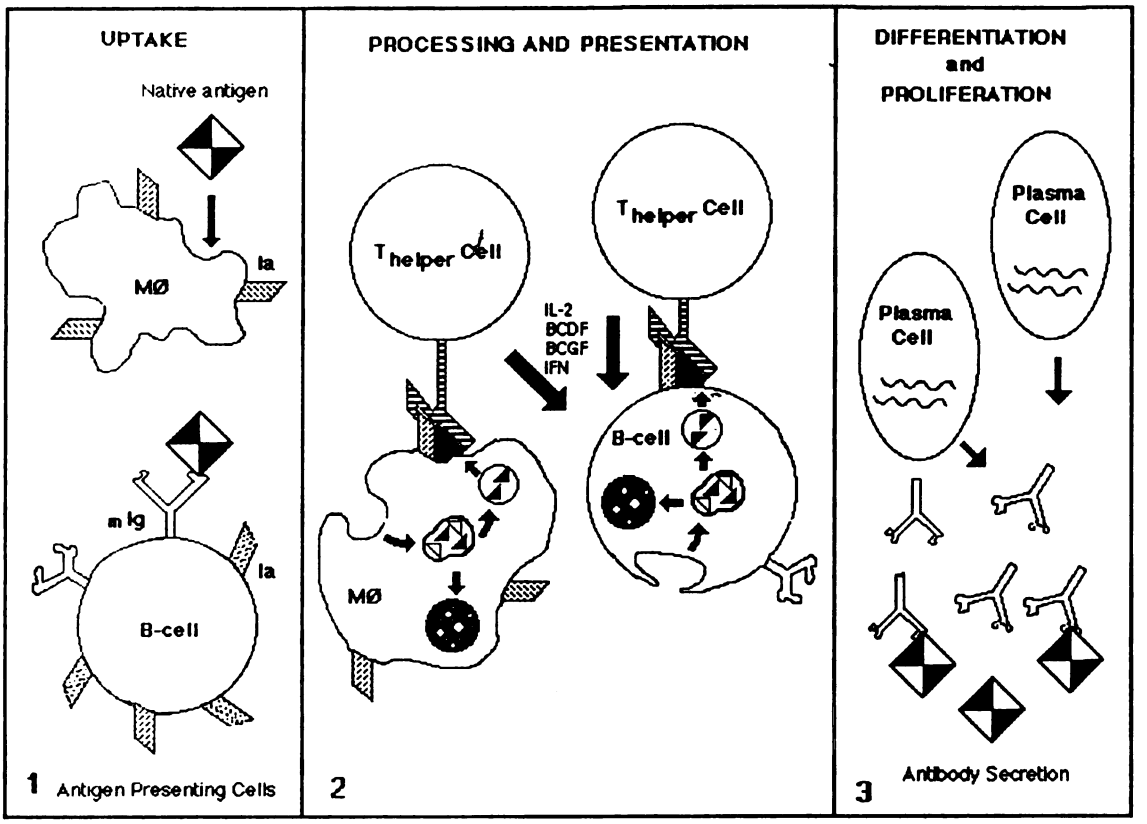


Figure I.5: The steps leading to the activation of B cells towards antibody secretion. **1**) Endocytosis of antigen (non-specific or receptor mediated) by APC. B cells recognize native antigen by their membrane surface immunoglobulin receptors (mIg); **2**) The antigen is processed and presented on the surface of the APC in conjunction with MHC-Class II (Ia) glycoprotein. Formation of ternary complex (processed antigen, Ia, and T helper cell receptor) stimulates T-helper cell to co-operate with B cell by secretion of non-specific factors (IL-2, BCGF. BCDF, etc), and other mechanisms; **3**) B cells are stimulated to proliferate and differentiate into antibody forming plasma cells which secrete antibody specific for antigen.

During the secondary response, memory B cells are present thus the antigen can be recognized with high efficiency by specific B cells. Also a greater number of antigen specific T cells are present so T-B encounters increase (Manca et.al., 1988).

I.2.9 VACCINATION

The goal of vaccination is to exploit two key elements of the immune response, i.e., specificity and memory. The principle is to alter an antigen in such a way as to make it innocuous without loss of its antigenicity. After vaccination the immune system will mount a much stronger response during a second encounter with antigen.

Synthetic vaccines present a more challenging problem due to the fact that most synthetic peptides are not in themselves immunogenic. In this case, the goal for developing vaccine systems is to identify fragments from the native antigen which represent the B and T helper cell stimulating epitopes. In most instances, an adjuvant is required to prolong the exposure of the antigen to the immune system. The requirements for generating an immune response to synthetic vaccines have been summarized by Ada (1988) and are listed in Table I.2. The live vector antigen was included to demonstrate that the requirements for immunogenicity are 'built in' to the native virus and adjuvants and/or targetting of the antigen are not necessary.

Table I.2: Requirements for generating an immune response

Antigen	T-cell responses carrier		activation	Adjuvant	Delivery system depot		controlled release	Targetting
peptide	+	or	+	+	+	or	+	+
protein	-		-	+	+	or	+	+
live vector	-		-	+	+	or	+	+

Modified from Ada, 1988.

I.2.10 ADJUVANTS

Adjuvants, as defined by Allison (1979), are agents that act nonspecifically to increase an immune response to a specific antigen. Some adjuvants exert their effects on a wide range of immune responses to an antigen, but in other cases the effects are more or less selective. Potentially, adjuvants could be picked based on the need for either a humoral or a cell mediated response in the immunization against an antigen, although there are limits to this. A major problem is that the findings which apply to one antigen when formulated in an adjuvant do not seem to apply to another. Also, species react differently from one another so that what works in mice may not in man.

Antigens can be incorporated into, or concomitantly injected with, an adjuvant. For instance, Freund's complete adjuvant (FCA) is used as a suspension of antigen and a water-in-oil emulsion containing mycobacterial lipids in the oil phase (Dresser and Phillips, 1973). Aluminum phosphate is another well known adjuvant consisting of adsorbed antigen on insoluble particles of aluminum phosphate (ibid). Table I.3 lists a series of adjuvants used in clinical settings and biomedical research. Several excellent and comprehensive reviews of adjuvants have been recently published (Warren et.al., 1986)

Table I.3: Common adjuvants used in the literature.*

Adjuvant
Vitamin A alcohol and palmitate
Squalene/squalene + emulsifying agent (Arlacel A)
Aluminum hydroxide and phosphate
Paraffin oil
Lipopolysaccharide (LPS), *Corynebacterium parvum*, *Bordetella pertussis*, lipid A
Muramyl dipeptide (MDP) and derivatives
Retinol
Beryllium
Saponins including Quil A and ISCOM's
Avridine
Silica
Liposomes
Slow release biodegradable capsules
Pluronic block polymer surfactants
SAF-1: pluronic polymer + squalene + tween 80 + MDP
RIBI formulation: monophosphoryl lipid A + trehalose dimycolate oil-in-water emulsion
Stearyl tyrosine and related structures
IMREG-1, lymphokines
Complete and Incomplete Freund's adjuvant

* Modified from Frost and Lance, 1973; Allison, 1973; Mitchell, 1988.

Section I.3: REVIEW OF THE LITERATURE: LIPOSOMES AS IMMUNE ADJUVANTS FOR PROTEIN ANTIGENS

It is the purpose of this section to review the literature regarding the in vivo use of liposomes as immunoadjuvants in the immune response against associated protein antigens. Immune responses against liposome coupled haptens are beyond the scope of this review and will be discussed only in instances where the responses reported are important in terms of aiding the understanding of the immunoadjuvant effect with protein antigens. Extensive studies on the immunogenicity of hapten-coupled liposomes can be found in reviews by Kinsky (1978; Kinsky et.al., 1982)

Table I.4 represents a compilation of most of the literature published to date. The studies are categorized by the type of antigen, the lipid composition, whether it is entrapped in or exposed on the surface of the liposome, the route of administration, and the presence of exogenous endotoxin or other adjuvant. Thorough reviews of liposomes in immunology have been published and the readers are referred to these for a more extensive literature review (Alving and Richards, 1983; Alving, 1987; van Rooijen and van Nieuwmegen, 1982; van Rooijen, 1988).

In general, the studies discussed in this review will demonstrate that association of protein antigen with liposomes enhances humoral immune responses so that specific antibody titers against proteins are greater and remain at

TABLE I.4: IMMUNOADJUVANT ACTIVITY OF LIPOSOMES: REVIEW OF THE LITERATURE

ANTIGEN	LIPID COMPOSITION	TYPE	LOCALIZATION (SURFACE OR ETRAPPED)	ENDOTOXIN OR OTHER ADJUVANT	ROUTE OF ADMIN.	REFERENCE
TOXINS						
diphtheria toxoid	EPC/CH/SA EPC/CH/PA EPC/CH/DCP	MLV	surface & entrapped	N.D.	iv., im., sc.	Allison & Gregoriadis, 1974
tetanus toxoid	EPC/CH DMPC/CH DPPC/CH DSPC/CH EPC/CH/PA EPC/CH/SA	MLV, DRV	surface & entrapped; covalently coupled to surface	N.D.	im.	Davis & Gregoriadis, 1987; Gregoriadis, et.al.1987
cholera toxin	DMPC/Avridine DMPC/CH	SUV	?	add avridine	intra-duodenal	Pierce & Sacci, 1984
cholera toxin	DPPC/CH/DCP	MLV	surface coupled via GM1	add lipid A	intra-duodenal	Pierce, et al, 1984
cholera toxin & peptide	DMPC/CH/DCP	MLV	surface coupled via GM1 or entrapped peptide	add lipid A, MDP	iv., sc.	Alving, et al, 1986

TABLE I.4 (continued)

ANTIGEN	LIPID COMPOSITION	TYPE	LOCALIZATION (SURFACE OR ETRAPPED)	ENDOTOXIN OR OTHER ADJUVANT	ROUTE OF ADMIN.	REFERENCE
VIRUSES						
HSV-1. proteins	EPC/CH	SUV	surface & entrapped	add lipid A test in C3H/HeJ	ip.. id.	Naylor, et al, 1982
hepatitis B surface antigen	various compositions	MLV	surface & entrapped 12-23% surface and adsorbed on surface (1.2 %)	N.D.	sc.	Manesis, et al, 1979
Encephalo-myocarditis virus, Semliki forest virus	DPPC/CH DPPC/CH/octyl-decylamine DPPC/CH/PA PA/CH	MLV	surface & entrapped	N.D.	ip.	Kraaijeveld et al, 1984
synthetic peptide from HSV-1 & HSV-2	EPC/CH/lysoPC	MLV	surface coupled via peptide-fatty acid conjugate	add, FCA	id.	Watari, et al,1987
hepatitis B virus intact & poly-peptide antigen	DPPC/CH/PA	MLV	surface & entrapped	FCA,. alum	im.	Sanchez,et al, 1980
hepatitis B virus peptide antigen	DLPC/CH/SA	MLV	surface coupled only	FCA,.	?	Neurath,et al, 1984

TABLE I.4 (continued)

ANTIGEN	LIPID COMPOSITION	TYPE	LOCALIZATION (SURFACE OR ETRAPPED)	ENDOTOXIN OR OTHER ADJUVANT	ROUTE OF ADMIN.	REFERENCE
VP1 subunit from FMD virus	EPC/CH/DCP EPC/CH	MLV	surface & entapped	N.D.	im.	Francis et al, 1985
Measles viral glyco-proteins	DSPC/CH/DCP	MLV	surface & entrapped	N.D.	sc.	Bakouche, et.al,1987b
VP1 subunit from FMD virus	EPC/CH/PS	MLV	surface & entrapped	add. FCA	sc.	Vasantha, et.al,1987
Epstein B. virus membrane gp340	EPC	ULV	surface & entrapped	add. lipid A; FCA	ip., iv., sc.	Morgan, et al, 1984 North,et.al 1982
influenza haemagglutinin	EPC/DCP EPC/PS PI	REV & virosomes	surface & entrapped	N.D.	id.	Oxford, et al, 1981
Adenovirus type 5 hexon	DPPC/CH/PA	MLV	surface & entrapped	tested in C3H/ HeJ mice	im., id.	Kramp, et al, 1979, 1982
SV40 tumor specific transplantation antigen	DMPC/SM/CH	MLV	surface & entrapped	add. FCA	sc.	Huet & Ansel, 1977

TABLE I.4 (continued)

ANTIGEN	LIPID COMPOSITION	TYPE	LOCALIZATION (SURFACE OR ETRAPPED)	ENDOTOXIN OR OTHER ADJUVANT	ROUTE OF ADMIN.	REFERENCE
TUMOR ANTIGENS						
human tumor membrane vesicles	EPC/CH/DCP	MLV	surface & entrapped	N.D.	sc.	Steele, et al, 1984
human colon tumor antigens	EPC/CH/PA	MLV	surface & entrapped 60-78% on surface	N.D.	iv.	Tom, et al 1982
human colon cancer antigen	SM/CH/DCP	MLV	surface & entrapped	N.D.	sc.	Steele, et al, 1984
human colon cancer antigen	EPC/CH/PA DSPC/CH/PA	MLV	surface & entrapped 54%-85% on surface	N.D.	in vitro	Raphael & Tom, 1984
human colon cancer antigen	EPC/CH/PA	MLV	surface & entrapped (54% surface)	N.D.	in vitro	Sengupta,et al, 1985
L2C tumor cells	EPC/PS/NBD-PE	ULV	surface & entrapped	BCG.	id.	Schroit & Key, 1983
MuLV-related tumor antigen	DPPC/CH/DCP	MLV	surface & entrapped 75% on surface	add lipid A	sc.	Gerlier, et al, 1983

TABLE I.4 (continued)

ANTIGEN	LIPID COMPOSITION	TYPE	LOCALIZATION (SURFACE OR ETRAPPED)	ENDOTOXIN OR OTHER ADJUVANT	ROUTE OF ADMIN.	REFERENCE
Gross cell surface antigen	DSPC/CH/DCP DSPC/DPPG/CH DSPC/DPPG/DCP/CH DPPG/DCP/CH DPPC/DCP/CH DPPE/CH/DCP	MLV	surface & entrapped	N.D.	sc.	Bakouche & Gerlier, 1986 a, b
Gross cell surface antigen	DPPC/CH/DCP	MLV	surface & entrapped 1.8-8% on surface	FCA	sc.	Gerlier, et al, 1980
tumor antigen	EPC/CH EPC/EPG/CH	MLV	surface & entrapped	?	sc.	LeGrue, 1984
SOLUBLE PROTEINS						
human serum albumin	EPC/CH	MLV	surface adsorbed only	N.D.	iv.	vanRooijen, et al, 1984
human serum albumin	EPC/CH/PA	MLV	surface adsorbed only & entrapped	N.D..	iv.	vanRooijen & vanNieuwmegen 1980a
human serum albumin	EPC/CH/PA	MLV	surface & entrapped	lyso-PC.	iv.	vanRooijen & vanNieuwmegen 1979

TABLE I.4 (continued)

ANTIGEN	LIPID COMPOSITION	TYPE	LOCALIZATION (SURFACE OR ETRAPPED)	ENDOTOXIN OR OTHER ADJUVANT	ROUTE OF ADMIN.	REFERENCE
human serum albumin	EPC	MLV	surface & entrapped	LPS	iv.	vanRooijen, et al, 1981 vanRooijen & vanNieuwmegen 1980c
human serum albumin & bovine gamma globulin	EPC EPC/SM EPC/SA	MLV	surface adsorbed only	N.D.	iv.	vanRooijen & vanNieuwmegen 1980b
horse radish peroxidase	EPC/PE	MLV	surface coupled	lipid A	sc., iv.	vanRooijen et al, 1982
bovine serum albumin	dialkylether PC/CH/DCP	MLV, ULV	surface & entrapped	N.D.	ip.	Shek, et al, 1986a
bovine serum albumin	DMPC/CH/DCP	MLV, ULV	surface & entrapped	N.D.	ip.	Shek, et.al, 1983
bovine serum albumin	EPC/CH/PA	MLV	surface & entrapped; surface adsorbed only	N.D.	ip.	Shek & Sabiston, 1982
bovine serum albumin	EPC/CH/PA	MLV	surface & entrapped	N.D.	ip.	Shek & Lukovich, 1982

TABLE I.4 (continued)

ANTIGEN	LIPID COMPOSITION	TYPE	LOCALIZATION (SURFACE OR ETRAPPED)	ENDOTOXIN OR OTHER ADJUVANT	ROUTE OF ADMIN.	REFERENCE
bovine serum albumin	EPC/CH/DCP	SUV	surface & entrapped (40% on surface)	N.D.	iv.	Beatty, et al, 1981, 1984
bovine serum albumin	EPC/CH/PA	MLV	surface & entrapped	N.D.	ip.	Shek & Sabiston, 1981
bovine serum albumin	EPC/CH	MLV	surface adsorbed only	N.D.	iv.	Classen, et al, 1987
bovine serum albumin	EPC/CH/MPB-PE	REV	surface coupled only	N.D.	ip.	Shek & Heath, 1983
keyhole limpet hemocyanin	EPC/CH	MLV	surface & entrapped	add saponin	sc.	Scott, et al 1985
ovalbumin	DPPC/CH/DCP DPPC/CH/SA	MLV	surface & entrapped	added MDP	ip., iv. topical (skin & cornea)	Kotani, et al, 1977
ovalbumin	EPC/CH/SA	MLV	surface coupled only; surface & entrapped	N.D.	iv., ip., sc.	Vannier & Snyder, 1988

TABLE I.4 (continued)

ANTIGEN	LIPID COMPOSITION	TYPE	LOCALIZATION (SURFACE OR ETRAPPED)	ENDOTOXIN OR OTHER ADJUVANT	ROUTE OF ADMIN.	REFERENCE
lysozyme	EPC/CH DSPC/CH EPC/CH/PA EPC/CH/SA DPPC/CH/SA DSPC/CH/SA	MLV	entrapped only	N.D.	sc.	Latif & Bachhawat, 1984
lysozyme	EPC/CH/PE	MLV	surface coupled only & entrapped only	N.D.	sc.	Latif & Bachhawat, 1987
ß-glucoronidase	DPPC/CH/PA	MLV	surface & entrapped	N.D.	sc., iv.	Hudson, et al, 1979
poly(LTyr,LGlu poly(DLAla) (LLys) synthetic peptide	DPPC/CH/DCP+ tocopherol	MLV	entrapped only	N.D.	ip., iv., id.	Lifshitz, et al,1981
MISCELLANEOUS						
Plasmodium falciparum	DPPC/CH	MLV	surface & entrapped	add. MDP	im.	Siddiqui, et al, 1978
R32tet32 tetrapeptide of CS protein in Plasmodium falciparum	DMPC/CH/DCP DMPC/DMPG/CH	MLV	surface & entrapped	lipid A, Alum	im., iv.	Richards, et al, 1988

TABLE I.4 (continued)

ANTIGEN	LIPID COMPOSITION	TYPE	LOCALIZATION (SURFACE OR ETRAPPED)	ENDOTOXIN OR OTHER ADJUVANT	ROUTE OF ADMIN.	REFERENCE
RT-1 transplantation antigen	DMPC DSPC DPPC	MLV	surface & entrapped	FCA.	ip.	Hedlund, et al, 1984
MDP	EPC/CH/PS	MLV	?	N.D.	iv., ip., sc., oral	Fraser-, Smith,et al 1983
ganglioside GM2	EPC/CH/SM EPC/CH/DCP	SUV	intercalated in bilayer?	add LPS, lipid A, FCA, BCG	sc.	Livingston, et al, 1987
O-antigen (Salmonellosis)	DPPC/CH EPC/CH	MLV	surface & entrapped	LPS is antigen	iv.	Desiderio & Campbell, 1985
estradiol	EPC/CH EPC/CH/DCP EPC/CH/SA	SUV	intercalated in bilayer?	N.D.	ip.	Latif & Mandal,1987
Streptococcus mutans poly-saccharide	EPC/CH/PA	MLV	surface & entrapped	N.D.	gastric int.	Bruyere,et al, 1987; Wachsman,et al, 1986; Wachsman,et al, 1985

TABLE I.4 (continued)

ANTIGEN	LIPID COMPOSITION	TYPE	LOCALIZATION (SURFACE OR ETRAPPED)	ENDOTOXIN OR OTHER ADJUVANT	ROUTE OF ADMIN.	REFERENCE
Streptococcus sobrinus ribosomal prep.	DPPC/CH/DCP	MLV	surface & entrapped	N.D.	gastric int.	Gregory, et al, 1986
Plasmodium yoelii	EPC	MLV	surface only & surface & entrapped	N.D.	sc.	Vinayak & Sharma, 1986

N.D. : not done or not determined.

Surface and entrapped antigen represents those cases where most of the antigen is inside the liposome but an unknown amount is on the surface.

high levels for longer times than unencapsulated protein. Small amounts of antigen induce an efficient immune response when associated with liposomes.

The use of liposomes in generating cytotoxic T cell responses will not be discussed in this review. This subject is treated in a review by Alving (1987). Induction of clinical immunity requires cell mediated responses. Humoral response to a viral infection, for example, may not be sufficient for protection.

The properties of the liposome carriers which are important in determining the adjuvant effect can be categorized into six factors, as shown in Figure I.6. The factors are interrelated, and, therefore, studies using the same antigen may show considerable divergence in results if all the conditions are not the same. The remainder of this section will focus on those studies which attempt to study each of the factors depicted in the figure.

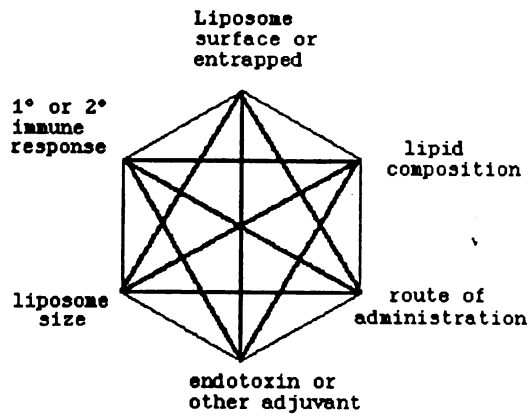


Figure I.6. Interrelationship of the important factors which have been found to influence liposome immunopotentiating activity in the immune response to protein antigens.

I.3.1 ENCAPSULATED AND SURFACE ATTACHED ANTIGENS: EFFECT ON IMMUNOADJUVANT PROPERTIES OF LIPOSOMES

The effect of the location of the antigen in the liposome on the resulting adjuvanticity is only now being resolved. Proteins have been covalently attached to, or non-specifically adsorbed to, the liposomal surface. Various techniques for the covalent coupling of proteins to liposomes have been developed. Excellent reviews have been written on these techniques (Machy and Leserman, 1987; Heath and Martin, 1986). The techniques fall into one of two categories: 1) coupling the protein to preformed liposomes or 2) coupling the protein to a lipid before formation of liposomes.

Proteins can also be entrapped within the aqueous spaces of the liposomes (Gregoriadis, 1984b). In either case, a portion of the protein may become embedded in the bilayer. The extent of bilayer association depends both on the charge and hydrophobicity of the protein and on the surface charge and lipid composition of the liposomes.

In studies before 1980, the distribution of protein antigen between the entrapped and surface bound forms was not determined. The first study which attempted to determine the amount of antigen remaining adsorbed to the surface of MLV was that using hepatitis B surface antigen (HBsAg) by Manesis and colleagues (1978; 1979). Entrapment of HBsAg resulted in 12-23% of the liposome associated material being adsorbed to the surface. The liposome-HBsAg preparations induced antibody production in guinea pigs earlier than free HBsAg. After a

second challenge the mean titer was 100 times greater in mice treated with liposome-HBsAg than in groups injected with free antigen.

Recognizing that most liposome encapsulated proteins consisted of a variable amount of surface adsorbed protein, experiments were done by van Rooijen and van Nieuwmegen (1980a) to investigate whether surface exposure or encapsulation of antigen was responsible for the immunoadjuvant properties of liposomes. Using human serum albumin (HSA) or bovine gamma globulin (BGG) as the antigens, the authors compared antibody titers in rabbits immunized with HSA or BGG free in solution, exposed on the surface of empty liposomes (adsorbed), or both exposed on and entrapped in liposomes (van Rooijen and van Nieuwmegen, 1982). Liposomes containing antigens in their trapped volume and a relatively low level of antigen on their surface induced an equivalent antibody response to that of antigen adsorbed to the surface of the liposome only. Six and colleagues (1980), using adenovirus purified type 5 subunits, proposed that only entrapped antigen is responsible for the enhanced response seen with liposomes. It may be that a small undetectable amount of protein remains associated on the surface of the liposome and is responsible for the response.

Shek and Sabiston (1982) attempted to reconcile the controversy between entrapped and surface presentation and suggested that only entrapped BSA elicited the enhanced antibody response. In their study, bovine serum albumin (BSA)

adsorbed to the surface of empty liposomes or BSA adsorbed onto empty liposomes and subsequently treated with trypsin, resulted in significantly lower antibody responses than BSA adsorbed on and entrapped in liposomes. The latter group was treated with trypsin to remove surface adsorbed BSA. Other studies in favor of the immunopotentiating effect of entrapped antigen are those of Nakao and coworkers using solubilized T2-phage antigens (Nakao et.al., 1981).

Since surface adsorption of protein molecules on liposome surface via hydrophobic and/or electrostatic interactions varies considerably, it is difficult to control the distribution of antigen on the liposome surface. Furthermore, nonspecifically adsorbed material may dissociate in vivo. This also makes interpretation of the results difficult.

The role of surface association of antigens on the immune response to liposomal antigen was further investigated by more stable, covalent liposome antigen preparations. Shek and Heath (1983) demonstrated a strong antibody response to BSA covalently coupled to the surface of liposomes. The antibody response to surface-coupled BSA was comparable to that obtained with encapsulated BSA (Shek and Sabiston, 1981) in terms of magnitude and kinetics. Davis and Gregoriadis (1987), using immunopurified tetanus toxoid, also found that equivalent responses were measured whether the antigen was covalently attached on the surface of liposomes or encapsulated. However, in both the latter (Shek and Heath,

1983) and former study (Davis and Gregoriadis, 1987), a small amount of surface adsorbed antigen remained in the preparation of liposome entrapped antigens.

Those studies which concluded that surface or encapsulated antigen gave a similar response were not supported by Latif and Bachhawat (1987) and Vannier and Snyder (1988). In the former study (Latif), lysozyme covalently coupled to the surface of liposomes resulted in greater antibody titers than entrapped or free lysozyme. In the latter study (Vannier), the antibody response to egg albumin encapsulated in or covalently bound to the surface of liposomes was dependent on the number of immunizations and the route of administration. In all cases but one, the highest titers of antibody were elicited with liposomes having egg albumin conjugated on their surface. In the case of multiple sc. administration, no difference was observed for free, entrapped in, or surface conjugated liposomes. Whether encapsulated or surface localized antigen yields the greater stimulation of antibody production, appears to depend upon the antigen studied and the route of administration.

I.3.2 EFFECT OF LIPID COMPOSITION ON THE ADJUVANT EFFECT OF LIPOSOMES

Manipulation of the phospholipid composition of liposomes is a useful and simple tool to study the adjuvant effect of liposome associated antigen. Among the earliest studies (Allison and Gregoriadis 1974; van Rooijen and van

Nieuwmegen, 1980b), lipids were chosen on the basis of providing a positive, neutral, or negative surface charge. In many cases, a large portion of the protein remained adsorbed to the surface of the liposome depending on the charge affinity of the protein. Diphtheria toxoid (DT), for example, was more efficient in eliciting antibody responses when associated with negative liposomes (Allison & Gregoriadis, 1974). It was proposed by van Rooijen (van Rooijen and van Nieuwmegen, 1982) that DT has a stronger affinity for negative surfaces thus more antigen adsorbs to these liposomes than to positive or neutral ones. Positively charged liposomes with very little adsorbed DT failed to elicit any response.

The most thorough studies dealing with the effect of liposome composition on the immune enhancing effect of liposomes were those of Bakouche and Gerlier (1986b), Davis and Gregoriadis (1987), and Gregoriadis et. al. (1987). Bakouche and Gerlier (1986b), using Gross cell surface antigen associated with liposomes, measured optimal antibody responses with bilayers composed of neutral phospholipids with high Tc as the major component (DPPC, DSPC). In addition, optimal immunogenicity required 20 mole% cholesterol and a negatively charged phospholipid as the minor component. Optimal responses were obtained when the neutral phospholipid was DSPC (Tc=54°C). These studies were supported by using human colon tumor cell membranes which demonstrated that the immunogenicity of the preparations was

greater for rigid than fluid liposomes (Raphael and Tom, 1982, 1984). However, in the latter studies, a cellular rather than a humoral response was studied. The requirements of the liposomal membrane may differ depending on the type of immune response desired. In addition, the latter studies were carried out in vitro.

The opposite effect of bilayer fluidity on the immune response to protein antigens was observed with tetanus toxoid (Gregoriadis et.al., 1987; Davis and Gregoriadis, 1987). The antibody response to tetanus toxoid, either entrapped or covalently anchored on the surface of liposomes, was similar when phospholipids with varying Tc from -32°C to 41.5°C but decreased significantly when DSPC (Tc=54°C) was used. The reason for the discrepancy, as suggested by Davis and Gregoriadis, may be due to differences in the lipid to protein ratios used in each of the studies. Davis and Gregoriadis (1987) demonstrated that the adjuvanticity of liposomes follows a bell shaped dose response curve. As the lipid to protein ratio of the dosage form increases, the antibody response increases until a lipid to protein ratio of 90,000 : 1 (w/w) is reached, above which the antibody response decreases. The nature of the antigens may also influence the requirements of phospholipid composition for optimal response.

In the case of hapten-N-substituted phosphatidyl-ethanolamine (PE) derivatives, such as DNP-Cap-PE, inserted in the bilayer of liposomes, humoral antibody responses have

been found to be dependent on a number of factors, including phospholipid composition. For DNP-Cap-PE containing liposomes, the rank order of phospholipids going from high anti-DNP responses to low response is: DSPC (Tc= 54°) > SM (Tc= 39°) > DMPC (Tc= 23°) > DLPC (Tc= 0°) > EPC(Tc= -10°) > DOPC (Tc= -22°). These authors suggested that the humoral response of DNP-Cap-PE sensitized liposomes is a function of bilayer fluidity with 'solid' liposomes being more effective immunogens than 'fluid' liposomes. The presence of 0-50% cholesterol did not alter this rank order (Kinsky, 1978; Yasuda et. al., 1977b; Dancey et. al., 1977). The latter result is in sharp contrast with tripeptide-enlarged DNP (J-PE) liposomes in which an 'intermediate fluidity' of the bilayer is required for optimal humoral responses to haptenated liposomes. J-PE liposomes prepared with DSPC were very poor immunogens (van Houte et. al., 1981b).

The results from the various studies indicate that different requirements for the optimal lipid composition exist depending on the type of antigen. However, consideration must be given to the fact that the studies discussed were all done with different routes of administration. The discrepancies discussed may have been due to varying the routes of administration rather than differences in lipid composition. Studies by Bakouche and Gerlier (1986b) were done sc., those by Davis and Gregoriadis (1987) and Gregoriadis and coworkers (1987) im., Kinsky (1978) ip., and van Houte et.al.(1981b) iv. The effect of the

route of administration on the liposome adjuvant effect will be discussed in the following section (III.3).

It has been observed that certain lipids suppress the immune response. For example, replacing phosphatidylcholine head groups with phosphatidylethanolamine headgroups, Bakouche and Gerlier (1986b) demonstrated that the immune enhancing effect of the liposome to Gross cell surface antigen is abolished. In the case of dipalmitoyl PE (DPPE) groups, it was suggested that toxicity for macrophages, in vitro, was responsible for the immune suppressive effect seen with liposomes. Dimyristoyl PE (DMPE), however, was non-toxic in vitro for macrophages. In a subsequent study, Bakouche et.al. (1987a), suggested that the inhibitory effect seen with DMPE containing liposomes was linked to the fact that DMPE induces the secretion of inhibitory prostaglandins by macrophages. The use of phosphatidylinositol (PI) (Wassef et.al., 1984) when inserted in liposomes specifically suppresses macrophage phagocytic function in vitro. It is possible that use of PI-containing liposomes could suppress the immunoadjuvant effect in vivo. In one case, cholesterol has been implicated in immunosuppression. Cholesterol can be readily oxidized to give the highly immunosuppressive steroid 25-hydroperoxycholesterol and as documented by Humphries (1981), liposomes containing cholesterol that is not pure and kept under N_2 can be immunosuppressive.

In an interesting study by Grover and Sundharadas (1986), evidence was provided in which cells enriched in B

cells but not T cells, were induced to proliferate in vitro in a manner resembling LPS. The proliferation of B cells depended on the lipid composition and the method of preparation. Liposomes composed of phospholipids with decreasing Tc (increasing membrane fluidity) demonstrated a corresponding decrease in mitogenic activity. In the same study, the liposome preparations were not tested for the presence of endotoxin, a potent B cell mitogen. It is possible that the results observed were due to endotoxin contamination.

In vivo applications in which an immune response to a liposome associated protein is not desired may warrant the use of immune suppressive lipid compositions. An example of this would be in the case where the goal is to target liposomes carrying anti-cancer drugs via surface coupled immunoglobulins specific for the target site.

I.3.3 EFFECT OF THE ROUTE OF ADMINISTRATION ON THE IMMUNOPOTENTIATING EFFECT OF LIPOSOMES

The effect of the route in which the liposome/protein complex is administered to animals on the immune response may be related to the in vivo disposition of liposomes(I.1.3). It is reasonable to suggest that differences in pharmacokinetic parameters as a result of the choice of administration will result in differences in immunoadjuvant properties.

Upon close inspection of Table I.4, it is clear that very few studies were done which compared routes of

administration on the immunoadjuvant effects of liposomes. The first such study utilized DT as the antigen (Allison and Gregoriadis, 1974; Gregoriadis, 1977). Administration of DT in negatively charged liposomes elicited greater immune responses in vivo than free DT when administered iv., im., or sc.. The most effective route of administration differed, depending on the type of immune response being observed. In the primary humoral response, all routes of administration elicited strong antibody responses, although the iv. route was the best. After a booster administration, detectable secondary responses were measured for sc. and im. routes only.

Vannier and Snyder (1988) suggested that the mechanism of liposome immunopotentiation may be different for different routes of administration. A single sc. injection of egg albumin bound to the surface of liposomes elicited prolonged antibody responses, suggesting a slow release or 'depot' effect for the liposome bound antigen. After multiple sc. injections, the results suggested that free antigen elicited stronger humoral responses than the same amount of antigen bound to the surface of liposomes. When multiple injections were made iv. or ip., however, the liposome bound antigen was superior. Furthermore, encapsulation of egg albumin significantly decreased the response to sc. or ip. administration.

Lifshitz and coworkers (1981) studied secondary responses to the synthetic polypeptide TGAL entrapped in

liposomes. The authors demonstrated that the ip. route elicited the greatest antibody responses followed by the iv. route. Intradermal (id.) administration was the least efficient route of administration except when FCA was co-administered. The results of Vannier and Snyder (1988) are consistent with those of Lifshitz and coworkers (1981), in that when the antigen was entrapped in the liposome the sc. route was the least efficient in immunopotentiation. The immunopotentiating properties of liposomes were dependent on whether the antigen was exposed on the surface of the liposome or entreapped. Furthermore, different patterns in immunopotentiation were observed depending on the route of administration.

The efficiency of the sc. route in the immunopotentiating effect of liposomes is also dependent on the liposome composition. The liposomes which stimulated the immune response most efficiently were those composed of postively charged lipid head groups, whether fluid or solid (Latif and Bacchaawat, 1984). In the case of lysozyme, a histological exam of the site of a sc. injection with lysozyme entrapped in liposomes demonstrated that positively charged liposomes, regardless of transition temperature, elicited granulomas at the site of administration. Non-charged liposomes whether fluid or solid, and negatively charged fluid liposomes, did not induce granulomas at the site of injection. The authors proposed that positive

liposomes are more slowly cleared from the site than are neutral or negatively charged fluid liposomes.

Clearance of liposomes from injection sites has been studied by Kramp and colleagues (1982). In their studies with adenovirus type 5 hexon, the clearance of antigen from the site of im. and id. injections was diminished by encapsulation in MLV liposomes. This result indicated a slow release or depot effect. This does not fully explain the immunopotentiation effect measured, since high antibody titers persisted much longer (56 days) than the persistence of the MLV-hexon in the injection site (16 days). Interestingly, an increase in macrophages in the site of injection was observed. The authors proposed that intact liposomes were taken up by these macrophages, thereby sequestering the antigen for slow release. The digested antigen might be either released at the site or in the draining lymph nodes after the cells migrated out of the injection site.

Several studies have demonstrated that orally administered liposomal antigens are effective in potentiating secretory IgA immune responses and protecting from a subsequent challenge dose of infectious agent (Gregory et.al. 1986; Wachsman et.al. 1985, 1986; Bruyere et.al., 1987). A more extensive review of this route of administration was published by Alving (1987).

I.3.4 EFFECT OF OTHER ADJUVANTS ON THE IMMUNOPOTENTIATING PROPERTIES OF LIPOSOMES

Among the most widely studied adjuvants which have been used in combination with liposomes are: 1) Freund's complete adjuvant (FCA), 2) Lipopolysaccharide (LPS) and its fragments such as lipid A, and 3) muramyl dipeptides (MDP) and derivatives.

In the majority of cases, optimal antibody responses were measured by combining FCA with liposome-associated protein antigen rather than without FCA (Neurath et.al., 1984; van Rooijen et.al., 1982; Gerlier et.al., 1983; Sanchez et.al., 1980; Watari et.al., 1987; Hedlund et.al., 1984). Due to serious adverse effects, including the induction of autoimmune reactions and the formation of granulomas, the use of FCA in vivo is not desirable (Alving, 1987).

The incorporation of endotoxin (LPS) or lipid A with liposomal protein antigens has been studied in various laboratories (Yasuda et.al., 1984; Morgan et.al., 1984; Naylor et.al., 1982; van Rooijen and van Nieuwmegen, 1980c; van Rooijen et.al., 1982; Alving et.al., 1986; Pierce et.al., 1984). Much of the work in this area has been done using hapten antigens and thorough reviews have been written on this topic (Kinsky, 1980). There is general agreement that in the case of either hapten or protein antigens, the immune response is considerably enhanced by incorporation of lipid A in liposomes. The enhanced response is a result of the simultaneous expression of the adjuvant and the antigen on

the same liposome structure. In some instances (van Rooijen and van Nieuwmegen, 1980c; 1982), co-administration of liposomes containing either LPS or protein antigen yielded an equivalent immune response to liposomes which simultaneously contained LPS and antigen.

Certain consequences of using LPS or lipid A in liposomal preparations to enhance immune responses must be considered when developing vaccine systems. On the positive side, the toxicity of LPS (lipid A) is diminished by incorporation with liposomes. For example, lipid A causes neutropenia in rabbits, but neutropenia did not occur with liposomes containing lipid A (Ramsey et.al., 1980). Other studies (Desiderio and Campbell, 1985; Richards et.al., 1988) also supported the decreased in vivo toxicity of liposome associated LPS. In a series of in vitro studies, it has been demonstrated that incorporation of LPS or lipid A in liposomes abolishes their ability to induce IL-1 activity (Dijkstra et.al., 1987; Bakouche et.al., 1987c). This finding may be related to the reduced in vivo toxicity of liposome encapsulated endotoxin.

On the negative side, LPS or lipid A, polyclonal B cell activators, incorporated in the liposomal bilayer, or even in the absence of liposomes, can render liposomes immunogenic by inducing anti-liposome antibodies (Banerji and Alving, 1981; Alving and Richards, 1983; Fogler et.al., 1987).

The use of lipophilic derivatives of MDP in conjunction with liposomal antigens has also been found to significantly

enhance the immune response to the antigen (Siddiqui et.al., 1978; Tsujimoto et.al., 1986; Kotani et.al., 1977; Alving et.al., 1986; Audibert and Chedid, 1980). MDP and its analogues have been shown to be potent inducers of macrophage activation (Nayar and Fidler, 1985; Fidler and Poste, 1982). Thus, the basis of the immune enhancement may be related to enhanced antigen presenting functions of the macrophage.

The nature of the antigen influences which adjuvant elicits the most efficient immune response. Sanchez and coworkers (1980) using HBsAg-derived antigens showed that aluminum gel appeared to be a more efficient adjuvant for the HBsAg particles than were liposomes. On the other hand, liposomes rendered the soluble polypeptide fragments of the virus more immunogenic than did aluminum gel. Richards and colleagues (1988) tested the immunogenicity of a recombinant peptide repeat sequence from the circumsporozite of malaria in various liposome formulations. Antigen entrapped in liposomes with and without lipid A, or liposomes with or without lipid A adsorbed on alum, were tested for immunogenicity. The strongest antibody response was obtained by administration of alum-adsorbed liposomes containing lipid A and the peptide antigen.

Liposomes have been also found to be effective vehicles for the delivery of other types of adjuvants such as saponin (Scott et.al. 1985) and avridine (Pierce and Sacci, 1984). In the case of avridine, an increased sIgA response and memory to cholera toxin was observed when incorporated in liposomes.

Approximately 5 to 7 fold less antigen was needed to induce antibody response when injected with Avridine containing liposomes than without. Saponin itself causes inflammatory reaction at the site of injection. When injected with liposomes rich in cholesterol, the inflammatory response to saponin is inhibited and the efficiency in stimulating the immune response is increased. Liposomes also abolished both the increased retention of antigen at the site of injection and increased localization in spleen but did not alter the adjuvanticity of the protein.

I.3.5 EFFECT OF LIPOSOME SIZE ON THE IMMUNOPOTENTIATING PROPERTIES OF LIPOSOMES

The size of the liposomes affects the ability of cells to take them up (Section I.1.3). This, in turn, will influence their circulation lifetime. Liposome size can also influence the rate of clearance from a sc. injection site. For example, a large liposome may allow the retention of liposome associated molecules at the site of injection more so than a smaller liposome. As a result, liposome size may influence the immunopotentiating properties in the immune response to protein antigens. Depending on the mechanism by which liposomes enhance immune responses and the cell types involved in the immune response, liposome size is yet another parameter to be considered in the development of liposomal vaccine systems.

Most studies which demonstrate immune enhancing properties for liposome-associated protein antigens fail to control liposome size. This may be due to limitations on the encapsulation efficiency of proteins in small liposomes. In most studies, MLV are used without extrusion and are large and heterogeneous in size. This permits high levels of protein incorporation. Francis and coworkers (1985) tested the antibody response to foot and mouth peptide fragment encapsulated in MLV or SUV liposomes. The authors did not measure the diameter of the liposomes nor determine how much peptide remained adsorbed to the surface of each liposome preparation. As discussed previously (section I.3.1), the amount of surface adsorbed antigen may influence the immune response. The authors proposed, without providing evidence, that SUV targeted the peptide to antigen presenting cells more efficiently than MLV due to their longer circulation lifetime. Shek and coworkers (1983), in a more carefully controlled study, demonstrated that the lamellar arrangement rather than the size of liposomes may play a role in affecting the magnitude of the antibody response. The vast majority of studies clearly demonstrate that a more thorough characterization of liposome preparations is in order before a definite statement can be made on the role of liposome size in the adjuvant effect of liposomes.

I.3.6 INDUCTION OF PRIMARY AND/OR SECONDARY ANTIBODY RESPONSES BY LIPOSOME-ASSOCIATED PROTEIN ANTIGENS IN VIVO

In the development of liposomes as carriers for vaccines, it is of practical importance to improve the immunogenicity in both primary and secondary responses. The induction of memory, required for long lasting protection, is regarded as a shift towards thymus dependence. The aim of this section is to list those studies which have measured the immunopotentiating properties of liposomes in inducing primary (1°) or secondary (2°) immunity to the protein antigen and whether the immune enhancement is general or specific for antibody subclasses.

Allison and Gregoriadis (1974) demonstrated that both the 1° and 2° immune response against diphtheria toxoid were enhanced when the toxoid was associated with liposomes. The booster injections (secondary immunizations) were done with the same form of antigen, either liposomal or free, as was done in the primary injection. In order to study the specific effect of liposomes on the generation of immunological memory, van Rooijen et.al. (1981) determined that priming with liposome associated HSA, elicited a stronger 2° response than priming with HSA free in solution. In these studies, booster injections were given as antigen free in solution whereas priming injections were given as either free in solution or liposome associated. In the case of horse radish peroxidase (HRP), a 2° response was found after priming only with liposome associated HRP, even though a 1° response for

the liposome associated HRP was not evoked (van Rooijen et.al., 1981).

Liposome-associated BSA was found to be a potent stimulator of immunological memory as demonstrated by the ability of liposomal-BSA primed animals to evoke a strong 2° response upon challenge with free BSA. Priming with free BSA or free BSA and empty liposomes failed to elicit a 1° or 2° response (Shek and Sabiston, 1981). Examination of the class of antibody induced in mice immunized with liposome-BSA showed an early low IgM and a sustained higher IgG response primarily due to IgG1 subclass (Beatty et.al., 1984).

The T cell dependency of BSA was not altered by its association with liposomes. Liposomal BSA or free BSA did not induce immune responses in Balb/c (nu/nu) mice (T cell deficient) (Shek and Sabiston, 1981; Beatty et.al., 1984).

In early studies by van Rooijen and coworkers (1981), the titers of antibody were measured by the haemagglutination technique which only measures IgM production. In subsequent studies, the authors measured both IgM and IgG antibodies after administration of liposome-associated HSA. Both IgG and IgM production against liposomal HSA is enhanced in the 1° response. After the priming injection with liposome associated HSA, liposomes did not further enhance the 2° response when also used in booster injections (van Rooijen and van Nieuwmegen, 1983; van Rooijen et.al., 1984). Control of the immunoglobulin isotype depended upon the location of the antigen in the liposome. Either a predominantly IgM or

IgG response could be induced depending on whether the antigen was administered on the surface of or entrapped in liposomes, respectively (van Rooijen, 1988).

Bakouche and Gerlier (1986b) and Davis and coworkers (1987) showed that the adjuvant effect of liposomal antigen was elicited during the 1° administration as measured by increased titers of antibody. In the latter study, 2° responses to tetanus toxoid were independent of the form in which the antigen was delivered (free or liposomal) but were a function of dose.

Oral administration of liposome associated *S. mutans* evoked a strong 1° response in salivary IgA. Upon boosting with appropriate antigen, a stronger 2° IgA response was also observed in saliva. Serum levels of IgA and IgG were comparable to those of salivary levels (Wachsman et.al., 1985; Gregory et.al., 1986).

In contrast to protein antigens, liposomes prepared by incorporation of the hapten modified PE group (DNP-Cap-PE) induce a thymus independent hapten-specific humoral response (Yasuda et.al., 1977a). Moreover, the immunogenicity of liposomal haptens is not altered when administered in mice lacking or deprived of thymus derived cells (van Houte et.al., 1979). Induction of IgM memory to haptenated liposomes was possible in normal mice but not in congenitally athymic mice (van Houte et.al., 1981).

SECTION I.4: MECHANISMS

The study of mechanisms of the mode of action of adjuvants requires: 1) The identification of the target cell(s) on which the adjuvant acts, 2) The distribution of the adjuvant in relation to the location of target cell(s), and 3) The target cell functions affected by the adjuvant.

A list of mechanisms which have been proposed for various types of adjuvants is shown in Table I.5. This information is taken from a review by Waksman (1979). Clearly, the number of possible mechanisms is great. Depending on the adjuvant, a wide range of immune responses to an antigen may be induced whereas for other types of adjuvants, more selective responses may be achieved (see also: Allison, 1979; Osebold, 1982).

The same situation exists in the case of the immunopotentiating mechanisms of liposomal adjuvants. The complexity of the in vivo immune response and that of the liposome antigen formulation itself create the possibility for various interdependent mechanisms in the enhancement of the immune response.

The major mechanisms for the liposome adjuvant effect can be categorized into two groups: 1) The direct or indirect action on immunocompetent cells or 2) Depot effects and/or slow release of antigen.

Table I.5: Targets of adjuvant action

Function of adjuvant	Examples
Depot, dissemination of antigen	Mineral oil
Selective antigen localization	Alkylamines, nonpolar cationic detergents
Particles bearing antigen	$Al(OH)_3$, $AlPO_4$, immune complexes, latex,
Depot function	bentonite, polymethyl methacrylate
Enhanced macrophage uptake	
Direct B-cell triggering	
Stimulation of accessory cells	Triolein, zymosan, carbonyl iron
Increased antigen uptake	BCG, CP, LPS, retinol, polyanions
Production of helper factors	polyelectrolytes, mineral oil
Production of factors affecting lymphocyte recirculation	
Production of colony-stimulating activity	
Production of complement components	
Enhanced lymphocyte flux into TDA	Pertussis, FCA, IFA, CP, retinol
Non-specific stimulation of T-cells	pA:U, MDP
Carrier-specific stimulation of T-cells	CFA, some viruses
Non-specific stimulation of B-cells	LPS
IgE switch of B-cells	Nipposstrongylus, other helminths
Enhanced maturation of T and B precursors	LPS, pA:U
Direct stimulation of NK cells	Arenaviruses, interferon, tolorone, statolone, lentinan
Direct stimulation of K cells	FCA
Cell damage and release of nucleotides	PHA, Con A, colchicine, silica
Elimination of supressor cells	X-ray, cyclophosphamide

Modified from: Waksman, 1979.

I.4.1 EFFECTS MEDIATED THROUGH IMMUNOCOMPETENT CELLS

I.4.1.A Macrophages

The role of macrophages in the stimulation of immune responses by adjuvants has been described by Unanue et.al. (1969). The ability of macrophages to phagocytose liposomal antigens and present the processed antigen to T-lymphocytes has been suggested to be responsible for the immunopotentiation of the humoral response to liposome-encapsulated protein antigens. Beatty and coworkers (1981) showed that liposomal BSA was taken up to a greater extent by macrophages than unencapsulated BSA. They proposed that the immune enhancement observed in vivo was due to the ability of the liposome to deliver more antigen to the macrophage. Later studies by the same group demonstrated that immunizing animals with liposomal BSA yielded an equivalent humoral response as immunization with peritoneal macrophages which had previously ingested liposomal BSA in vitro (Beatty et.al., 1984). In these cases, the liposome facilitated the uptake of antigen by macrophages. Similar conclusions were reached by Gerlier et.al. (1983).

Bakouche and Gerlier (1986b) proposed that phospholipid composition directly affected the uptake and processing of antigen by macrophages, thereby influencing the magnitude of the antibody response. Drastic differences in the rate and extent of processing of liposomal antigen vs. unencapsulated antigen by macrophages were measured in vitro. Liposome compositions which resulted in the greatest

immunopotentiation in vivo demonstrated the greatest degradation of liposome associated antigen when incubated with the J774.1 macrophage tumor cell line in vitro (Bakouche et.al., 1987b).

Early studies by van Rooijen and van Nieuwmegen (1979), and more recently by Zigterman et.al. (1987), proposed a different theory. Zigterman et.al. suggested that enhanced antibody responses are achieved by diminishing the ability of the macrophage to degrade the antigen. In the former study, inhibition of macrophage lysosomal activity was presumably achieved with the addition of lyso-PC to the liposome composition, while in the latter, a nonionic block polymer, which is cytotoxic to macrophages, reduced the elimination of the liposome entrapped antigen.

The effect of certain lipid compositions, such as DMPE and PI, on macrophages were discussed in a previous section. These studies reinforce the importance of the liposome-macrophage interaction in the generation of immune responses to protein antigens.

In vitro systems and the use of animals depleted of particular cell types were introduced by various researchers as a means to further delineate the cell types involved in the immune response to liposomal antigens.

Raphael and Tom (1984) demonstrated that the deletion of spleen adherent cells from an in vitro assay system which measures T cell cytotoxic activity completely abrogated the immune response. The response could be reconstituted by

adding spleen adherent cells, but only from mice which were histocompatible with the T cytotoxic cells. Similar results were reported by Bakouche and Gerlier (1986a). These studies show that antigen presentation in an MHC-restricted fashion requires adherent cells (macrophages). The results imply that the liposome acts by modifying the uptake and processing of the antigen by macrophages to promote specific stimulation of lymphocytes.

Establishment of the direct involvement of macrophages in the immune response to liposomal protein antigen was demonstrated in vivo by the administration of carrageenan (CGN) prior to the liposome antigen administration (Shek and Lukovich, 1982). As a result of pretreating the animals with CGN, the antibody response to BSA administered entrapped in liposomes was abolished. The CGN sensitive cells were morphologically and functionally macrophage-like. No effect on T or B cells was observed. The authors are careful to point out that the effects of CGN on other antigen presenting cells (such as dendritic cells) could not be excluded.

I.4.1.B: Antigen presenting cells other than macrophages

In a series of studies by Classen et.al. (1987), a unique approach to study the role of macrophages on the liposome adjuvant response was employed. The rationale was to specifically eliminate the cells which are responsible for taking up liposomal antigens without influencing other

immunocompetent cells and then testing these 'compromised' animals for their ability to make an antibody response to liposomal antigen. Dichloromethylene diphosphonate (DCMDP) entrapped in liposomes and injected iv. could eliminate cells which take up liposome. When the DCMDP was co-entrapped with BSA, and injected iv., cells which are involved in the uptake of liposomal antigen were eliminated. The authors discovered that local macrophages were important for inducing immune responses and the uptake and processing of liposomal antigen. They found that the primary anti-BSA response was markedly depressed but the secondary immune response remained unchanged. They concluded that the adjuvant activity (and memory function) of liposomes with surface exposed antigen occurs irrespective of the presence or absence of splenic or liver macrophages. This implied that macrophages at sites other than the spleen and liver or other antigen presenting cells such as B cells or dendritic cells were responsible for processing and presentation of liposomal antigen. Cells other than those involved in liposomal uptake played a significant part in the immune response to liposomal antigen (van Rooijen and Su, 1989).

The involvement of cell types in the immune enhancing effects of liposomal antigen other than macrophages was demonstrated with a similar approach, in which the anti-proliferative agent methotrexate (MTX) was encapsulated in liposomes which had BSA covalently coupled on their surface. In vivo administration of these liposomes resulted in a

suppressive effect on the antibody response to BSA. It was concluded by the authors that administration of liposomes with BSA on the surface does not affect macrophages (non-proliferating cell) and thus could have affected lymphocytes directly (Shek et.al., 1986b).

The ability of liposomes to interact with B lymphocytes directly had been demonstrated for the T-independent hapten-modified phospholipids, such as DNP-Cap-PE, on the surface of liposomes (Kinsky, 1978; van Houte et.al., 1979). It was suggested by Davis et.al. (1987) that direct interaction between a T-dependent protein antigen and an immunoglobulin surface receptor on B lymphocytes may occur and the chance of this would be greater if the protein was exposed on the surface of the liposome. Covalent attachement of tetanus toxoid to the surface of liposomes failed to elicit an increased secondary response over entrapped antigen. The authors suggested that it is only the primary response in which the adjuvant effect was observed, i.e., when macrophages are the predominant antigen presenting cell type.

I.4.1.C Effects on T cells

The encapsulation of protein antigens in liposomes maintains the T cell dependency of the protein antigen. This was demonstrated independently by Shek and Sabiston (1981) and Beatty et.al.(1984). Using thymectomized or congenitally T cell-deficient mice, administration of liposome BSA failed to induce an antibody response to the liposomal antigen

whereas normal mice or mice reconstituted with syngeneic T cells showed normal and enhanced antibody responses over unencapsulated antigen.

There have been a few reports in which direct stimulation of T helper cells was achieved with liposomes with covalently coupled antigen at a high density on their surface. For instance, in studies by Walden and coworkers (1985, 1986a, 1986b), pure T cells could be stimulated, in vitro, without the need for antigen presenting cells. This phenomenum has not been well studied and may not play a significant role in vivo.

In more recent studies (Oth et.al., 1987; Mansour et.al., 1988; Perrin et.al., 1988), antigen specific T helper cell responses were measured in vitro as a result of the administration of liposome associated antigen. It was demonstrated that production of IL-2 was enhanced after administration of liposomal antigen, but not by free antigen.

I.4.2 MECHANISMS BASED ON DEPOT/SLOW RELEASE OF ANTIGEN

The mechanism of action of the enhancement of humoral immunity induced by liposomes appears to be due in part to their function as a depot for antigen. Liposome adjuvant effects due to depot or slow release of antigen can be further divided into two types, namely depots of antigen at the site of injection and depots of antigen within intracellular sites.

Depots of antigen at the site of injection which are slowly released are, conceptually, similar to mechanisms proposed for adjuvants like Complete Freund's adjuvant. Various authors have proposed depot-based mechanisms for liposomes (Sarkar et.al., 1982; Gerlier et.al., 1980; Lifshitz et.al., 1981; Latif and Mandal, 1987; LeGrue, 1984; Latif and Bachhawat, 1984; Kramp et.al., 1982). In general, association of antigen with liposomes promotes retention of antigen at the injection site or other extracellular sites. The antigen is then slowly released from the site. Immuno-competent cells may be recruited to the site, thereby prolonging the effect on the immune response. As discussed by Kramp et.al. (1982), the depot effect does not fully explain the immunopotentiation, since the immune enhancement can sometimes be detected for a longer time than the antigen can be detected at the injection site. It has been suggested that some of the antigen migrates, via macrophage-like cells which infiltrate the site and endocytose the liposomal antigen, to distant lymph nodes where the antigen stays at intracellular sites for prolonged periods. Thus, the depot is not physically at the same site where the adjuvant was injected.

Intracellular depots of antigen have been favored in several studies (Desiderio and Campbell, 1985; Beatty et.al., 1981; Gregoriadis et.al., 1987; Zigterman et.al., 1987; Gerlier et.al., 1983; Kramp et.al., 1982). These mechanisms are based on the fact that the interaction between macrophages and liposomes is a critical step in the immune

stimulation observed in vivo. After endocytosis of liposomal antigens by macrophages the antigen is concentrated within intracellular sites. These cells can transport the liposomal antigen to sites rich in lymphocytes, degrade the liposomal carrier, and process the antigen for stimulation of lymphocytes. Again, the rate and extent of each step is dependent on the liposomal characteristics.

I.4.3 OTHER MECHANISMS

There must, however, be other mechanisms of action than the ones described in the previous sections. Antigens entrapped in liposomes, unlike antigens injected with aluminum salts or oil emulsions, can elicit cellular as well as humoral immunity (Lawman et.al., 1981; Sanchez et.al., 1980). In the case of the immunopotentiation of cytotoxic T cells (CTL) by liposomal antigen, a mechanism proposed by various researchers involves the ability of the liposomal surface to mimic the membrane topography of the natural antigen. The incorporation of membrane tumor antigens, for example, on the surface of liposomes may resemble the configuration of the antigen found on a tumor cell. The liposomal antigen may stimulate the CTL directly without the need for APC. Stimulation of CTL requires the co-recognition of MHC-encoded Class I glycoprotein and the antigen on the surface of APC for stimulation to occur. Thus, liposomal antigens would have to be delivered along with Class I macromolecules. It may be that the CTL response to liposomal

antigen is due to crudely prepared tumor antigens which may carry a significant amount of contaminating Class I glycoprotein.

Section I.5: RATIONALE FOR USING IN VITRO ANTIGEN PRESENTATION SYSTEMS TO STUDY THE LIPOSOME ADJUVANT EFFECTS

In his review of the mechanism(s) of adjuvant action, Waksman (1979) pointed out that the complexity of adjuvant effects has been underestimated due to the complexity of the immune response itself. As discussed in section I.3, the adjuvant effect of liposomes on the immune response depended on: 1) whether the antigen was surface exposed or entrapped in the liposome, 2) the lipid composition, 3) the route of administration, 4) whether the response was primary or secondary, 5) whether additional adjuvants were present in the liposome preparation, and 6) the size of the liposome. Comparisons between studies was difficult since the factors are interdependent.

In the last decade, advances in the study of antigen presentation by cellular imunologists have been made using in vitro systems. These are composed of purified cell lines which can be easily maintained in vitro. Most cell lines can be obtained through a commercial source (such as American Type Culture Collection). In those cases where there is no commercial source, techniques are available to isolate cells of interest.

The use of an in vitro antigen presentation system in the study of the liposome adjuvant effect is novel and, to date, the first of its kind in the literature. The antigen presentation system which we used in the current studies allows one to separate out those effects dependent on the function of the immunocompetent cells from effects due to the in vivo distribution of liposomes (pharmacokinetic). A better understanding of the cellular requirements and functions involved in the liposomal adjuvant effect will allow researchers to carry out better designed in vivo studies.

In a recent review by van Rooijen and Su (1989), the authors correctly pointed out that the study of the immune system belongs in vivo and not in vitro. However, a thorough understanding of the immune response is necessary to interpret the in vivo results. This understanding will be established by the utilization of simple, reproducible, and reliable in vitro systems such as those which have been developed by cellular immunologists to study the details of antigen presentation.

Section I.6. SCOPE OF THIS THESIS

An in vitro antigen presentation system was established to study the effect of liposome association of two model antigens, ovalbumin and cytochrome-c, on the IL-2 response by antigen specific T-helper cell hybridomas. The in vitro system was characterized for ovalbumin in terms of specificity, APC to T cell ratio, and Ia expression. The

effect of liposome type on the IL-2 response by cOVA-specific T cells was also measured (Chapter II). A more detailed evaluation of the liposome effect was undertaken using cytochrome-c as the model antigen. We studied: 1) the effect of liposome encapsulation on the IL-2 response when the liposomal antigen is presented by peritoneal macrophages or B-cell tumors (Chapter III), 2) The biochemical fate of the liposomal antigen when exposed to either macrophages or B cells (Chapter III), 3) the effect of lipid composition on the IL-2 response when the antigen is presented by peritoneal macrophages (Chapter IV), 4) the IL-2 response when the antigen was presented by macrophages from various sources and states of activation (Chapter V), and 5) antigen presentation by macrophages or B cells as a function of whether PCC, or its antigenic fragment, was exposed on the surface of the liposome or entrapped in the liposome internal volume (Chapter VI).

Chapter II: ESTABLISHMENT OF AN IN VITRO ANTIGEN PRESENTATION SYSTEM USING OVALBUMIN AS THE MODEL ANTIGEN

II.1: INTRODUCTION

A necessary first step in the induction of immune responses is the presentation of antigen by APC to T lymphocytes. The interaction between these cells is restricted by the MHC I-region and requires that the antigen be presented in conjunction with the Class II (Ia) glycoprotein on the surface of the presenting cell.

We established an in vitro antigen presentation assay to study the effect of encapsulation of protein antigens in liposomes. The assay system was originally developed by Zlotnik and coworkers (1983). The assay system is composed of an APC, an antigen-specific T-cell hybridoma, and a bioassay for the detection of IL-2.

The APC employed was the P388D1 macrophage tumor line. This cell line does not express Class II glycoprotein unless induced with gamma-interferon (γ-IFN)-containing supernatants from activated T cells. Expression of Class II by many macrophage cells has been found to be under regulatory control (Beller and Unanue, 1981). The antigen/Class II complex on the surface of the P388D1 is presented to an Ia^d-restricted chicken ovalbumin c(OVA)-specific T cell hybridoma (3DO54.8). Successful presentation results in the secretion of IL-2 by the antigen/Ia-stimulated T cell.

Our first objective was to characterize the antigen

presentation assay and confirm those results obtained by Zlotnik and coworkers (1983). Herein we show: 1) an excellent correlation between the expression of Ia on the P388D1 surface and its ability to induce IL-2 secretion by the T cell hybrid in the presence of antigen, 2) the specificity of the interaction was established using specific antibodies for the Ia glycoprotein and T cell hybrids of relevant and irrelevant specificity, 3) IL-2 response was dependent on the amount of antigen present in the culture well and the ratio of presenting cell to T cell hybrid. The optimal ratio was (2 : 1) P388D1 : 3DO54.8.

We next studied the presentation of cOVA when present in soluble form or encapsulated in liposomes. We demonstrated that the efficiency of the IL-2 response was enhanced by the encapsulation of cOVA in liposomes. The enhancement was dependent on the step which one adds the antigen. Increased efficiency of presentation was only observed when the liposomal antigen was preincubated with the APC and subsequently removing unbound liposomes from the medium before adding the T cells. Finally we demonstrated that the liposome is not unique in increasing the efficiency of presentation. A similar level of increased efficiency is also observed with denatured antigen.

II.2: MATERIALS AND METHODS

II.2.1: Chemicals and solutions:

Cholesterol (CH) was obtained from Sigma Chemical Co. (St. Louis, MO); egg phosphatidylcholine (EPC) and egg phosphatidylglycerol (EPG) from Avanti Polar Lipids, Inc.(Birmingham, Al). All reagents are of analytical purity or better. All buffers were filter sterilized through 0.22 μm filters (Millipore).

II.2.2: Protein antigens, mitogens, and antibodies

Concanavalin A (Con A) was obtained from E-Y Laboratories (San Mateo, CA); LPS Re595 mutant from *Salmonella minn.* (List Biological Laboratories, Campbell, CA); FITC-F(ab')2 fragment of goat anti-mouse IgG (heavy and light chain specific) was purchased from Cappel/Cooper Biomedical, Malvern, PA. Nonspecific IgG was a gift from Dr. T. Heath (UCSF Cancer Research Institute). Ovalbumin (cOVA) (type VII crystallized and lyophilized, free of S-ovalbumin), keyhole limpet hemocyanin (KLH), and BSA were obtained from Sigma. The peptide, P101, was synthesized by the Merrifield procedure (Merrifield, 1969). This peptide corresponds to a 17 amino acid region of native cOVA (Shimonkevitz et.al., 1984). The sequence of the peptide is H_2N-Ile-ser-gln-ala-val-his-ala-ala-his-ala-glu-ile-asn-glu-ala-gly-arg-COOH.

II.2.3: Liposome preparations

Liposomes containing cOVA were composed of EPC, EPG, and CH at a molar ratio of 9:1:8 were prepared by two different methods: 1) a modification of the REV method of Szoka and Papahadjopoulos (1978) extruded through 0.4 µm polycarbonate membranes (Nucleopore, Pleasenton, CA.) and 2) the dehydration-rehydration method (DRV) (Kirby and Gregoriadis, 1984). Briefly, the DRV procedure was as follows: Forty (40) µmoles of the lipid mixture was dried down as a thin film in a 50 cc round bottom flask under high vacuum at room temperature for 1 hour. The lipid was rehydrated with 2 ml of sterile water and vortexed for 5-10 minutes followed by sonication until clear suspension (~45 minutes). Freshly prepared cOVA solution (2 ml of 20 mg/ml in water) was added to the liposomes and then frozen as a thin shell using an isopropanol dry-ice bath. The material was lyophilized overnight and rehydrated with 200 µl water for 30 minutes followed with 2 ml of PBS. The liposomes were centrifuged at 10,000 X g for 15 minutes (2X) each time discarding the supernatant. The pellet was finally reconstituted with 2 ml phosphate buffered saline (PBS) and extruded through 1.0 µm membranes under high pressure. Non-encapsulated cOVA was separated from the vesicle-associated cOVA by gel filtration through a Bio-Gel A0.5M (Bio-Rad, Richmond, CA.) (1 X 10 cm) column. The liposomes were assayed for lipid content by phosphorous analysis (Bartlett, 1959) and protein content was measured by a modified Lowry assay (Markwell et.al., 1978).

II.2.4: Preparation of rat spleen cell Con A supernatants

The procedure for preparing the spleen cell supernatants (RCS) was a modified method of Steeg and coworkers (1980). Briefly, spleens were aseptically removed from female 250 gram Sprague Dawley rats (Charles River, Wilmington, MA). The spleens were pressed through sterile wire mesh screens with a plunger from a sterile 10cc syringe. The cells were collected and washed twice with ice cold HBSS. The cells were spun down and resuspended in 2 ml of red blood cell lysis buffer (90 ml of 0.16M NH_4Cl + 10 ml of 0.17M Tris pH 7.65, adjust to final pH 7.2 with HCl) for 2 minutes. Wash the cells 2 times with HBSS and resuspend at 1 X 10^6 cells/ml in 50cc conical tissue culture tubes. Add Con A to 2.1 µg/ml final concentration and incubate 48 hours, 37°C, 5% CO_2, 95% relative humidity. Spin the tubes, add supernatant to sterile bottles containing pre-swollen Sephadex G50 (Pharmacia, 1 ml/100 ml supernatant). This procedure will remove the Con A. Incubate at 37°C while stirring, spin, and filter through 0.45µm sterile filters. Divide into 10 ml aliquots and store at -20°C.

II.2.5: Cells, medium and incubation conditions

The cOVA-specific T-cell hybridoma, 3DO54.8 (H-2^d) and KLH specific BDK23.1 (H-2^d) T-cell hybridoma (Shimonkevitz et.al., 1983) were generously provided by Dr. Philippa Marrack (Department of Medicine, National Jewish Hospital and Research Center, Denver, CO.). The P388D1 (H-2^d) was obtained from the UCSF Cell Culture Facility and originally isolated

from a methylcholanthrene-induced neoplasm (P388) of a DBA/2 ($H\text{-}2^d$) mouse (Koren et.al, 1975). The CTLL-20 line, an IL-2 dependent cell line, was provided by Dr. Daniel Stites (University of California at San Francisco, CA). The MKD6 B-cell hybridoma which produces the anti-Ia^d antibody, was obtained from American Type Culture Collection.
The cell lines were tested and found to be free from mycoplasma contamination.

The P388D1 and the 3DO54.8 were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, INC. Logan,Utah), 2 mM glutamine, 25 mM N-2-hydroxy-ethylpiperazine-N'-2-ethane sulfonic acid, 1 mM pyruvate, 5×10^{-5} M 2-mercaptoethanol, and antibiotics (RPMI-CM). Fresh cultures of the cell lines were initiated from frozen (liq. N_2) samples of early passages every 20-25 passages. This was crucial since the IL-2 response was lost after prolonged subculturing (not shown). CTLL-20 were grown in Dulbecco's minimum essential medium supplemented with 5% FBS and 40% RCS as a source of IL-2. Media and FBS were obtained from the Cell Culture Facility, University of California at San Francisco. All incubations, unless otherwise stated were done at 37°C, 5% CO_2, 95% relative humidity.

II.2.6: <u>Induction of Ia: Fluorescence activated cell sorter measurements</u>

The P388D1 cells were seeded at 1×10^6 cells per dish

(35mm diameter tissue culture dish, Costar, Camridge, MA) in 1 ml of RPMI-CM. Four fold dilutions of the RCS in RPMI-CM were prepared and added to dishes with cells in duplicate culture per condition (1 ml /dish). The cells were incubated for 24 hours, washed, scraped with a rubber policeman, centrifuged, and reconstituted with 5 ml of RPMI-CM.

Incubations with anti-Ia^d antibody (5 µl of a ~ 1mg/ml solution in PBS) were done in an ice bath for 30 minutes. The antibody was isolated from ascites fluid of Balb/c mice injected *i.p.* with MKD6 B cell hybridomas using Protein A sepharose (CL4B, Pharmacia) column chromatography. After the incubation with the anti-Ia^d antibody, the cells were washed with 4%FBS-PBS and incubated with a dilution of FITC-goat anti-mouse IgG for 30 minutes. The cells were washed thoroughly and analyzed by fluorescence activated cell sorting (FACS).

II.2.7: Antigen presentation assay

Unless other wise stated, the P388D1 were seeded at 1 X 10^5 cells/well (96-well, flat bottom wells; Costar) in 100 µl RPMI-CM. The cells were induced for expression of Ia with RCS to the desired level as determined by the FACS assay. After overnight incubation, the cells were washed twice with ice-cold HBSS and 100 µl of fresh RPMI-CM was added to each well. The 3DO54.8 were added to the P388D1 culture at 1 X 10^5 in 100 µl volume. Two different antigen pulsing techniques were used: 1) graded doses of cOVA antigenic forms were added to

the P388D1 in 50 µl aliquots simultaneously with the RCS addition. The incubation was washed after 24 hours to remove unbound antigen. This is referred as the 'preincubation' method; 2) graded doses of cOVA antigens were added in 50 µl aliquots simultaneously with the addition of the T cells, after the induction of P388D1. This is referred as 'co-incubation' method. At least three cultures per condition were included in each experiment. The P388D1/T cell culture was incubated for 24 hours and 100 µl of the culture supernatant was analyzed for IL-2 using 1 X 10^4 CTLL-20 per well in a 24 hour assay (Gillis et.al., 1978). The cells were pulsed in the final 4 hours of the incubation with ^{3}H-thymidine(TdR), 1 µCi/well, and subsequently harvested on glass fiber discs and counted.

II.3: RESULTS

II.3.1: CTLL-20 proliferation as a function of IL-2 concentration

The use of cytotoxic T cell lines for in vitro assays of IL-2 has been reviewed by Smith and Ruscetti (1981). The assay is based on the ability of IL-2 to stimulate proliferation of these cells.

The dependency of CTLL-20 cell line on IL-2 is shown in figure II.1. The data is plotted as ^{3}H-thymidine incorporation observed at each corresponding dilution of the IL-2-containing RCS. Control spleen cell suspensions

incubated without Con A did not induce proliferation of CTLL-20. A standard titration curve such as that shown in figure II.1 is included each time unknown samples are tested. The standard curve provides a relative measure of the IL-2 content in the culture supernatant. The assay is quantitative, sensitive, and reproducible with a coefficient of variation under 5%

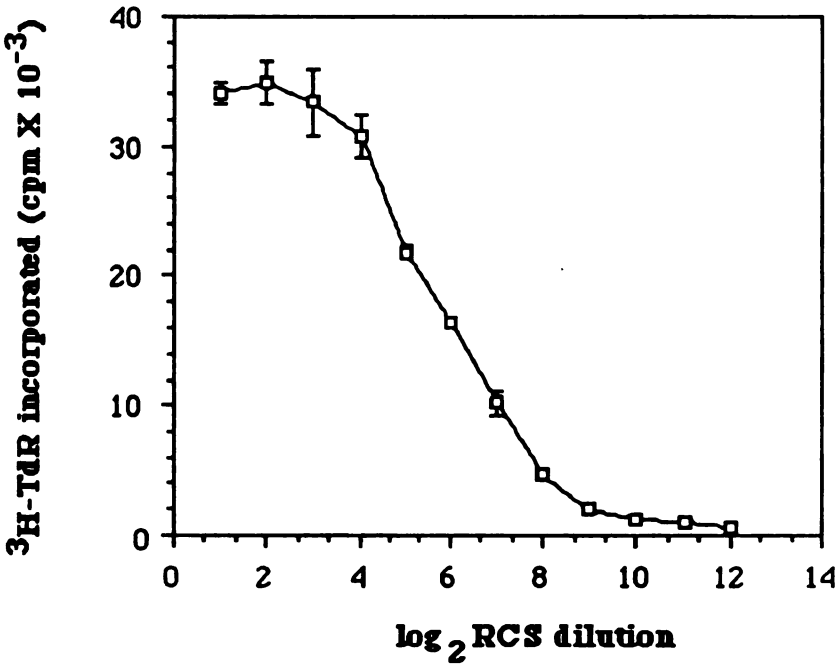


Figure II.1: Dependency of CTLL-20 cell proliferation on IL-2 containing medium. Incorporation of ^{3}H-TdR during the last 4 hours of a 24 hour incubation in the presence of RCS dilutions. Each point represents an average of triplicate cultures ± standard deviation.

Optimal CTLL-20 ^{3}H-TdR incorporation was obtained by subculturing the cells every 4 days at 2 X 10^4 cells/ml/well in 24 well plates (Costar) in the presence of 40% (v/v) RCS. The cells were thoroughly washed to remove the supplemented IL-2 prior to testing unknown samples.

IL-2 activity of RCS remained unchanged 1 year after storage at -20° C. The RCS could be stored at 4°C for as long as 3 months without loosing activity. The IL-2 activity was not diminished by at least one freeze-thaw cycles.

II.3.2: Induction of P388D1 Ia with RCS

The induction of Class II glycoproteins with Con A stimulated spleen cell supernatants has been studied by various laboratories (Steeg et.al., 1980; Birmingham et.al.,1982). It was later demonstrated that the factor responsible for the induction was γ-IFN (King and Jones, 1983; Zlotnik et.al., 1983).

We examined the induction of Ia^d on P388D1 cells with serial dilutions of RCS. Our results demonstrate that uninduced P388D1 are approximately 40% Ia^+. Measurable expression of Ia could be obtained with a 1 : 128 dilution of RCS. Approximately 85-88% of the cells express Ia^d when incubated with a 1 : 2 dilution of RCS for 24 hours. The results are shown in figure II.2.

Zlotnik and coworkers (1983) demonstrated that in the case of the cOVA/P388D1/3DO54.8 assay, the induced state is maintained at least for 48 hours. One must consider that

surface Ia expression is temporal and will eventually decrease. Decrease in Ia expression by induced macrophage lines commonly occurs (Emerson and Cone, 1979).

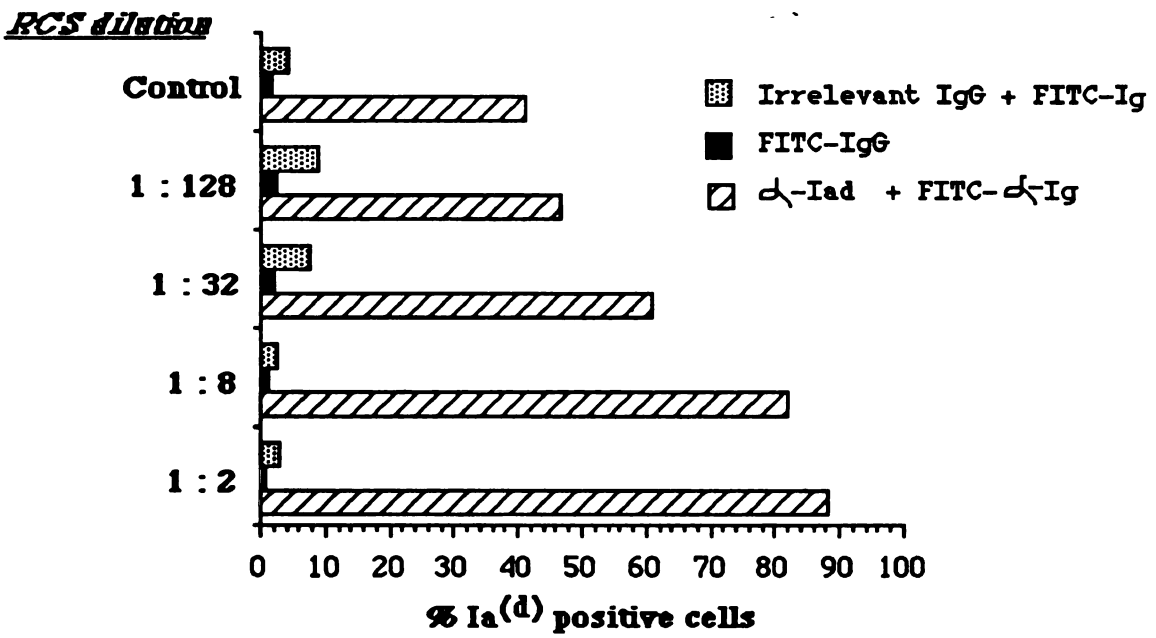


Figure II.2: Induction of Ia^d expression on P388D1 with RCS. Cells were incubated with dilutions of RCS for 24 hours as indicated in Materials and Methods. Expression of Ia was measured by double antibody staining using FACS technology. Nonspecific staining was measured with an antibody of irrelevant specificity.

II.3.3: Induction of cOVA/Ia^d presenting activity of P388D1 by RCS

A flow diagram of the in vitro antigen presentation assay system is shown in figure II.3. The first step involves the induction of the P388D1 for Ia^d expression with dilutions of RCS for 24 hours. Pulsing with antigen can be done prior to adding the T cells (pre-incubation) or simultaneously with T cells (co-incubation). After addition of T cells, aliquots of the P388D1/T-cell culture supernatants are tested for the presence of IL-2.

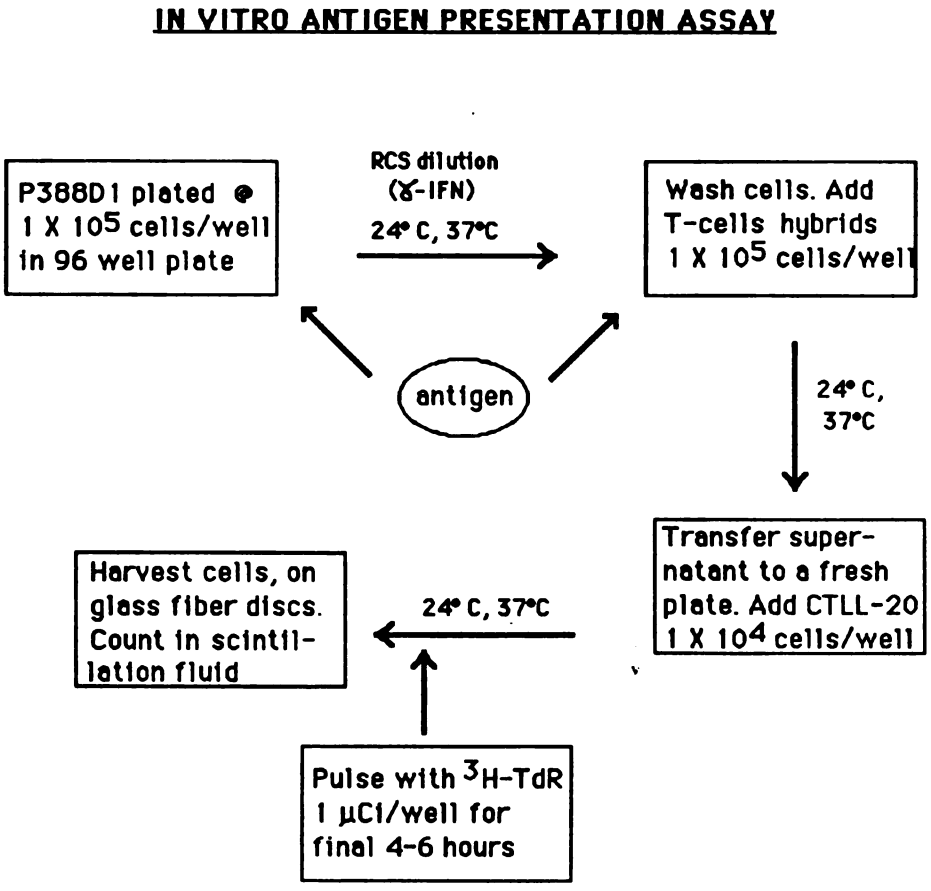


Figure II.3: Schematic of the antigen presentation assay

Incubation of P388D1 with decreasing concentrations of RCS at a constant concentration of cOVA (pre-incubated or co-incubated) correlates with decreasing levels of ^{3}H-TdR incorporation by the CTLL-20 cells (figure II.4). Maximal incorporation was observed with a 1/2 or 1/4 dilution of RCS. Excellent correlation of IL-2 production and Ia^{d} expression was observed. This finding confirms those of others (Steeg et al 1980; Zlotnik et al 1983). Interestingly, uninduced P388D1 were not able to present cOVA at the concentration tested. This is in spite of the fact that 40% of the population is Ia^{+} (figure II.2).

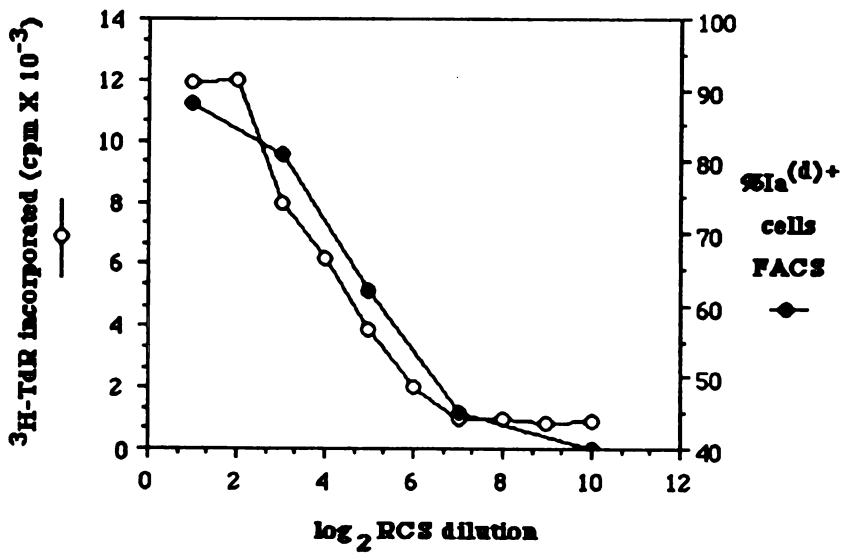


Figure II.4: Correlation of Ia expression and production of IL-2 by the 3DO54.8 T cell hybrids. The P388D1 cells were incubated with serial dilutions of RCS simultaneously with a 30 μM cOVA final concentration for 24 hours (pre-incubation method). IL-2 was measured as described in Materials and Methods.Incubations without T cells or without antigen did not induce IL-2 (not shown). Values for ^{3}H-TdR incorporation represent the average of triplicate cultures. FACS measurements of Ia are those shown in figure II.2.

II.3.4: Antigen presentation as a function of P388D1 to T cell ratio

We tested the ability of P388D1 induced with RCS to detemine if the IL-2 response was affected by the ratio of APC to T cell while keeping the antigen concentration constant. Indeed, figure II.5 demonstrates that incubation of cOVA with various ratios of P388D1 to 3DO54.8 yielded a bell shaped response curve. Maximal responses were obtained with a ratio of (2 : 1) or (3 : 1), P388D1 to T cell hybridoma. The response is diminished by either increasing or decreasing this ratio. Similar shifts in IL-2 response have been observed by others and reflect the competition of the T cell receptor for antigen-Ia complexes (Ashwell et.al., 1986).

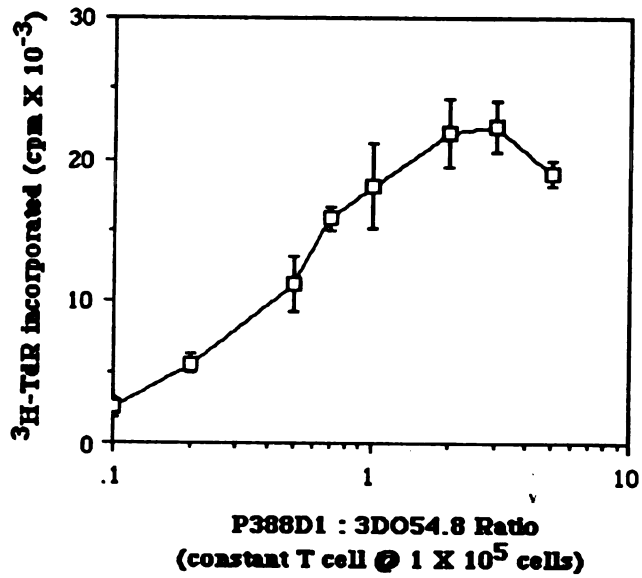


Figure II.5: The effect of the ratio of the antigen presenting cell (P388D1) and the T-cell hybridoma on the IL-2 response in the presence of 95 μM cOVA. P388D1 were induced with a 1 : 4 dilution of RCS. cOVA and T cells were added simultaneously.

II.3.5: Specificity of antigen presentation assay

The specificity of the assay system was tested using monoclonal antibodies against Ia and T cells of irrelevant antigen specificity. The results are shown in figure II.6 and can be summarized as follows: Measurable IL-2 responses were achieved with induced P388D1, antigen, and T cell hybridomas specific for the same antigen. The addition of monoclonal antibodies specific for Ia^d on the P388D1 inhibited antigen presentation whereas addition of anti-Ia for a different haplotype had no effect. These results confirm the genetic restriction of antigen presentation demonstrated by other laboratories (Zlotnik et al, 1983; Kappler et al, 1981; Larsson et al, 1984). T cell hybrids specific for cOVA responded only to cOVA/Ia^d whereas KLH specific T cell hybrids responded only to KLH/Ia^d. LPS at 1 µg/ml final concentration did not alter the response. The response to liposomal cOVA are shown for comparison and will be discussed in the following section.

II.3.6: Effect of encapsulation of cOVA in liposomes on the IL-2 response by 3DO54.8

The encapsulation efficiency for cOVA in DRV liposomes varied between 3 and 20% of the initial cOVA amount added. The protein to lipid ratio of the final preparation was between 0.004 to 0.006 µmole cOVA/ µmole lipid and depended on the amount of cOVA added.

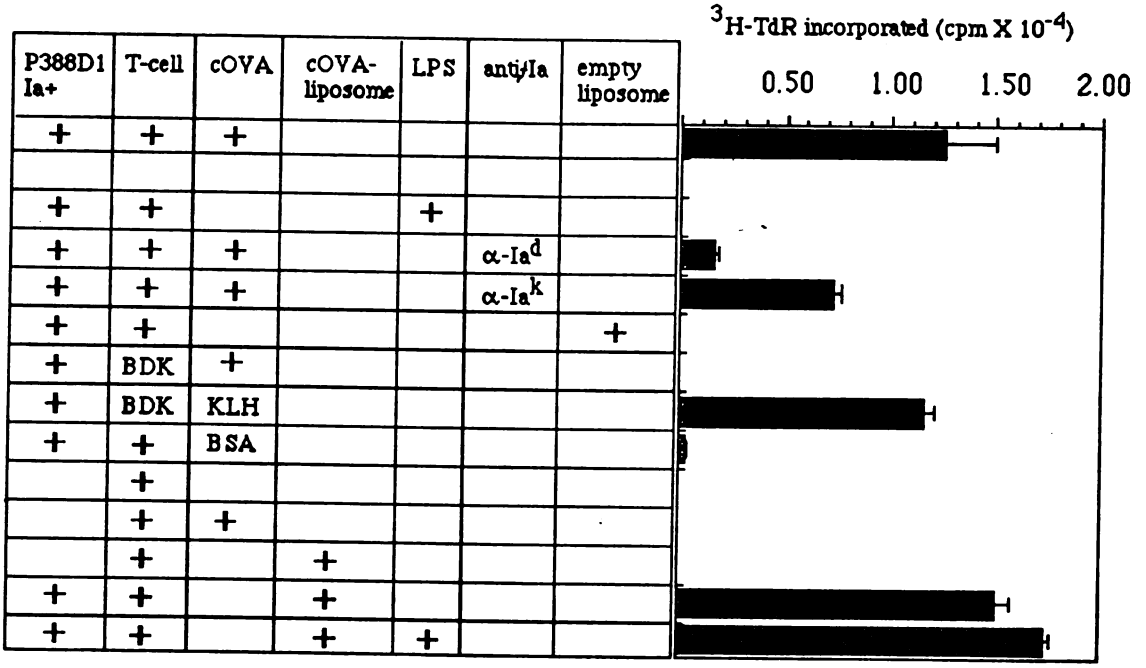


Figure II.6: Specificity of the antigen presentation assay. Plus sign (+) sign signifies the inclusion of the factor in the assay. The 3DO54.8 T cell hybridoma was used in all conditions except in the cases where the KLH-specific hybrid, BDK23.1, was used. Free cOVA final concentration used was 11.9 μM, liposomal cOVA was 8.7 μM. Lipid amounts used either as empty liposomes or cOVA containing DRV, was 700 nmoles per assay well. LPS was added to a final concentration of 1 μg/ml. KLH and BSA were added to a final concentration of 1 mg/ml in the culture well.

We tested the ability of the Ia^+ P388D1 to present cOVA-DRV liposomes to the T cell hybridomas. Two conditions were tested: 1) the liposomal antigen was pre-incubated with the P388D1 for 24 hours followed by washing to remove unbound antigen prior to the addition of the T cells, and 2) the liposomal antigen was added simultaneously with the T cells. In both cases, control samples were added in which equimolar concentrations of lipid in the form of empty liposomes were added in the presence of free cOVA.

The results are shown in figure II.7.A &II.7.B. Co-incubation of liposomal cOVA with T cells was comparable to free cOVA. The response to free cOVA was significantly diminished when equimolar concentrations of lipid in the form of empty liposomes were added (figure II.7.A).When the antigenic forms were pre-incubated with the P388D1 and washed to remove unbound material, the efficiency of the response to liposomal cOVA was increased 5-10 fold over free cOVA. The inhibitory effect of empty liposomes on the response to free cOVA was absent when pre-incubated with the P388D1. No significant change in response to free cOVA was observed in the presence or absence of empty liposomes (figure II.7.B). These results suggested that the liposomes themselves may inhibit the interaction between the macrophage and the T cell. Pre-incubation and thorough removal of unbound antigen abolished the inhibitory effect. As a result of these studies, subsequent experiments with liposomal antigen were carried out by the pre-incubation method.

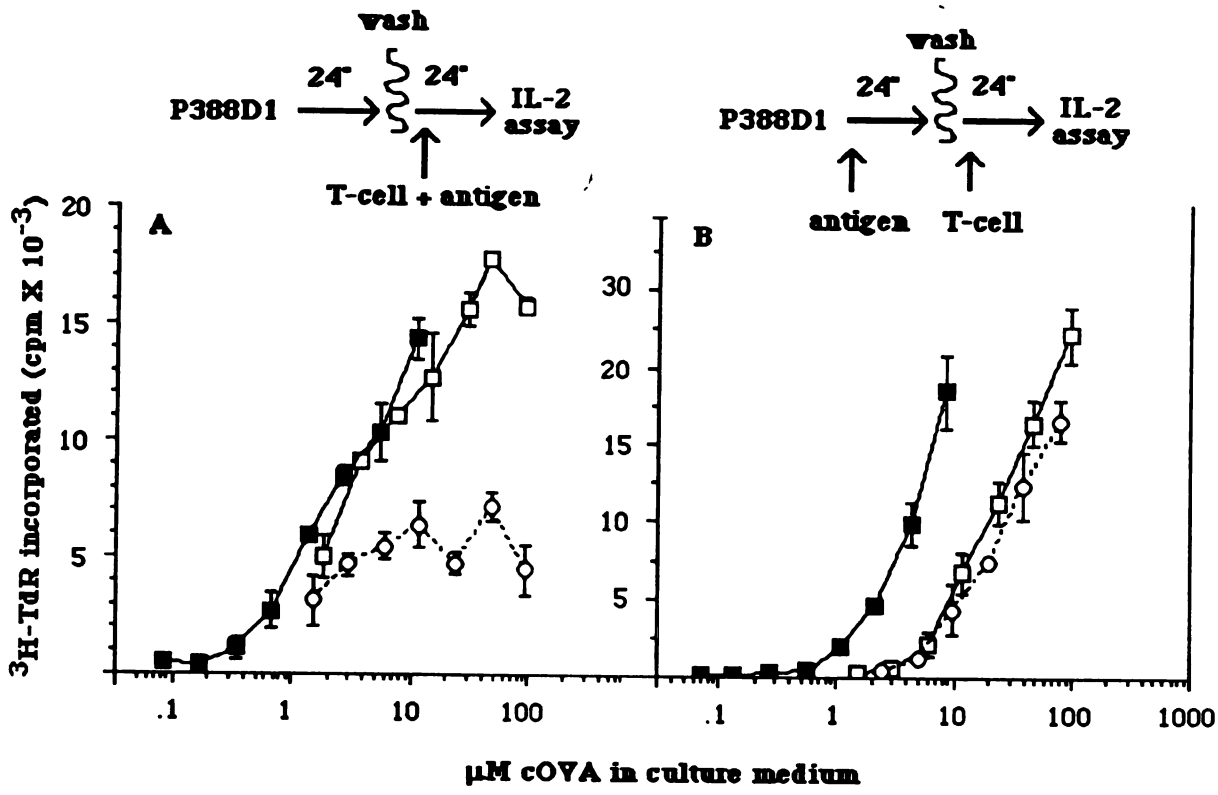


Figure II.7: Dose response curve for the presentation of cOVA in DRV liposomes by Ia^{d+} P388D1 cells. A) P388D1 were initially induced with RCS and subsequently incubated with antigen and T-cells simultaneously (pre-incubation) or B) P388D1 and antigen were preincubated for 24 hours in RCS supernatant and the T-cells were added simultaneously with antigen. Symbols: □, soluble cOVA; ○, soluble cOVA in the presence of empty liposomes (855-918 nmoles lipid per culture); ■, cOVA encapsulated in DRV liposomes (762-872 nmoles lipid per culture). Each point represent the average ± standard deviation of triplicate cultures.

II.3.7: Comparison of presentation of cOVA encapsulated in DRV or REV liposomes

The presentation of antigen may be influenced by the type of liposomal carrier employed. We compared the ability of P388D1 (Ia^{d+}) to present cOVA encapsulated in DRV or REV liposomes. DRV liposomes are oligolamellar and have a large size distribution whereas REV are unilamellar and have a smaller diameter than the DRV. The REV were extruded through o.4 μm membranes whereas the DRV were extruded through 1.0 μm membranes.

Four preparations of each liposome type were made. The final protein to lipid ratios of the REV liposomes ranged from 0.00128 to 0.007 μmole cOVA/ μmole lipid. The encapsulation efficiency of the REV liposomes was between 6.03 and 10.1% of the initial amount of cOVA added (10-40 mg/ml). The DRV liposomes consisted of 0.0004 to 0.006 μmole cOVA/ μmole lipid with a final encapsulation efficiency of 2.18-20.20% of the initial amount of cOVA added (1-40 mg/ml).

The results of the antigen presentation assay are shown in figure II.8. Successful presentation of cOVA delivered in liposomes requires a critical protein to lipid ratio of approximately 0.004 μmole cOVA/ μmole lipid. Significant induction of IL-2 was not observed at lower protein to lipid ratios for either preparation. As the protein to lipid ratio is increased above 0.004 μmole/μmole, the DRV liposomes were more efficient carriers of antigen than the REV.

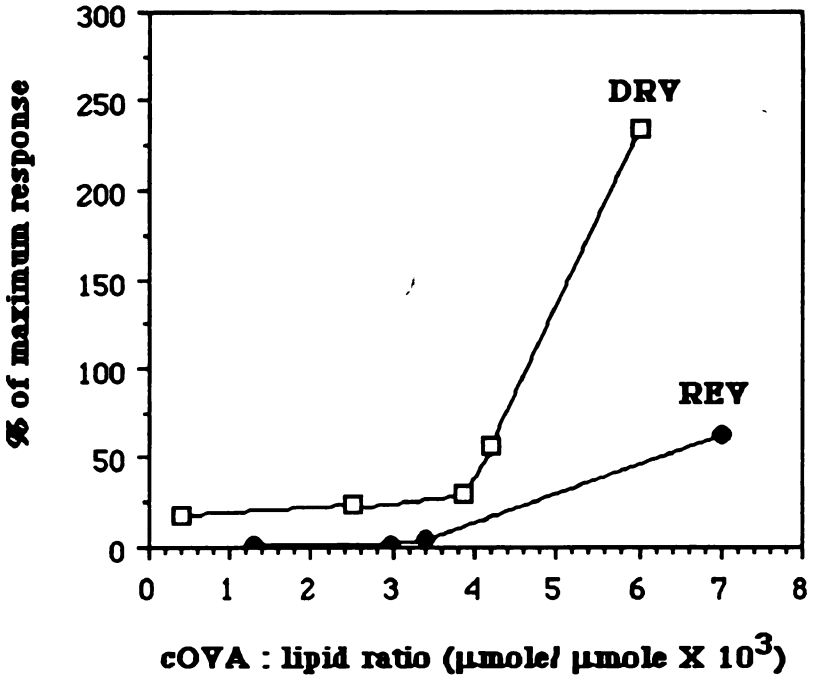


Figure II.8: Comparison of presentation of cOVA in DRV or REV liposomes by P388D1. The lipid amounts used in each assay well was 100 nmoles total lipid.

II.3.8: Antigen presentation by P388D1 with free cOVA, denatured cOVA and cOVA encapsulated in REV

The emulsification of lipid with the agent to be entrapped is an cruicial step in the production of REV liposomes. Since many macromolecules are irreversively denatured when exposed to organic solvents, we were interested in comparing the IL-2 response of native cOVA, cOVA denatured in diethyl-ether, and cOVA encapsulated in REV.

The cOVA was treated exactly as when REV liposomes were prepared except that the lipid was omitted. The cOVA formed large aggregates when exposed to the diethyl-ether. The aggregates were partially broken up by passing the protein through 0.45 µm filters. The protein content of the filtrate was determined prior to the antigen presentation experiment.

The IL-2 response curves for the three antigenic forms of cOVA are shown in Figure II.9.

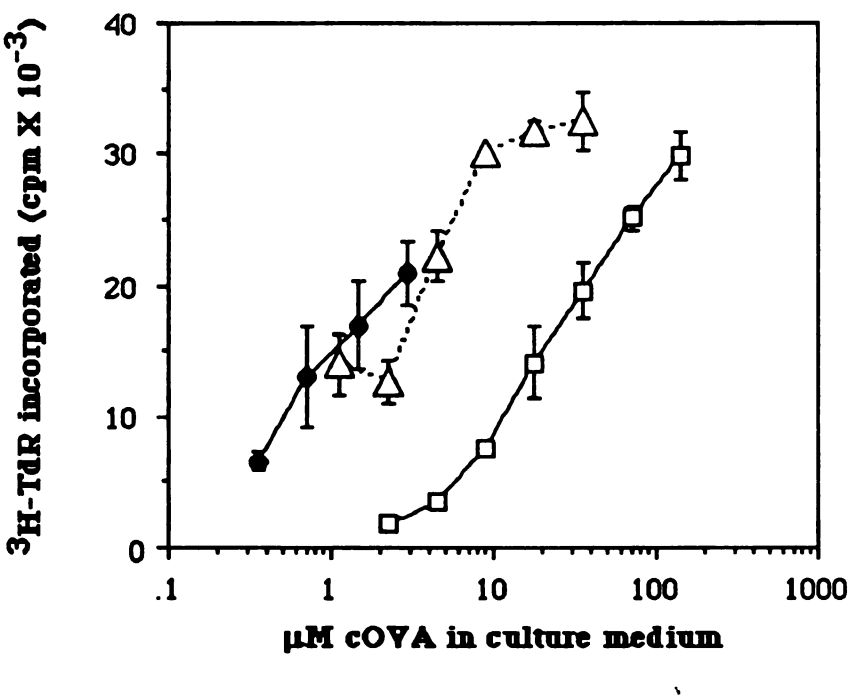


Figure II.9: The induction of IL-2 secretion by P388D1 which had been exposed to soluble cOVA, denatured cOVA, and cOVA encapsulated in REV liposomes. The REV preparation consisted of 0.007 µmole cOVA/µmole lipid. Dilutions of liposomal antigen were made with empty liposomes to maintain the final lipid concentration costant. Symbols: ● , REV; ·△· , denatured cOVA; □, native cOVA.

Denatured cOVA or cOVA encapsulated of in REV liposomes show superimposible IL-2 dose response curves. Denatured cOVA or liposome-encapsulated cOVA are more immunogenic than free cOVA. This suggests that immunogenicity can simply be increased by denaturing the protein and in this case liposome structures are not necessary.

II.3.9: Antigen presentation of cOVA and a synthetic peptide representing a cOVA 17 amino acid fragment

The processing function of macrophages may be bypassed by using tryptic fragments of protein antigens (Shimonkevitz et.al., 1984; Unanue, 1984). We synthesized a 17 amino acid peptide segment of cOVA which had been identified as the immunogenic region responsible for stimulating the 3DO54.8 T cell hybrid in conjunction with Ia^d.

The fragment was tested in the antigen presentation assay and compared to native cOVA (Figure II.10). The dose response curve is shifted so that on a molar basis 100 fold less peptide is required to achieve an equivalent IL-2 response as compared to native cOVA.

II.4 CONCLUSIONS

In these studies we describe the establishment of an in vitro antigen presentation assay. Briefly, the antigen is delivered to APC for processing, the APC presents the procesed antigen to antigen-specific T cells, and production of IL-2 by the activated T cell is determined. The assay is

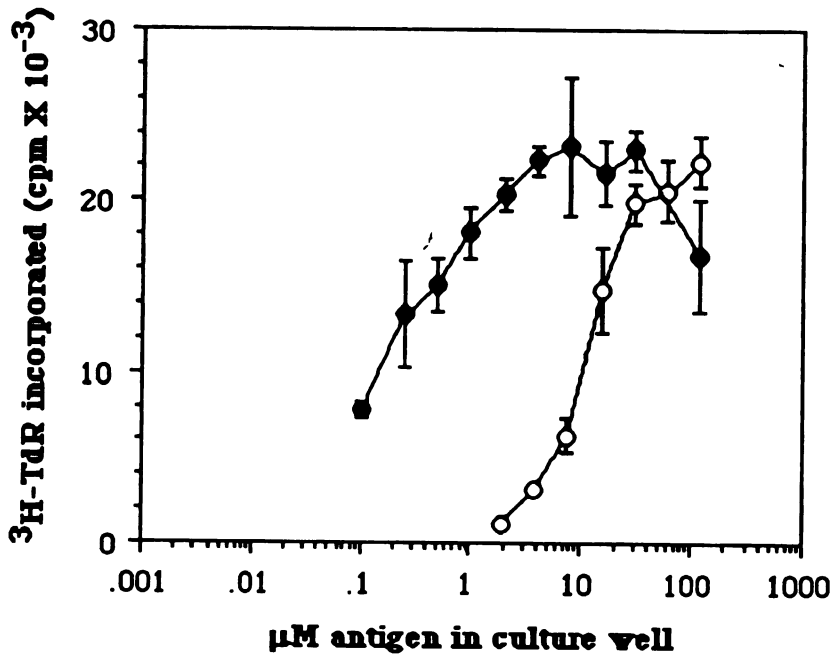


Figure II.10: Comparison of the IL-2 response when cOVA (○) or P101 (●) is presented by P388D1

sensitive, reproducible, and relatively simple. The major findings from the experiments outlined in this chapter are summarized as follows:

1) Successful antigen presentation is determined by the IL-2 response of the T cells and depends on the amount of antigen, the level of Ia expression by the APC, and the ratio of APC to T cell.

2) Varying levels of Ia expression by P388D1 may be induced with RCS. The level of expression dictates the

ability of the cell to present antigen to antigen specific T cells.

3) Encapsulation of cOVA in liposomes enhances the efficiency of presentation 5-10 fold over native cOVA. This was only observed in the case where the antigen was pre-incubated with macrophages.

4) The minimal cOVA to lipid ratio which elicits a significant response is 0.004 µmole cOVA per µmole lipid.

5) Encapsulation of cOVA in DRV liposomes is more efficiently presented by P388D1 than in REV liposomes.

6) Equivalent IL-2 response was observed with cOVA encapsulated in REV liposomes or cOVA denatured with diethyl-ether.

7) Antigen presentation is approximately 100 fold more efficient with a 17 amino acid peptide fragment representing the T cell epitope of cOVA as compared to native cOVA.

CHAPTER III: EFFECT OF LIPOSOME ENCAPSULATION ON ANTIGEN PRESENTATION IN VITRO: COMPARISON OF PRESENTATION BY PERITONEAL MACROPHAGES AND B-CELL TUMORS

Section III.1: INTRODUCTION

The association of a protein antigen with phospholipid vesicles (liposomes) has been shown to potentiate the antibody response to that antigen when injected into animals. This finding has been observed with various antigens including; diphtheria toxoid (Allison and Gregoriadis, 1974), hepatitis B surface antigen (Manesis et.al., 1979), cholera toxin (Alving et.al., 1986), tetanus toxoid (Davis et.al., 1987), and tumor cell antigens (Raphael and Tom, 1982) For a recent review on the liposome adjuvant effect see Alving (Alving, 1987).

A central role in the *in vivo* immune enhancement observed for liposomal antigens has been ascribed to the macrophage (Shek and Lukovich, 1982; Beatty et.al., 1981). Macrophage involvement in the stimulation of immune responses has also been shown for other non-specific adjuvants (Unanue et.al., 1969; Watson and Sljivic, 1976) due both to its function as an antigen presenting cell (APC) (Unanue and Allen, 1987) and to its capacity to phagocytose particulate material. Indeed, liposomes are avidly taken up by macrophages both *in vivo* (McDougall et.al., 1974; Patel and Ryman, 1981) and *in vitro* (Raz et.al., 1981; Hsu and

Juliano, 1982) which makes this cell type a prime candidate for mediating the liposomal adjuvant effect. Beatty and co-workers found that liposome encapsulation of bovine serum albumin (BSA) stimulated T-helper cells *in vivo* and proposed that the stimulation is dependent on liposome uptake by macrophages (Beatty et.al., 1984). Furthermore, by *in vivo* depletion of the macrophage population, Shek and Lukovich found a dramatic decrease in the ability of mice to mount an antibody response to liposome associated BSA (Shek and Lukovich, 1982). The authors conclude that the immune enhancement observed by encapsulation of protein antigens in liposomes is due to the accumulation of greater amounts of antigen in the macrophage than that achieved in the absence of the liposomal carrier.

B lymphocytes have recently been shown to present soluble antigen in a manner analogous to that of macrophages (Chesnut and Grey, 1986) and could also be involved in the liposomal adjuvant effect. The liposome might mediate its effect by direct contact with B lymphocytes, perhaps releasing antigen at the lymphocyte surface and thus becoming available for antigen presentation.

The optimization of the liposome as an adjuvant for immunization depends upon understanding both the cell types and mechanisms involved in the liposome mediated immune enhancement. However, the mechanism of the liposome induced immune enhancement for protein antigens is complex and not easily studied *in vivo* . We utilized an *in vitro* antigen

presentation assay in which presentation of PCC by either macrophages or a B-cell was assessed by measuring IL-2 production by a PCC-specific T-helper hybridoma. Experiments were designed with the following goals in mind : 1) To determine whether liposome encapsulation of a soluble PCC would result in an increase in presentation by two types of antigen presenting cells, the elicited peritoneal macrophage (PEC) and the LK35.2 B-cell hybridoma. 2) To correlate the uptake and degradation of PCC in the encapsulated and non-encapsulated form with the level of presentation.

We show that a liposome encapsulated protein antigen can be taken up, processed, and presented to antigen-specific T cells by macrophages but not by the B cell hybridoma. The relevance of these observations to the use of liposomes as immunological adjuvants is discussed.

Section III.2: MATERIALS AND METHODS

III.2.1: Mice

Healthy C_3H (Ia^k) male mice, 6-8 weeks old, were purchased from Simonsen Laboratories (Gilroy, CA).

III.2.2 Antigens

PCC (Type XIII from pigeon breast muscle) and BSA (Fraction V) were obtained from Sigma Chemical Co. (St.Louis,MO). Radiolabelled PCC was prepared by a modification of the method of Greenwood et al (Greenwood

et.al., 1963) using [^{125}I]-NaI (Amersham, Arlington Heights, IL). Briefly, 5 mCi of [^{125}I]-NaI, 350-600 mCi/ml, was mixed with 20 µl of 0.5M phosphate buffer pH 7.5. To that, 10 µl of a 821.5 µM solution of PCC in 0.05 M phosphate buffer was added. Chloramine-T prepared immediately prior to the reaction (200 µg in 20 µl 0.05 M phosphate buffer) was added and the reaction was allowed to proceed for 50 seconds at room temperature with intermittent vortexing. The reaction was stopped by adding 20 µl of a 25 mg/ml solution of sodium metabisulfite in 0.05 M phosphate buffer, pH 7.5 prepared immediately prior to the reaction. Potassium iodide (50 µl of 33 mg/ml in 0.05 M phosphate buffer) and PCC (250 µl of 2.46 mM solution in 0.05 M phosphate buffer) were added as carriers. Unbound ^{125}I was removed by passing the reaction mixture through a Bio-Gel P6-DG (1 X 7 cm) column which had been pre-saturated with PCC and extensively washed with 10 mM N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) and 150 mM NaCl pH 7.4 buffer (TES/NaCl).Specific activities between 3.04 and 6.08 µCi/nmole were usually obtained. Prior to each experiment, the radiochemical purity of the protein was tested by precipitating an aliquot of the labelled protein in CH_3CN (85% v/v final concentration) along with 100-200 µg BSA as carrier protein. The labelled PCC was used only when greater than 88% of the radioactivity existed as CH_3CN-precipitable counts.

III.2.3: Liposome preparations

Liposomes containing PCC were prepared by a modification of the method of Szoka and Papahadjopoulos (Szoka and Papahadjopoulos, 1978). The phospholipid vesicles consisted of dipalmitoyl-phosphatidylcholine (DPPC) (Avanti Polar Lipids, INC., Birmingham, AL), dipalmitoylphosphatidyl-glycerol (DPPG) (Avanti) and cholesterol (CH) (Sigma) in a molar ratio of 9:1:8. Briefly, 20 μmoles of total lipid dissolved in chloroform were added to a 25 ml long neck round bottom flask. After removal of the solvent by rotary evaporation, the lipids were further dried under high vacuum for 1 to 1.5 hours. The lipids were then resuspended in 1 ml of a mixture composed of trichlorotrifluoroethane (Burdick and Jackson Laboratories, INC., Muskegon,MI.) : isopropyl ether (Fisher Scientific, Fairlawn, NJ) 1:2 (v/v) and dissolved by warming the flask to 45-50°C under a nitrogen atmosphere. While keeping the flask at 45°C, 0.33 ml of PCC solution in TES/NaCl buffer was added to the lipids followed by a brief (10-15 second) sonication using a bath type sonicator. After the brief sonication, the flask and its contents were immediately placed in a rotary evaporator and the organic phase was slowly removed under reduced pressure while maintaining the water bath at 45°C. Within 10 minutes the system formed a viscous gel which was disrupted by vortexing to form a fluid aqueous suspension. Evaporation at slightly higher vacuum was continued for 20 minutes to remove any residual organic solvent after which 0.66 ml of

additional protein solution was added to make the total volume to 1 ml. When [^{125}I]-PCC containing liposomes were made, [^{125}I]-PCC was diluted with unlabelled PCC (821.5 to 1643 µM in 10 mM N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) and 150 mM NaCl pH 7.4, TES/NaCl buffer). Final specific activities ranged between 38.96 to 274.32 µCi/µmole. For degradation studies, [^{125}I]-PCC of higher specific activities was used (984.8 µCi/µmole). Non-encapsulated PCC was separated from the vesicle-associated PCC by flotation through discontinuous metrizamide gradients (Heath, 1987) or gel filtration through a Bio-Gel A0.5M (Bio-Rad, Richmond, CA.) (1 X 10 cm) column. The liposomes were assayed for lipid content by phosphorous analysis (Bartlett, 1959) and protein content was measured spectrophotometrically (O.D. 550 nm, E^{1mM} = 28) under reducing conditions (excess dithionite) (Margoliash et.al., 1959) in the presence of 0.02% Triton X-100. Liposome diameter was measured by laser light scattering on a Nanosizer model N-4 (Coulter Electronoics, Hialeah, FL.).

III.2.4: Pronase treatment of liposomes

The amount PCC on the liposome surface was assessed by measuring the extent of digestion of protein after treatment of the vesicles with pronase (Boehringer Mannheim GmbH, W. Germany). Liposomes containing [^{125}I]-PCC prepared by the standard method described above were treated with pronase (1 mg/ml final) for 35 minutes at room temperature. The digested

PCC was determined by precipitating an aliquot with acetonitrile as described above. The amount of CH_3CN soluble radioactivity represents digested PCC accessible to pronase.

III.2.5: Cell lines

The PCC specific T-cell hybridoma, 2B4 ($H\text{-}2^k$) (Samelson and Schwartz, 1983) and the B-cell hybridoma, LK35.2, were generously provided by Dr. Ron Schwartz (National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). The antigen presenting B-cell hybridoma, LK35.2 ($H\text{-}2^{d/k}$), was developed and described by Kappler and colleagues (Kappler et.al., 1982). The CTLL-20 line, an interleukin-2 (IL-2) dependent cell line, was provided by Dr. Daniel Stites (University of California at San Francisco, CA). The cell lines were tested and found to be free from mycoplasma contamination. PEC were used as a source of antigen presenting macrophages. These cells were harvested from C_3H mice by peritoneal lavage with ice-cold Hank's balanced salt solution 4-5 days after an intraperitoneal injection of 4 ml of thioglycollate medium (3% in water). The cultures were enriched for macrophages by allowing the cells to adhere to plastic culture dishes for 3-4 hours with subsequent washing of the monolayers. The purified macrophages were used immediately for the in vitro assays. Both of the antigen presenting cells (APC), the B-cell tumor and the PEC, were cultured in vitro in RPMI-1640 supplemented with 10% fetal calf serum (FCS, HyClone

Laboratories, INC. Logan,Utah), 2 mM glutamine, 25 mM N-2-hydroxy-ethylpiperazine-N'-2-ethane sulfonic acid, 1 mM pyruvate, 5 X 10^{-5} M 2-mercaptoethanol, and antibiotics (RPMI-CM). CTLL-20 were grown in Dulbecco's minimum essential medium supplemented with 5% FCS and 40% Concanavilin A-stimulated rat spleen cell supernatant as a source of IL-2 (Steeg et.al., 1980). The 2B4 cell line was cultured in MEM Eagle's with Hank's balanced salt solution (EHAA) and RPMI 1640 (1:1 v/v) supplemented with 10% FCS, glutamine, non-essential amino acids, and antibiotics (EHAA:RPMI). Fresh cultures of 2B4 and LK35.2 cell lines were initiated from frozen (liq. N_2) samples of early passages every 20-25 passages. Media and FCS were obtained from the Cell Culture Facility, University of California at San Francisco.

III.2.6: Functional Assay

The basis of the functional assay was the determination of IL-2 secretion by the 2B4 when co-cultured with antigen pulsed APC.

The LK35.2 was incubated with either liposomal or free antigen in suspension culture in 15 ml conical culture tubes (Costar, Cambridge,MA) at 2.6 X 10^6 cells per culture in 1 ml of RPMI-CM and gently rocked throughout the incubation period. The PEC were plated as monolayers on 96 well plates (flat bottom, Costar) at 2.15 X 10^5 cells per well in 200 µl RPMI-CM. After 24 hours at 37°C, the cells were washed 3-4 times (LK35.2 were washed by centrifugation) to remove any

unbound antigen. The antigen-pulsed APC (2 X 10^5) were subsequently incubated with the T-cell hybridoma, 2B4 (1 X 10^5), in a volume of 200 µl EHAA:RPMI in 96 well culture plates. After 24 hours at 37°C, a 100 µl aliquot of the supernatant from each well was removed and assayed for IL-2 content as determined by the incorporation of tritiated thymidine ([^{3}H]-TdR) by the IL-2 dependent cell line CTLL-20 (Gillis et.al., 1978). All titrations for IL-2 activity were performed in triplicate. Results are expressed as mean ± SD. Standard deviations from the mean were generally between 5 and 15%.

III.2.7: Antigen uptake studies

Binding of the antigen to the APC was determined by adding radiolabelled PCC solution (free or liposomal) in TES/NaCl buffer. Usually 2 X 10^6 to 1 X 10^7 LK35.2 or 4.1 X 10^5 to 2 X 10^6 PEC per culture were used in the experiments. The LK35.2 were incubated as described above. The PEC were cultured as monolayers on 35mm diameter culture dishes (Costar). After varying time intervals the cells were washed 4-6 times in ice-cold phosphate buffered saline (PBS; 0.2 g/liter KCl, 0.2 g/liter KH_2PO_4, 8 g/liter NaCl, 1.15 g/liter Na_2HPO_4, pH 7.4). The washed cells were then solubilized with 1 ml of 0.1M NaOH-0.4% sodium deoxycholate (NaOH/DOC) and assayed for total cell associated radioactivity by gamma scintillation counting. All assays were performed in duplicate. Results are expressed as mean ± range. Controls

were included to determine non-specific binding of antigen to cell culture dishes or tubes and less than 5-15% binding was observed. This level of non-specific binding was subtracted from culture vessels containing cells.

III.2.8: Determination of antigen catabolism after initial uptake by APC

A schematic of the degradation assay is shown in figure III.1. For the catabolism of PCC by both of the APC, [^{125}I]-PCC (liposomal or free) was added to the APC cultures and allowed to incubate for 3 hours at 37° C. After this pre-incubation period, the unbound PCC was washed off as described above . Fresh RPMI-CM was added and the cells were further incubated at 37°C for varying periods of time. At designated time periods, the cells were separated from the culture medium. The cells were lysed in NaOH/DOC and the total radioactivity associated with the lysate and the medium was assessed. Degradation of PCC was determined by precipitating the cell or medium sample in CH_3CN as described. The radioactivity which is CH_3CN soluble (degraded PCC) and CH_3CN insoluble (precipitable PCC) was measured by gamma scintillation counting.

The molecular weight distribution of the CH_3CN precipitable material is shown in figure III.2. We selected a T= 19 hour sample from an incubation of liposomal PCC with PEC. The sample was precipitated as described above and the pellet was reconstituted with NaOH/DOC. The sample was eluted

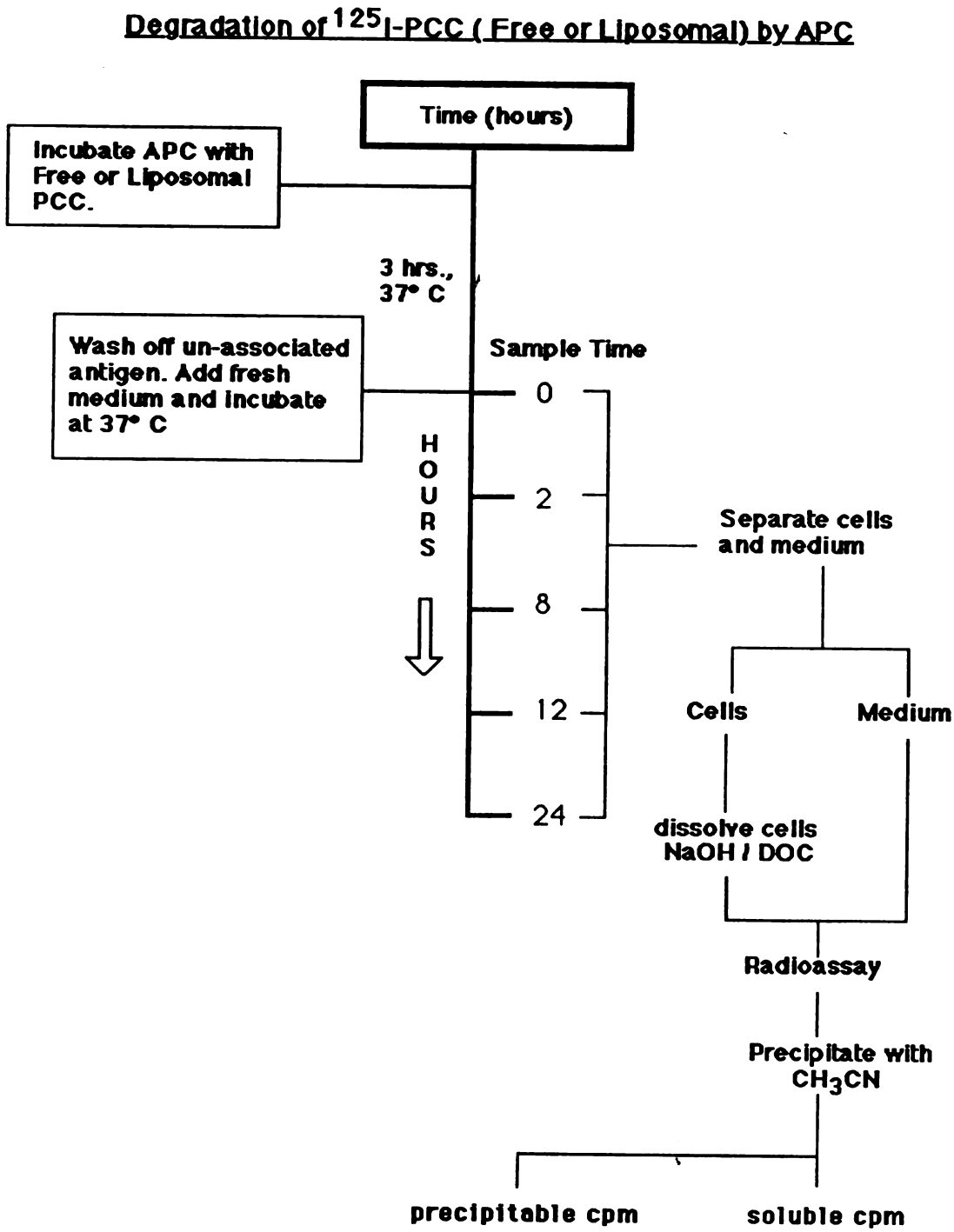


Figure III.1: Scheme of degradation assay

through a precalibrated Bio-Gel P6 column (1 X 27 cm) and the fractions collected and counted for radioactivity. The results indicate that the CH_3CN-precipitable material is composed of fragments greater than 2500 molecular weight.

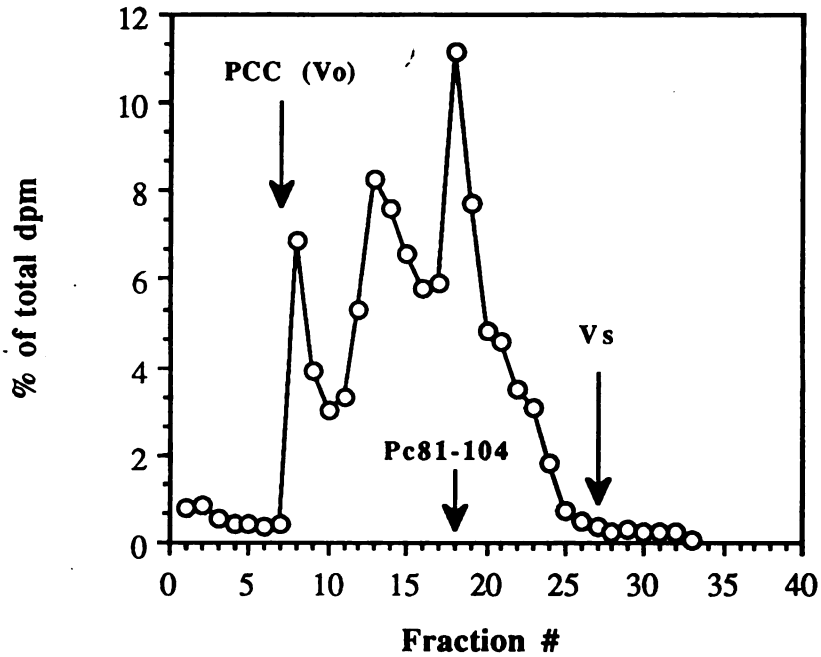


Figure III.2: Molecular weight distribution of CH_3CN-precipitable radioactivity. The void volume (Vo) was determined by eluting radiolabeled PCC (mw. 12,173) and the salt peak (Vs) with potassium dichromate. A radiolabeled peptide, Pc81-104 (2647 mw) was used for calibration purposes.

The increase in CH_3CN soluble radioactivity during the incubation provided a measure of the PCC digested. All measurements were performed in duplicate. Results from a single experiment are expressed as mean ± range.

III.3: RESULTS

III.3.1: Characterization of liposomes

All liposome preparations used in these studies were composed of the high transition temperature (Tc) synthetic phospholipids DPPC (Tc= 41°C) and DPPG (Tc= 41°C), and cholesterol.

The encapsulation efficiency of PCC in the liposomes ranged between 3.3% and 8.1% with a mean of 6.1%. This was dependent on the initial concentration of PCC used. On the average, the liposomes consisted of 85 μg PCC per μmole of total lipid. The majority of the PCC was entrapped within the vesicles as determined by the ability of the digestive enzyme, pronase, to degrade [^{125}-I]-PCC associated at vesicle surface. In 3 preparations tested, an average of 16.5% of the protein was found accessible to pronase digestion and in one preparation approximately 40% of the liposome associated protein was degraded. The liposome preparations were sized by dynamic laser light scattering and found to be a homogeneous population with an average diameter of 0.312 μm ± 0.116.

III.3.2: Presentation of soluble and liposomal antigen to antigen specific T cell

We compared the ability of peritoneal cells and B-cell hybridomas to present non-encapsulated PCC and liposome-encapsulated PCC to the PCC-specific T-cell hybridoma, 2B4.

The results of a representative experiment in which the IL-2 response to a series of titrations of either form of antigen are shown in figure III.3. Under the experimental conditions, the dose response curve to free PCC presented by PEC (figure III.3.A) and LK35.2 (figure III.3.B) are sigmoidal in shape for the concentration range studied. Moreover, the dose response to free PCC was similar for both presenting cells and saturation was generally observed at PCC amounts of greater than 7-10 μM per culture. The saturation was not due to saturation of the IL-2 sensitive cell line as was demonstrated by assaying serial dilutions of the IL-2 containing test supernatants (not shown). In this system, the B-cell tumor is as efficient as PEC macrophages in presenting the non-encapsulated antigen.

When the antigen is introduced in a liposomal carrier, the ability of the B-cell to stimulate IL-2 secretion by the T-cell is totally abolished (figure III.3.B). For the case of the PEC, a modest shift to the left in the dose response curve is seen as compared to the non-encapsulated PCC (figure III.3.A). In 5 separate experiments with PEC, the effect of liposome encapsulation of PCC was to slightly increase the potency in four cases and to slightly decrease the potency in the fifth experiment. The enhancement is more pronounced at the lower concentrations of PCC while at higher concentrations no enhancement in IL-2 secretion is observed. We cannot say with certainty if the response to liposomal PCC reaches the same plateau as that of free PCC.

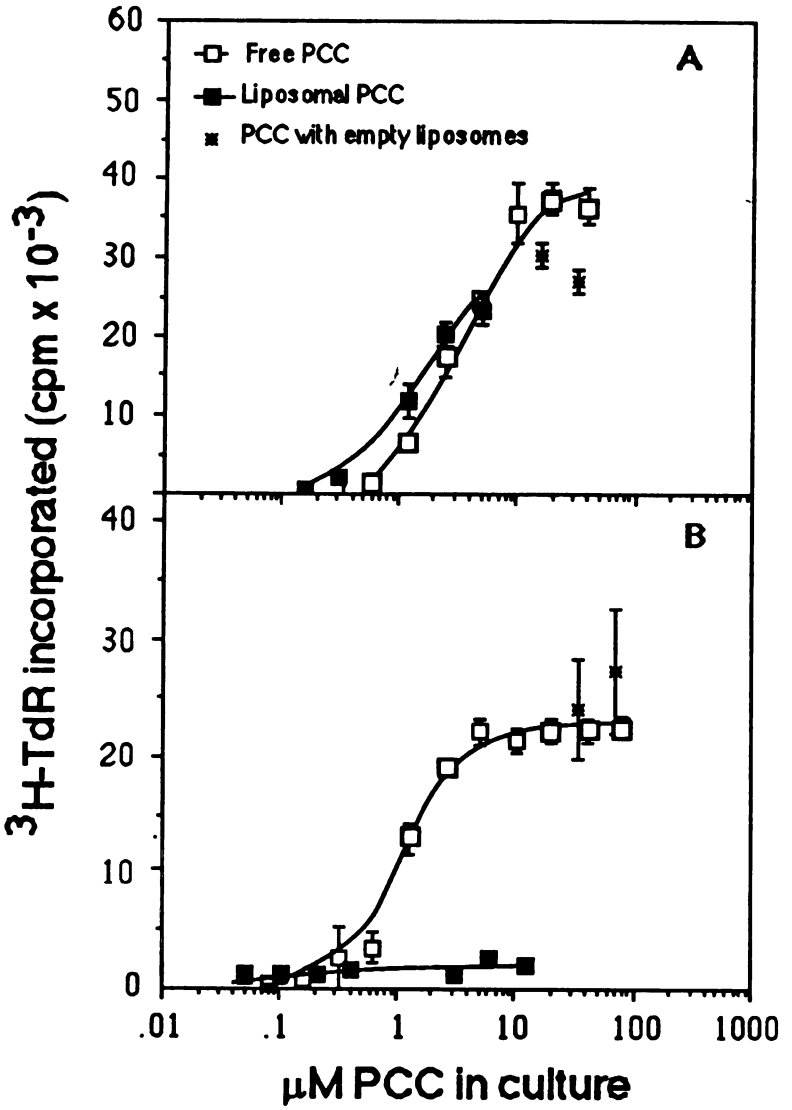


Figure III.3 : The response of PCC specific T-cell hybridoma (2B4) to free or liposomal antigen by peritoneal cells (A) and LK35.2 (B). The IL-2 content (y-axis) of a 1/4 dilution of the culture supernatant was determined as described in Materials and Methods. The data points represent the average ± standard deviation of triplicate cultures. The lipid concentration used for the case of the liposome was kept constant for all titrations of PCC and ranged between 100-158 nmoles of lipid. Empty liposomes, at the same concentrations, had a slight (peritoneal cells) or no effect (LK35.2 cells) on the response to free PCC (*). Error bars not shown fall within the symbol.

In our experiments we were limited by the encapsulation efficiency of the liposomes and thus could not assess greater concentrations of liposomal PCC.

Co-incubation of PCC with empty liposomes did not alter the IL-2 response to free antigen with either APC (figure III.3 A & B). IL-2 activity was not detected when empty liposomes alone were used in the antigen presentation assay and liposomes with or without antigen did not induce IL-2 activity when incubated together with 2B4 in the absence of APC (not shown). Moreover, lipid concentrations used in these experiments was well below saturation of vesicle association by the APC (unpublished result). The addition of 1 μg/ml of Salmonella minnesota lipopolysaccharide (List Biological Laboratories. Campbell, CA.) to the antigen presentation assay with free antigen did not alter the IL-2 response (not shown). Finally, non-encapsulated PCC treated via the same procedure to prepare the liposomes, but in the absence of lipids, yielded similar presentation curves to that of untreated PCC (not shown).

III.3.3: Quantitation of antigen uptake by LK35.2 and PEC

An early step required to elicit a T-cell response during antigen presentation is the binding and uptake of the antigen by the presenting cell. Approximately twice as much non-encapsulated [^{125}I]-PCC became associated with the PEC than with the LK35.2 (data not shown). In the PEC, cell association of free antigen begins to saturate at

approximately 70 μM PCC in culture after 24 hours. Association of free PCC in the LK35.2 gradually increases and no evidence of saturation at levels as high as 60 μM free PCC is seen (not shown). The IL-2 response (figure III.3) saturated at lower PCC levels than did the uptake of the non-encapsulated antigen.

The second issue of interest was a comparison of cell associated radioactivity after incubating the cells with liposomal-PCC. A dose range covering the logarithmic phase of the dose-response curve shown in figure 1 was chosen for study. The results for the PEC are shown in figure III.4.A and III.4.B. Regardless of the concentration of PCC in the culture medium or the duration of the incubation, approximately 2-fold more of the liposomal PCC becomes cell associated than non-encapsulated PCC. It is also of interest to note that cell association appears to saturate after approximately 24 hours of culture (figure III.4.A), the time used to pulse the APC in the antigen presentation assay. With the PEC, between 0.04% and 0.05% of the added PCC became cell associated in the non-encapsulated form and between 0.08% and 0.1% of the liposome encapsulated PCC became cell associated at 24 hr when 14.5 μg PCC was added.

Cell association of free and liposome encapsulated PCC was compared in the LK35.2 cell. It was observed that less non-encapsulated PCC associated with LK35.2 than with PEC. Encapsulation of PCC in liposomes did not decrease binding to the cells from that of free PCC (figure III.5 A & B). Similar

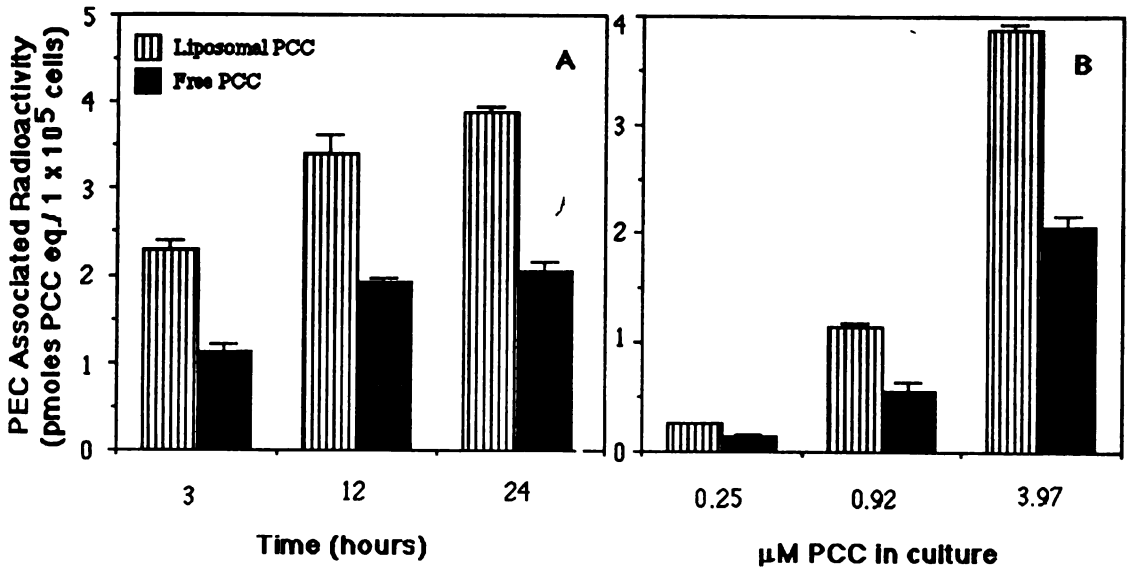


Figure III.4 : (A) Kinetics of the uptake of liposomal or free [^{125}]I-PCC by PEC. Adherent cells were incubated at 37° C with 4.8 nmoles (200 µl) of [^{125}]I-PCC (sp. act. 95,000 dpm/nmole) or 4.8 nmoles of [^{125}]I-PCC in liposomes (1.5 µmoles total lipid). At various times, the cultures were washed and the amount of cell-associated radioactivity was determined as described in Materials and Methods . Data represents the mean of duplicate cultures ± range. (B) Concentration dependence of the uptake of liposomal or free [^{125}]I-PCC by PEC. Adherent cells were incubated at 37° C with varying amounts of [^{125}]I-PCC or [^{125}]I-PCC in liposomes for 24 hours at 37°C. Total lipid concentration ranged from 150 nmoles at the lowest concentration of PCC to 2 µmoles lipid for the highest concentration of PCC used. Similar results are observed (not shown) when constant lipid concentrations are used in each well. Cell asssociated radioactivity was determined as described above.

levels of cell associated radioactivity were found for either form of PCC and in some cases more PCC became cell associated when in liposomes. This trend was observed for all time points (figure III.5.A) and concentrations (figure III.5.B). With the LK35.2, between 0.01% and 0.015% of the non-encapsulated PCC became cell associated and between 0.01% and 0.02% of the liposome encapsulated PCC became cell associated at 24 hr when added at 21.6 μg/ml.

III.3.4: Fate of [125I]-PCC in liposomes or free, after uptake by LK35.2 and macrophages

Based on results of the cell association of antigen, the lack of an IL-2 response to the liposomal PCC when the LK35.2 was the presenting cell must be due to a step subsequent to cell association. Three explanations appear plausible: 1) lack of internalization of surface bound antigen, 2) rate limitation in the degradation and processing of the PCC prior to presentation, or 3) interference in the binding of the processed peptides to cell surface Ia molecules or the interaction of the T-cell hybridoma with the LK35.2 . To test the third possibility, a dose range of free PCC was incubated in the presence of either empty liposomes (figure III.3.B) or PCC-containing liposomes (not shown). The IL-2 response was not significantly different than that measured in the absence of the liposomal carrier. Also, the presence of empty liposomes did not interfere with the binding of radiolabelled free PCC (not shown). This suggests

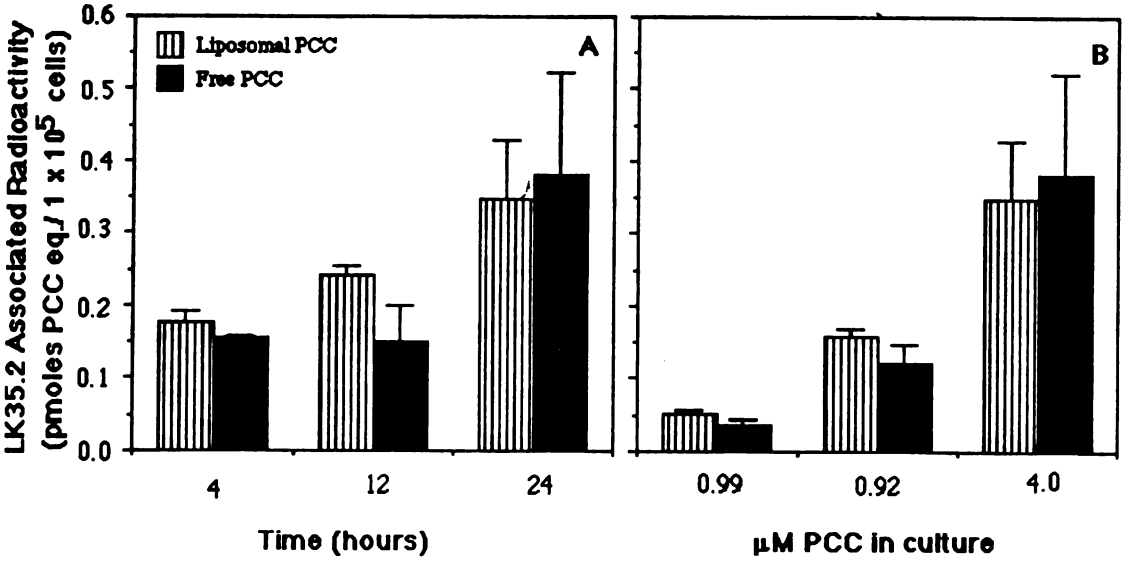


Figure III.5 : (A) Kinetics of the uptake of liposomal or free [^{125}]I-PCC by LK35.2. Cells were incubated at 37° C with 4.6 nmoles (300µl) of [^{125}]I-PCC (sp. act. 86,500 dpm/nmole) or 4.6 nmoles of [^{125}]I-PCC in liposomes (1.54 µmoles total lipid). At various times, the cultures were washed and analyzed for cell-associated radioactivity as described in Materials and Methods . Data represents the mean of duplicate cultures ± range. (B) Concentration dependence of the uptake of liposomal or free [^{125}]I-PCC by LK35.2. Cells were incubated at 37° C with varying amounts of free [^{125}]I-PCC (sp. act. 609,000 cpm/nmoles; 300 µl) or [^{125}]I-PCC in liposomes for 24 hours at 37°C. Total lipid concentration ranged from 127 nmoles at the lowest concentration of PCC to 2 µmoles lipid for the highest concentration of PCC used. Similar results (not shown) are observed when constant lipid concentrations are used in each well. Cell asssociated radioactivity was determined as described above.

that the third possibility, interference in the "processing" step or interference in APC-T-cell contact by the liposomal carrier, is not the reason for the lack of presentation of the liposomal PCC by the LK35.2 line.

Since it is difficult to quantitate the extent of internalization of surface bound liposomes, the rate of degradation of the PCC and the fraction of intact PCC that remained cell associated after incubation with the two cell types was measured. The extent of PCC catabolism to small fragments over time was determined by quantitating the CH_3CN soluble and CH_3CN precipitable radioactivity found associated with the cells or expelled into the medium during an incubation at 37°C. The disappearance of CH_3CN precipitable radioactivity from the cells is due to the release of undegraded PCC into the medium, as well as to the appearance of iodopeptides in the medium.

The results for PEC show that similar patterns for the rate of disappearance of precipitable PCC from cells and appearance of precipitable PCC in the medium are obtained for either form of antigen delivery (figure III.6.A). Differences in degradation profiles for liposome and free PCC arise when one observes the extent of formation of CH_3CN-soluble PCC in the medium (figure III.6.B). Approximately 60% of the initially bound radioactivity exists as degraded protein in the medium after 20 hours for the liposomal PCC whereas only about 40% of the radioactivity is released as small PCC fragments into the medium for free PCC over this same

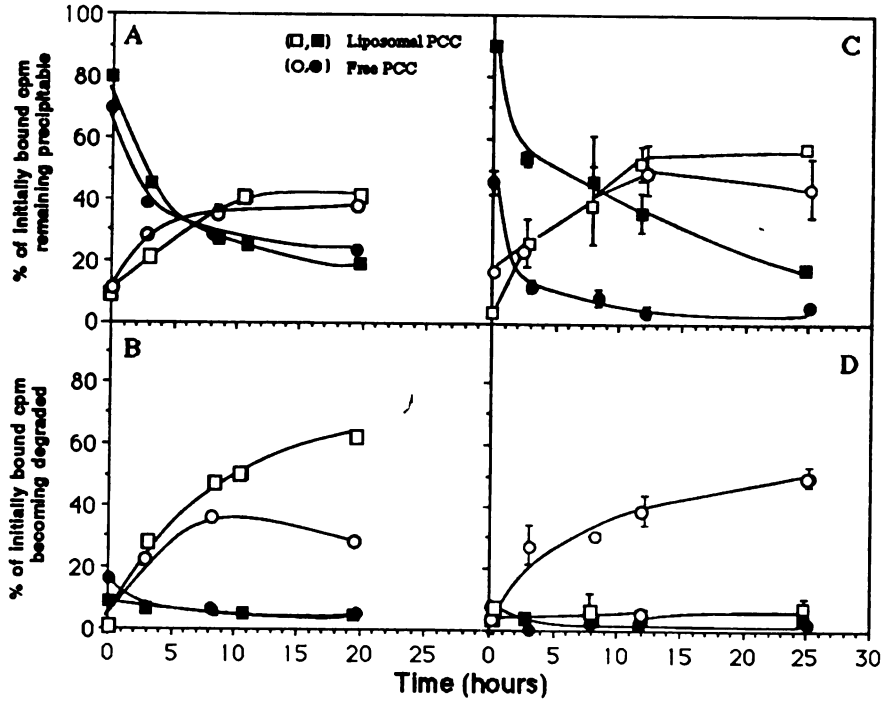


Figure III.6 : The metabolism of [^{125}I]-PCC (liposomal and free) by PEC **(A,B)** and LK35.2 **(C,D)**. PEC: 2.25 X 10^6 PEC/ 962 mm^2 in 1.5 ml was incubated with either 4.04 nmoles (300 µl; sp. act. 2.18 X 10^6 dpm/nmole) [^{125}I]-PCC in liposomes or 10.36 nmoles soluble [^{125}I]-PCC (200 µl; same sp. act.); LK35.2: 1.83 X 10^7 cells/ 1 ml in 15 cc. culture tubes incubated with 4.04 nmoles (300 µl; sp. act. 2.16 X 10^6 dpm/nmole) for both free or liposomal PCC. The ^{125}I-antigens were allowed to bind to the cells for 3 hours at 37° C. Cells were washed thoroughly, and were incubated at 37° C for varying periods of time. Duplicate samples of each culture were then tested for the amount of CH_3CN-precipitable **(A,C)** and CH_3CN-soluble **(B,D)**. Filled symbols represent cell associated material, open symbols represent material in the culture fluid. Results from PCC added as the non-encapsulated or liposome encapsulated form are given. The results are expressed as the percentage of the total initial counts bound to the cells during the initial 3 hour incubation. The total initial counts cell associated were: PEC- 102,672 ± 161 and LK35.2- 11,566 ± 242 cpm. Error bars not shown fall within the symbol.

interval. When the data are plotted as the net digestion of PCC during the experimental period, approximately 60% of the cell associated PCC is digested when delivered in liposomes as compared to 40% digestion without the carrier (figure III.7.A).

The LK35.2 show a more striking difference in the fate of liposomal versus free PCC. For the liposomal PCC, approximately 60% of the initially bound radioactivity is released into the medium in a precipitable form. The remainder, within experimental error, is left associated with the cells also in the precipitable form (figure III.6.C). The [^{125}I]-PCC in the medium was shown to co-migrate with the lipid when separated on a metrizamide gradient centrifugation (not shown). For free PCC, about 50% of the initially bound material is released into the medium as the precipitable form. Approximately 10% remains cell associated as the precipitable form by the end of the 24 hour period. In the case of the liposomal PCC there was no CH_3CN-soluble counts in either the medium or the cells after 24 hours (figure III.6.D). With the free PCC, however, 40% of the initially bound radioactivity is released into the medium as CH_3CN-soluble counts. Net digestion in the B-cell amounts to approximately 95% of the initial cell associated material for the free PCC whereas less than 5% net digestion is observed for the liposomal PCC (figure III.7.B).

The rate of digestion of PCC, as estimated from the initial slope during the first 2 hours, is greater for the

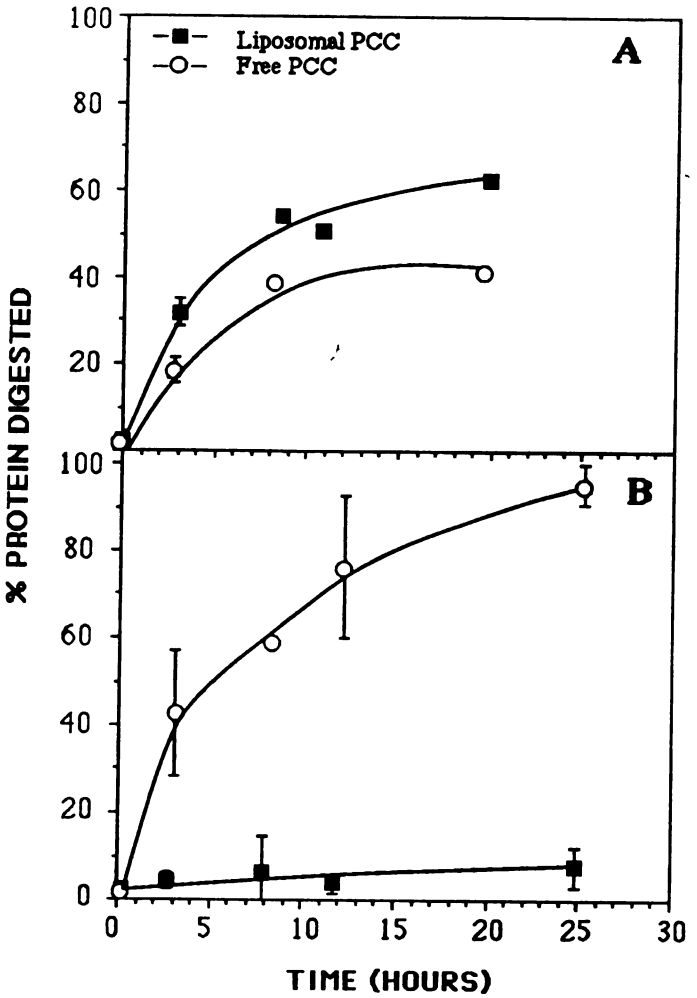


Figure III.7 : Kinetics of degradation of [^{125}I]-PCC, free and liposomal, expressed as the percent of exogenous cell-associated PCC, digested as a function of time in PEC (A) or LK35.2 (B). Net production of CH_3CN-soluble radioactivity gives an indication of the amount of initially bound protein that was digested and is calculated as follows: **[(CH_3CN-soluble cpm in cells + CH_3CN-soluble cpm in culture fluid) - (initial CH_3CN-soluble cpm in the cells)]**. This value is then divided by the initial CH_3CN-precipitable cpm to obtain the amount of PCC digested at each time point. Error bars not shown fall within the symbol.

LK35.2 (18% / hr) than the macrophage (8% / hr). The rate of degradation in macrophages is slightly faster for liposomal PCC than free PCC whereas the degradation in B-cell tumors is totally abolished when encapsulated in liposomes (figure III.7 A & B).

III.4: DISCUSSION

We have demonstrated that encapsulation of PCC in phospholipid vesicles will stimulate the *in vitro* immune response to PCC by peritoneal macrophages at the level of antigen presentation. In contrast, presentation of PCC by a B-cell hybridoma is abolished by incorporating the antigen into liposomes. Our findings suggest that the difference in antigen presenting function of the two APC to the liposome encapsulated antigen is due to the capability of the macrophage to internalize and degrade liposomes which is absent in the B-cell tumor.

To our knowledge, this is the first study that correlates the antigen-specific IL-2 response to liposomal antigen with the uptake and processing of liposomes by APC *in vitro*. Recently, Oth and coworkers measured an *in vitro* antigen-specific IL-2 response after restimulation of splenocytes from mice immunized with either complete inactivated rabies virus or the purified viral glycoproteins incorporated in liposomes (Oth et.al., 1987). However, the IL-2 response was not correlated with the interaction of the liposomal antigen with immunocompetent cells.

III.4.1: Response of peritoneal elicited macrophages to antigen in liposomes

Using the antigen presentation assay, we demonstrated that PEC can efficiently present PCC to PCC-specific T-cell hybridomas as measured by IL-2 production (figure III.3.A). This finding is consistent with other studies on the APC function of elicited PEC (Schwartz, 1985). We further demonstrated that liposome entrapped PCC leads to efficient antigen presentation by PEC *in vitro* (figure III.3.A). The dose-response curve was variable from experiment to experiment but in general, the increase in potency of antigen presentation by liposomal entrapment was only 2 fold. A possible explanation for the variability in the results could be due to the fact that peritoneal exudate cells express low and variable levels of Ia glycoprotein on their surface (Beller et.al., 1980; Beller and Unanue, 1982). While the total population of the PEC are involved in the uptake of the antigen, only the fraction of the population which express Ia are actually able to present the antigen. It is also possible that the variability in the response was due to differences in the amount of PCC on the surface of the liposomes from preparation to preparation.

When the antigen was entrapped in liposomes, approximately 2 times more antigen was cell associated compared to the non-encapsulated PCC (figure III.4). This is not surprising since it is well known that peritoneal exudate cells can take up liposomes (McDougall et.al., 1974; Patel

and Ryman, 1981; Raz et.al., 1981; Hsu and Juliano, 1982). The increased uptake correlates with the modestly enhanced IL-2 response seen for liposomal antigen. Furthermore, the rate and extent of degradation of PCC is slightly enhanced by entrapping the antigen in liposomes (figure III.7.A). Our findings suggest that the slightly increased potency of the liposomal antigen resulted from delivering proportionally greater amounts of PCC to the macrophage, which was more rapidly degraded than non-encapsulated PCC.

III.4.2: Response of a B cell hybridoma to liposome encapsulated antigen

It has been recently reported that cells classically defined as non-phagocytic, might be able to process and present particulate forms of antigens (Ratcliffe et.al., 1984; Malynn et.al., 1985; Lombardi et.al., 1987). For this reason, we studied the ability of a B-cell hybridoma (LK35.2) to process and present liposomal PCC. The LK35.2 was a suitable APC for comparison to the PEC due to the following qualities: 1) It is an efficient presenter of PCC to PCC-specific T-cell hybridomas (Kovac and Schwartz, 1985; Lakey et.al., 1986), 2) LK35.2 behaves as a fully activated B-cell and expresses high levels of I-A (>95% $I\text{-}A^+$) (Kim et.al., 1979), and 3) it has a high endocytic index for soluble protein as compared to resting B-cells (Chesnut et.al., 1982).

The LK35.2 is as efficient in presenting non-

encapsulated PCC as are the PEC (figure III.3.B). This observation is consistent with findings on the antigen presentation ability of other B cell types. For instance, in the BAL lymphoma, antigen presentation to keyhole limpet hemocyanin -specific T-cell hybrids is as effective as that for a macrophage line (P388D1) or normal PEC's (Chesnut et.al., 1982). In contrast, the LK35.2 is totally ineffective in presenting the liposome encapsulated antigen to the 2B4 cells, as measured by the production of IL-2 (figure III.3.B). We demonstrated that the lack of IL-2 response was not due to the inability of liposomal PCC to associate with the B-cell. Cell association was similar or greater when PCC was delivered in liposomes than when free (figure III.5). Moreover, empty liposomes did not influence the response to free PCC, suggesting that the lipid vesicles do not interfere with the processing activity of the LK35.2.

Our findings suggest that the liposomal antigen was not degraded because it was not internalized, in accord with previous studies on the interaction of liposomes with lymphocytes, where internalization was found to be low and dependent on the size of the liposomes (Machy and Leserman, 1983). Liposomes adhered to the surface of the LK35.2, but continued incubation resulted in a slow release of radioactivity into the medium (figure III.6.C). This material was precipitated by acetonitrile and co-migrated with the lipid fraction when placed on a metrizamide gradient suggesting that intact liposomes were desorbing from the

surface of the APC. The fact that significant levels of acetonitrile-soluble radioactivity were not found with cells incubated with the liposomal PCC indicates that little or no degradation of the encapsulated PCC had occurred, in marked contrast to the fate of non-encapsulated PCC, which was efficiently degraded by the LK35.2 (figure III.6.C,D & III.7.B). The ability of LK35.2 to degrade soluble forms of PCC is consistent with studies by Kovac and Schwartz using a different PCC-specific T-cell hybridoma (Kovac and Schwartz, 1985).

In the antigen presentation system studied here, only one liposome composition was tested. Although we believe these results to be generally representative, other lipid compositions, or liposome types, might behave differently. Recently it has been shown that phospholipid composition may influence an immune response to a liposome-associated tumor antigen (Bakouche and Gerlier, 1986). The authors speculated that macrophage uptake and/or processing could be modified by the lipid composition of the liposomal preparation.

III.4.3: Relevance to liposome adjuvant effect in vivo

The in vivo adjuvant effect of liposomes may be due to a greater fraction of the dose of antigen delivered to the APC because liposomes have a smaller volume of distribution than the non-encapsulated antigen (Hwang, 1987) and are avidly taken up by macrophage-like APC (Beatty et.al., 1981; Hsu and Juliano, 1982). In addition, liposomes not taken up by APC

may serve as a depot to prolong antigenic stimulation of the immune system by slowly releasing the entrapped antigen (Kramp et.al., 1982), this effect occurring either at the site of injection or in lymph node tissue rich in APC. Moreover, due to the high internal concentration of antigen in the liposome, a greater amount of antigen may be delivered to individual APC. Since this would occur at the early stages of the immune cascade, a small effect may be considerably amplified. Finally, liposomes may contain immunomodulators such as bacterial lipopolysaccharides or lipid A (Alving and Richardson, 1984) added either intentionally or accidentally during liposome preparation, resulting in enhanced humoral and cellular responses.

The *in vitro* system is relevant at the level of the individual APC and demonstrates that macrophages but not B cells can present liposome-encapsulated antigen. Liposome encapsulation did not dramatically enhance antigen presentation with the responsive APC when compared to non-encapsulated antigen. Therefore, it seems unlikely that liposome encapsulation and delivery of antigens turns the individual APC into a super efficient presenting cell *in vivo*. This suggests that the liposome adjuvant effect is probably due to one or a combination of the other mechanisms outlined above; pharmacokinetic properties, depot effect or to the presence of an immunomodulator in the liposome.

Chapter IV: PRESENTATION OF CYTOCHROME C ENCAPSULATED IN LIPOSOMES BY PERITONEAL MACROPHAGES: TIME DEPENDENCE AND EFFECT OF LIPID COMPOSITION

IV.1: INTRODUCTION

Liposomes have been found to be useful as adjuvants of immune responses to protein antigens in vivo. (Morein and Simons, 1985). The enhancement of an antigen-specific antibody response observed when the antigen is associated with liposomes has been attributed to the ability of accessory cells such as macrophages, to interact with the liposome carriers (Shek and Lukovich, 1982; Gerlier et.al., 1983; Bakouche et.al., 1987). Moreover, the immune enhancement is dependent on the phospholipid composition of the liposomes (Heath et.al., 1976; Dancey et.al., 1978; van Houte et.al., 1981; Bakouche et.al., 1987).

We have previously used an in vitro antigen presentation system to study the ability of peritoneal macrophages to bind, process, and present unencapsulated protein antigen, PCC, and PCC encapsulated in solid liposomes (Chapter III). We demonstrated that encapsulation of PCC resulted in: 1) Approximately a 2 fold increased association of PCC with PEC as compared to unencapsulated PCC; 2) the rate and extent of degradation of PCC was slightly enhanced over the unencapsulated form; and 3) the efficiency of presentation by PEC was modestly enhanced as a result of encapsulation of PCC. Based on our findings with one liposome type, we became

interested in using our antigen presentation system to study the effect of different lipid compositions.

We studied the ability of peritoneal macrophages to present PCC when encapsulated in liposomes of various lipid compositions. The kinetics of association of the antigenic forms with the peritoneal macrophages and the time course of IL-2 response by the T-cell hybridoma (2B4) were measured. Two lipid compositions were tested, 1) a fluid phase preparation composed of egg phosphatidylcholine (EPC)/egg phosphatidylglycerol (EPG) and 2) a solid phase preparation composed of synthetic phospholipids dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG) and cholesterol (CH).

We demonstrate that both the rate and extent of cell association and the presentation depends on the antigenic form in which PCC is delivered. PEC are more stimulatory when PCC is delivered in solid liposomes than in fluid liposomes or unencapsulated in soluble form. Our results support in vivo studies by Bakouche and Gerlier in which the immunogenicity of tumor antigens was increased by liposome entrapment depending on the lipid gel-liquid crystalline transition state temperatures (Bakouche and Gerlier, 1986; Bakouche et.al., 1987).

IV.2: MATERIALS AND METHODS

IV.2.1: Antigens

PCC (Type XIII from pigeon breast muscle) and BSA (Fraction V) were obtained from Sigma Chemical Co. (St.Louis,MO). Radiolabelled PCC was prepared by a modification of the method of Greenwood et al (Greenwood et.al., 1963) using [^{125}I]-NaI (Amersham, Arlington Heights, IL). Specific activity of 0.995 μCi/ nmole was obtained.

IV.2.2: Liposome preparations

Liposomes containing PCC were prepared by a modification of the method of Szoka and Papahadjopoulos (1978). The procedure was described in Chapter III. Two lipid compositions were used: 1) DPPC (Avanti Polar Lipids, INC., Birmingham, AL), DPPG (Avanti) and CH (Sigma) in a molar ratio of 9:1:8; 2) EPC/ EPG molar ratio 9 : 1. Non-encapsulated PCC was separated from the vesicle-associated PCC by gel filtration through a Bio-Gel A0.5M (Bio-Rad, Richmond, CA.) (1 X 10 cm) column. The liposomes were assayed for lipid content by phosphorous analysis (Bartlett, 1959) and protein content was measured spectrophotometrically (O.D. 550 nm, E^{1mM} = 28) under reducing conditions (excess dithionite) (Margoliash et.al., 1959) in the presence of 0.02% Triton X-100. Liposome diameter was measured by laser light scattering on a Nanosizer model N-4 (Coulter Electronoics, Hialeah, FL.).

IV.2.3: Cell lines

The PCC specific T-cell hybridoma (2B4) and the IL-2-dependent cell line (CTLL-20) were used. These cells are described in Chapter III. The cell lines were tested and found to be free from mycoplasma contamination.

PEC were used as a source of antigen presenting macrophages. These cells were harvested from C3H/HeJ male mice (Ia^k) (6-8 weeks old, Charles River, Wilmington, Delaware) by peritoneal lavage with ice-cold Hank's balanced salt solution 4-5 days after an intraperitoneal injection of 4 ml of thioglycollate medium (3% in water). The cultures were enriched for macrophages by allowing the cells to adhere to plastic culture dishes for 3-4 hours with subsequent washing of the monolayers. The purified macrophages were used immediately for the in vitro assays. The PEC were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS, HyClone Laboratories, INC. Logan,Utah), 2 mM glutamine, 25 mM N-2-hydroxy-ethylpiperazine-N'-2-ethane sulfonic acid, 1 mM pyruvate, 5 X 10^{-5} M 2-mercaptoethanol, and antibiotics (RPMI-CM). CTLL-20 and 2B4 were cultured as described in Chapter III. Media and FCS were obtained from the Cell Culture Facility, University of California at San Francisco.

IV.2.4: Antigen presentation assay

Antigen presentation was determined by measuring the induction of IL-2 secretion by the 2B4 cells when co-cultured with antigen pulsed APC. The PEC were plated as monolayers on

96 well plates (flat bottom, Costar) at 2.5 X 10^5 cells per well in 200 µl RPMI-CM. A single concentration of PCC in the antigenic forms studied was added to each well containing adherent PEC. Each set of triplicate cultures was incubated for varying periods of time at 37° C, 5% CO_2, 95% R. H. The cells were washed 2 times with phosphate buffered saline (PBS; 0.2 g/liter KCl, 0.2 g/liter KH_2PO_4, 8 g/liter NaCl, 1.15 g/liter Na_2HPO_4, pH 7.4) to remove any unbound antigen and subsequently fixed with glutaraldehyde (0.05% v/v final concentration) for 80 seconds at room temperature. The PEC were incubated with 0.2 M lysine in PBS, pH 7.4 for 80 seconds and thoroughly washed. The antigen-pulsed fixed PEC may be stored for up to 1 week at 4°C until addition of T cell hybridomas.

The antigen-pulsed APC were subsequently incubated with the 2B4 cells (1 X 10^5) in a volume of 200 µl EHAA:RPMI in 96 well culture plates. After 24 hours at 37°C, a 100 µl aliquot of the supernatant from each well was removed and assayed for IL-2 content as determined by the incorporation of tritiated thymidine ([^{3}H]-TdR) by the IL-2 dependent cell line CTLL-20 (Gillis et.al, 1978). All titrations for IL-2 activity were performed in triplicate. Results are expressed as mean ± SD. Standard deviations from the mean were generally between 5 and 15%.

IV.2.5: Cell association of encapsulated or nonencapsulated PCC

PEC, washed to remove nonadherent cells, were cultured on 35mm diameter culture dishes (Costar) at 1.8 X 10^6 cells per dish in 1 ml of RPMI-CM. A single concentration of radiolabelled PCC, encapsulated or nonencapsulated, in TES/NaCl buffer was added to the PEC in 0.2 ml aliquots. After varying time intervals the cells were washed 10 times in ice-cold PBS. The washed cells were then solubilized with 1 ml of 0.1M NaOH-0.4% sodium deoxycholate (w/v) and assayed for total cell associated radioactivity by gamma scintillation counting. All assays were performed in duplicate. Results are expressed as mean ± range. Controls were included to determine non-specific binding of antigen to cell culture dishes or tubes and less than 5-15% binding was observed. This level of non-specific binding was subtracted from culture vessels containing cells.

IV.3: RESULTS

The two phospholipid compositions studied in this report consisted of the "solid" or gel state liposomes (DPPC, DPPG, and CH) and a "fluid" or liquid crystalline liposome (EPC/EPG) preparation. The liposomes exhibited a net negative charge on their surface due to the negatively charged head group on phosphatidylglycerol and were similar in size. The major difference in the preparations was in the amount of PCC which was encapsulated. Solid liposomes encapsulated 2.1% of

the added protein whereas fluid liposomes encapsulated 9% of the added PCC. The result of this difference is reflected on the 3-5 fold increase in payload of PCC for the fluid liposomes over the solid liposomes. The liposome characteristics are summarized in (Table IV.1).

IV.3.1: Kinetics of IL-2 production by 2B4 cells

PEC were incubated with PCC forms for varying lengths of time, washed free of excess antigen, fixed with glutaraldehyde, and tested for their ability to stimulate 2B4 cells to secrete IL-2. All the antigen-pulsed fixed PEC were co-cultured with 2B4 cells at the same time.

Regardless of the form in which PCC is delivered to PEC (free or liposome encapsulated), there exists an initial lag period of approximately 2 hours where no IL-2 can be detected (Figure IV.1). Subsequently, a rise in IL-2 production can be measured. The rate of the rise during the first 6-8 hours is similar, within experimental error, for all antigenic forms of PCC. In the case of the unencapsulated PCC and PCC encapsulated in fluid liposomes, maximal stimulation is reached by 8 hours, and remain at this level through to the last sample at 24 hours. In contrast, the IL-2 stimulatory activity of PEC to PCC encapsulated in solid liposomes continues to increase beyond 8 hours and gradually levels off at 24 hours. The level of [^{3}H]-TdR incorporation at 24 hours is approximately 2 fold greater for the PCC encapsulated in solid liposomes than that of unencapsulated PCC.

Table IV.1: Liposome preparations used in the study

Liposome	**Solid**	**Fluid**
I) **Lipid composition**	DPPC/DPPG/CH	EPC/EPG
mole ratio:	9 : 1 : 8	9 : 1
II) **Diameter** (nm)		
a) IL-2 study	178 ± 48	161 ± 46
b) cell association	200 ± 69	183 ± 50
III) **Protein:Lipid ratio**		
(μmole PCC/ μmole lipid)		
a) IL-2 study	1.90×10^{-3}	9.24×10^{-3}
b) cell association	1.45×10^{-3}	3.78×10^{-3}
i) μCi/ μmole PCC	54.20	54.20
IV) **Encapsulation efficiency***	2.06%	9.55%

* Based on the amount of PCC added to prepare the liposomes

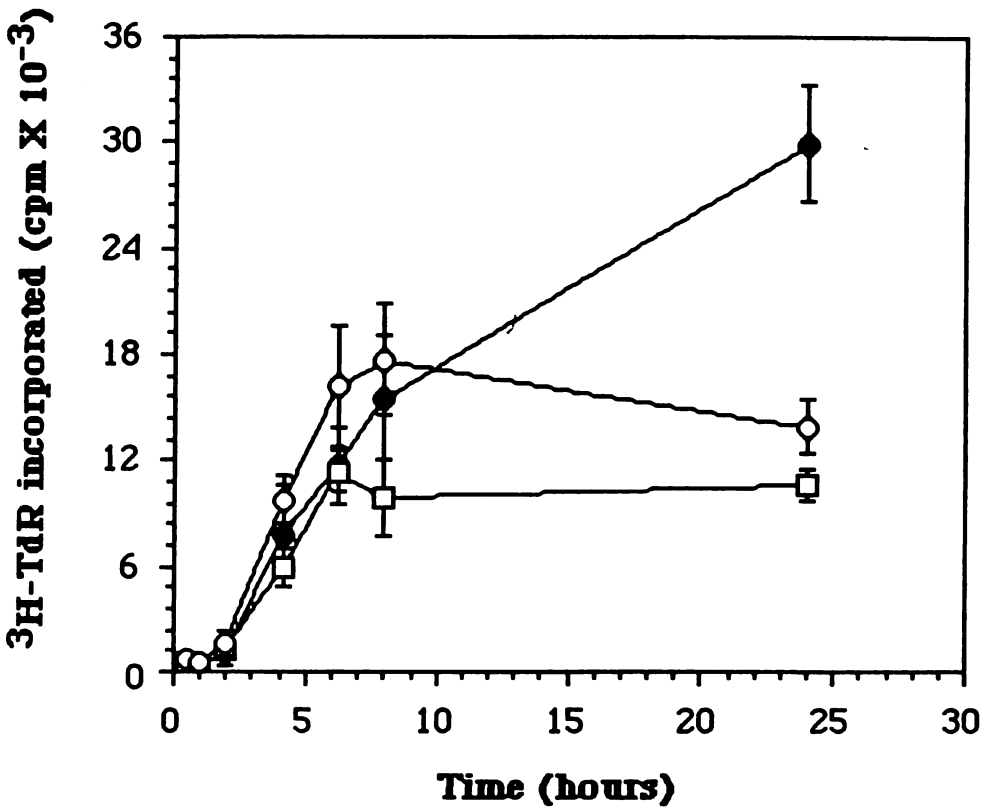


Figure IV.1: Kinetics of the IL-2 response by 2B4 cells when PCC antigenic forms are presented by peritoneal macrophages. The PEC were incubated with 1.81 μM PCC either in the unmodified form, ○; encapsulated in solid liposomes, ●; or encapsulated in fluid liposomes, □ for varying periods of time and subsequently fixed in 0.05% glutaraldehyde before culturing with 2B4 cells. IL-2 response by 2B4 was measured as described in the Material and Methods. Cultures with no antigen resulted in less than 250 cpm incorporation. Each value represents the average of triplicate cultures. The standard deviation was less than 10%.

IV.3.2: Time course in cell association of PCC antigens with PEC

The cell association of PCC and PCC-loaded liposomes is shown in figure IV.2. The rate and extent of cell association is influenced by the form in which the antigen is delivered. For the unencapsulated PCC, the amount of cell associated PCC increased gradually up to 0.25 pmoles PCC per 1 X 10^5 cells at 23 hours. Encapsulation of PCC in fluid liposomes resulted in approximately 2.5 times more PCC cell associated by the first time point tested than the unencapsulated PCC. The extent of association was increased 2.4 times over that of unencapsulated PCC at the end of the 23 hour incubation. A dramatic increase in both the rate and extent of cell association was demonstrated by the PCC entrapped in solid liposomes. At the earliest time point tested, association of PCC increased 8 fold over the unencapsulated antigen. Incubation of PCC encapsulated in solid liposomes for longer periods did not significantly alter cell association levels from those seen at early times. At 23 hours, the level of PCC cell associated was 3-4 fold greater when encapsulated in solid liposomes than when unencapsulated.

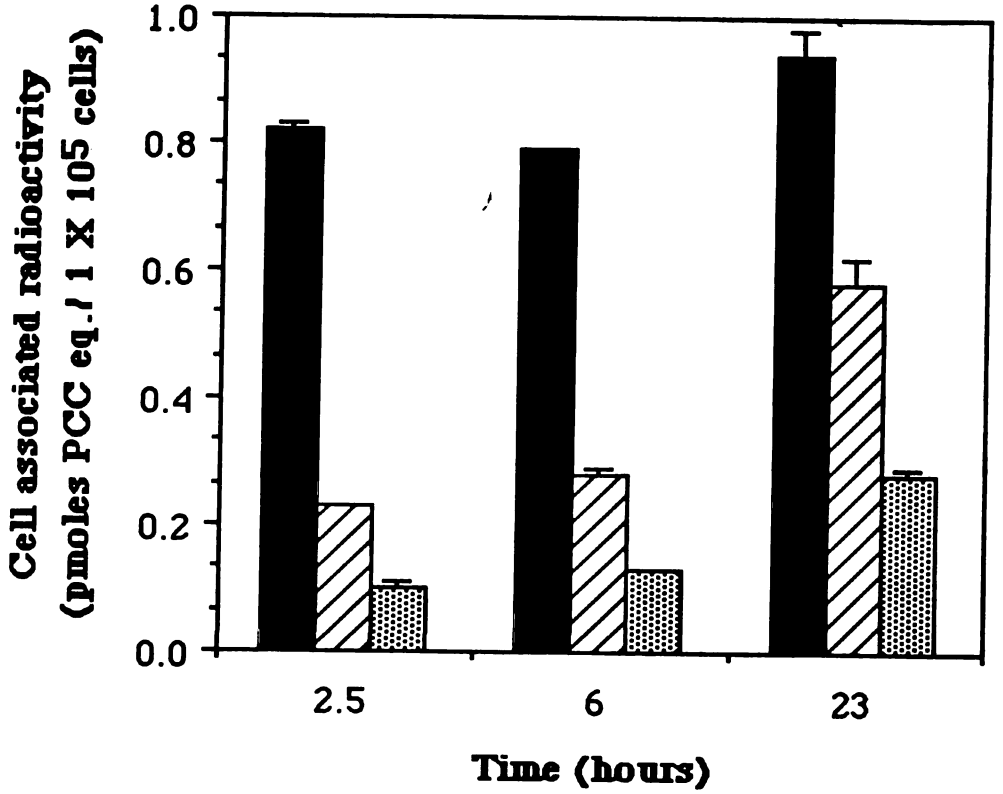


Figure IV.2: Cell association of PCC antigenic forms with PEC as a function of time. PEC were incubated with 0.5 μM [^{125}I]-PCC in the unmodified form, ▨ ; encapsulated in solid liposomes, ■ ; and encapsulated in fluid liposomes, ▨ . Each value represents the mean ± range of duplicate cultures. Non-specific binding was less than 10%.

IV.4: DISCUSSION

In this report, using an in vitro antigen presentation system, we studied the effect of encapsulating PCC in liposomes of different composition on the time course of two key events of antigen presentation; the association of antigen with the antigen presenting cell and the presentation of processed antigen to antigen-specific T helper hybridomas.

We demonstrated that after an initial 2 hour lag period in which no IL-2 production can be detected, PEC become stimulatory to 2B4 cells. During the initial 6-8 hours of incubation, the kinetics of the response was similar for all forms of PCC. Maximal stimulatory activity for PCC and PCC encapsulated in fluid liposomes was observed at approximately 6 to 8 hours. We can not offer any explanation for the finding that unencapsulated PCC stimulates higher IL-2 levels than the PCC encapsulated in fluid liposomes. This result was from a single experiment. In our other studies (Chapter II and VI), the encapsulated antigen always gave a response equal to or greater than the unencapsulated antigen. Interestingly, for the solid liposomes, the level of stimulation increased beyond the 6-8 hour point up through 24 hours to levels of incorporation approximately 2 fold greater than the unencapsulated PCC or PCC in fluid liposomes.

Similar kinetics of presentation for unencapsulated PCC have been observed by Lakey and colleagues using B cells as the antigen presenting cell (Lakey et.al., 1988). The kinetic pattern is a result of of the time required for

internalization, partial degradation, and presentation of the antigen to the T cell in conjunction with Ia on the surface of the APC (Ziegler and Unanue, 1981; Ziegler and Unanue, 1982).

We demonstrated that the amount of cell associated PCC as a function of time was always greater for the encapsulated antigen rather than the unencapsulated. The measurements were done at 37°C thus the amount of cell associated material represents bound and internalized antigen. Partial degradation of PCC may have occured by the time the sample was tested. However, in spite of the enhanced cell association of PCC with the liposome carriers, the unencapsulated PCC had an equivalent or slightly greater IL-2 response during the first 8 hours than the liposomal forms.

Two possible scenarios may explain these results; 1) the liposomal PCC binds to the cell surface but is not internalized as rapidly as unencapsulated PCC. Due to the antigen carrying capacity of the liposomes, very few liposomes need to be internalized to achieve intracellular PCC concentrations equivalent to those of the unencapsulated PCC, 2) the liposomes are internalized and deliver high concentrations of PCC however, the rate of degradation of the liposomal bilayers is rate limiting and dependent on lipid composition.

It is possible that both scenarios play significant roles in the responses observed. Uptake and processing of liposomes by macrophages is dependent on lipid composition.

Liposomes composed of lipids with high gel-liquid transition state temperatures (Tc) are usually taken up more efficiently than those with low Tc. In addition, liposomes composed of high Tc lipids are less susceptible to degradation by membrane phospholipases (Senior and Gregoriadis, 1982; Kirby et.al., 1980). Solid liposomes, being more stable than fluid ones, may allow an intracellular depot of encapsulated antigen which slowly becomes degraded to release antigen. Other explanations are possible and further work is needed to elucidate the mechanism(s) involved.

We can conclude from these studies that the amount of antigen available at intracellular sites for processing, is limited by the rate and extent of internalization of liposomes and the rate at which PCC is released from the liposomes once inside the cell. Furthermore, internalization and/or liposome degradation is a function of lipid composition.

CHAPTER V: IL-2 PRODUCTION BY T-CELL HYBRIDOMA (2B4) AFTER PRESENTATION OF LIPOSOMAL OR SOLUBLE PCC BY DIFFERENT MACROPHAGE POPULATIONS.

V.1: INTRODUCTION

Antigen presenting cells (APC) were originally identified as macrophages. Since then, the number of cells reported to function as APC has grown to an impressive list (Erb et.al., 1984). All APC express I-region associated (Ia) gene products on their surface. Any Ia-bearing cell is able to induce antigen specific T helper cell activation although to a variable degree. For instance, spleen adherent cells (SAC) can efficiently present rabbit anti-mouse immunoglobulin to antigen specific T cells (Kakiuchi et.al., 1983).

In our previous studies (Chapter III, IV), we focused on the ability of thioglycollate-elicited peritoneal exudate cell (PEC) to function as APC for unencapsulated and liposome encapsulated antigen. A review of the literature shows that the properties of PEC can vary depending upon how they are obtained: 1) Intraperitoneal injection of agents such as mineral oil, peptone, or thioglycollate induce an increase in the number of macrophages at the site of injection while the proportion of Ia^+ macrophages remains similar to that of a resident macrophage population (1-5% Ia+ cells) (Ziegler et.al., 1984; Johnson and Zwillig, 1985); 2) Peritoneal macrophages rapidly loose the ability to synthesize and

express Ia antigens in culture regardless of how they are elicited. The vast majority of Ia+ macrophages become Ia- by the second day of culture (Beller and Unanue, 1982); 3) Ia+ cells cultured in vitro become Ia- by day 2 of culture even in the presence of Con A stimulated T cell supernatants. Re-expression of Ia can occur on day 3-6 of the in vitro culture by incubating with fresh Con A-stimulated T cell supernatants (Beller and Unanue, 1982); and 4) Intraperitoneal injection of Con A elicits a peritoneal exudate cell population with 50-60% of the cells being Ia+ (Friedman and Beller, 1987)

The purpose of these experiments was to determine the effect on the IL-2 response when PCC (encapsulated in liposomes or unencapsulated) is presented by APC prepared by different methods of isolation.

V.2: MATERIALS AND METHODS

V.2.1: Antigens and other reagents

PCC (Type XIII from pigeon breast muscle) was obtained from Sigma Chemical Co. (St.Louis,MO); Con A (E-Y Laboratories)-stimulated rat spleen cell supernatants (RCS) was prepared as described in Chapter II. The RCS had been stored for approximately 9 months at -20°C and thawed to conduct these experiments.

V.2.2: Mice and Treatments

C3H/HeJ male mice 5-6 week old (Charles River, Wilmington, Delaware) were injected ip. with 1.5 ml thioglycollate broth (3% in H_2O) or with 1.5 ml Con A (30µg/ml in sterile saline).

V.2.3: Liposome preparations

PCC encapsulated in liposomes composed of DPPC : DPPG : CH, molar ratio 9:1:8, were prepared by a modification of the method of Szoka and Papahadjopoulos (1978) as described in Chapter III. Final liposome preparations consisted of 3.53 µmole/ml lipid and 40.2 µM PCC. Empty liposomes of the same lipid composition were prepared in the same manner in the absence PCC.

V.2.4: Cells

The following APC were tested:

1) Fresh PEC(Thio): Thioglycollate stimulated (ip, 4 day prior) PEC, plated, wash off non-adherent at 3 hours, add antigen, incubate 24 hours, wash, add T cells.

2) Fresh PEC(Thio) + RCS : Same as above except add 50% RCS for the 24° incubation period.

3) 5 DAY PEC(Thio) + RCS : Thioglycollate stimulated as above. Plate for five days in the presence of RCS (50%). Pulse with antigen final 24 hours.

4) Fresh PEC(ConA) : Con A stimulated PEC (5 day prior ip.). Harvested and plated. Washed off non-adherent after 3 hours,

pulse with antigen and incubate 24 hours. Wash off unbound antigen, add T-cells. We noticed that the number of cells collected after peritoneal lavage from mice stimulated with Con A was considerably lower than that obtained with thioglycollate and comparable to the cell number found in non-elicited mice.

5) 5 DAY PEC(ConA) + RCS : Same as 4, except that PEC are plated for 5 days in the presence of RCS (50%) and pulsed with antigen in the final day (Day 4).

6) 21° SAC : Whole spleen cell suspension incubated with antigen for 21 hours. At the end of this period the cells (and the antigen) was thoroughly washed off. Remaining cells are 21° spleen adherent cells (SAC). The T-cells were then added.

7) 3° SAC : Whole spleen cells plated, wash off non-adherent cells after 3 hours, add antigen and incubate 21 hours, wash off unbound antigen, add T-cells.

All of the PEC were obtained from C3H/HeJ mice by peritoneal lavage with ice cold HBSS. The cells were washed thoroughly and plated on 96 well plates at 2.6×10^5 cells per well. Spleens were aseptically removed and placed in ice-cold HBSS. Cell suspensions were made by passing the spleens through wire mesh. The cells were washed twice and resuspended at 3×10^7 cells /ml in NH_4Cl lysis buffer containing 2% FBS at 4°C in order to lyse the RBC. The cells were centrifuged through 100% FBS, washed twice and resuspended at 7.05×10^6 cells/ml. A volume of 200 µl per

well was plated on 96 well plates. The APC were cultured in RPMI-CM.

The PCC specific T-cell hybridoma, 2B4 ($H\text{-}2^k$) (Samelson and Schwartz, 1983) and the CTLL-20 line, an interleukin-2 (IL-2) dependent cell line were described in Chapter III. Culture conditions for the 2B4 and the CTLL-20 are described in Chapter III.

V.2.5: Antigen presentation assay

The antigen presentation assay is based on the determination of IL-2 secretion by the 2B4 when co-cultured with antigen pulsed APC. The method is described in Chapter III. The antigen (unencapsulated or liposomal) was added in 50 µl aliquots. Serial dilutions of liposomes were done with empty liposomes to maintain constant lipid concentration in the assay well. Final lipid concentration in all wells containing liposomes (empty or PCC loaded) was 173 nmoles per well.

V.3: RESULTS AND DISCUSSION

V.3.1: Antigen presentation by PEC

The antigen presenting function of thioglycollate-elicited PEC was compared with freshly isolated cells and cells which were cultivated in vitro for 5 days in the presence of RCS. The results are shown in figure V.1.A,B and can be summarized as follows:

1) PEC cultivated for 5 days were significantly less efficient than fresh PEC even in the presence of RCS. This was the case for the unencapsulated and encapsulated PCC (fig. V.1.B).

2) Fresh PEC were efficient presenters of PCC whether in liposomes or free (fig V.1.A). In this experiment, the efficiency of presentation of liposomal PCC is slightly lower than that described in Chapter III. The difference in the two studies is more pronounced at higher PCC concentrations. The reasons for this discrepancy are unknown.

3) The addition of RCS to the cultures did not alter the ability of fresh PEC to present free PCC. The response to liposomal PCC was significantly reduced in the presence of RCS (fig. V.1.A).

The results from these studies indicate that the ability of PEC to present free or liposomal PCC is diminished by prolonged incubation. The APC function of 5 day PEC could not be induced with RCS. Moreover, addition of RCS to fresh PEC inhibited their ability to present liposomal PCC.

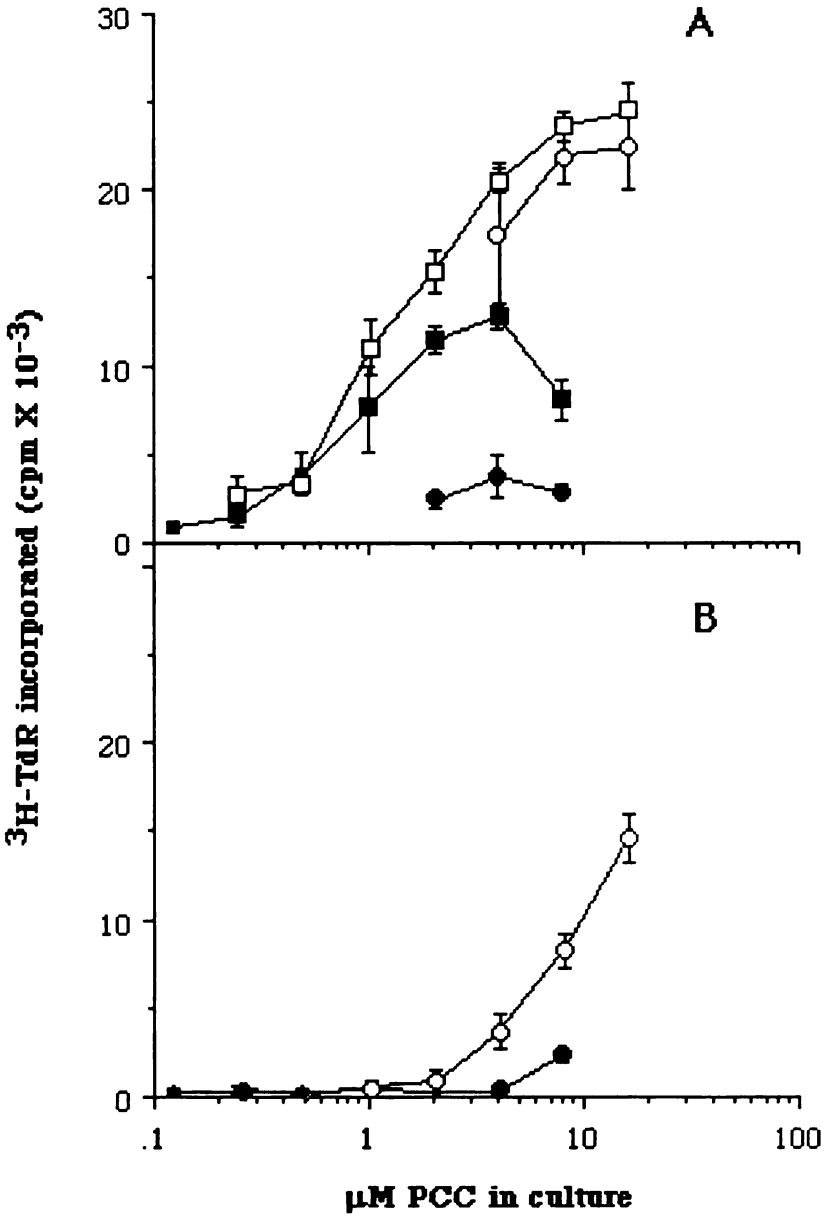


Figure V.1 : Antigen presenting function of thioglycollate-elicited PEC used (A) fresh or (B) after 5 days of cultivation in the absence (squares) or presence (circles) RCS. Presentation of free PCC (open symbols) or REV (filled symbols).

V.3.2: Comparison in antigen presenting function of thioglycollate-elicited PEC, Con A-stimulated PEC, and SAC

V.3.2.A: Presentation of unencapsulated PCC

The ability of various APC populations to present unencapsulated PCC is shown in figure V.2. The freshly isolated thioglycollate-elicited PEC was the most efficient APC followed by fresh Con A-stimulated PEC and SAC isolated from 3° cultures. The IL-2 response was significantly reduced by prolonging the cultivation of the APC. In the case of the Con A-stimulated PEC, the response after prolonged cultivation was totally inhibited.

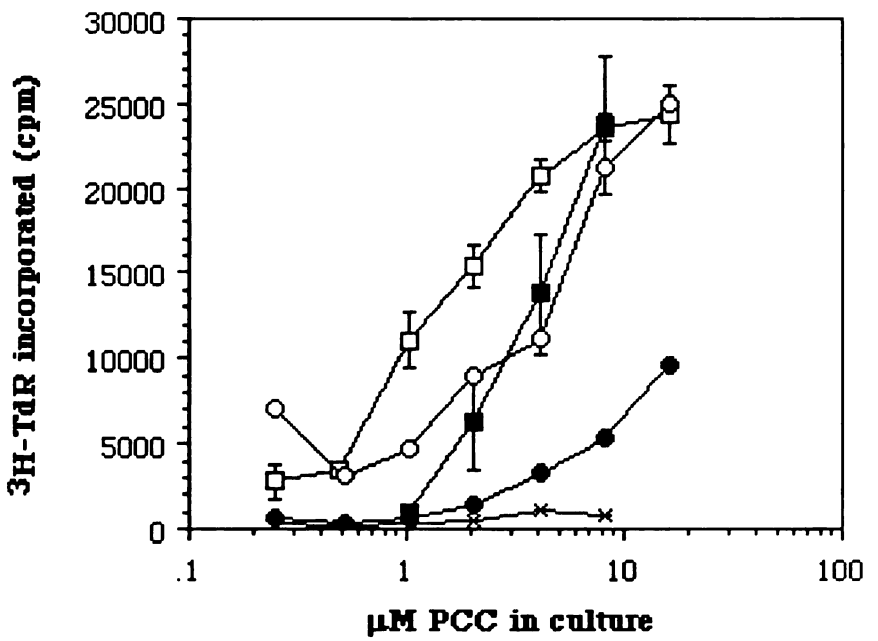


Figure V.2 : Comparison in the IL-2 response when unencapsulated PCC is presented by: fresh thioglycollate-elicited PEC (□), fresh Con A-stimulated PEC (■), 3° SAC (○), 21° SAC (●), and Con A stimulated PEC from 5 day cultures (*). The ability of 3° SAC to present free PCC is not altered by the addition of empty liposomes (not shown).

V.3.2.B: Presentation of liposomal PCC

The IL-2 response measured when liposomal PCC was presented by the various APC is shown in figure V.3. The freshly prepared thioglycollate-elicited PEC are the moct efficient APC. Surprisingly, the ability of fresh Con A-stimulated PEC and 3° SAC to present unencapsulated PCC was almost completely abolished by encapsulating the antigen.

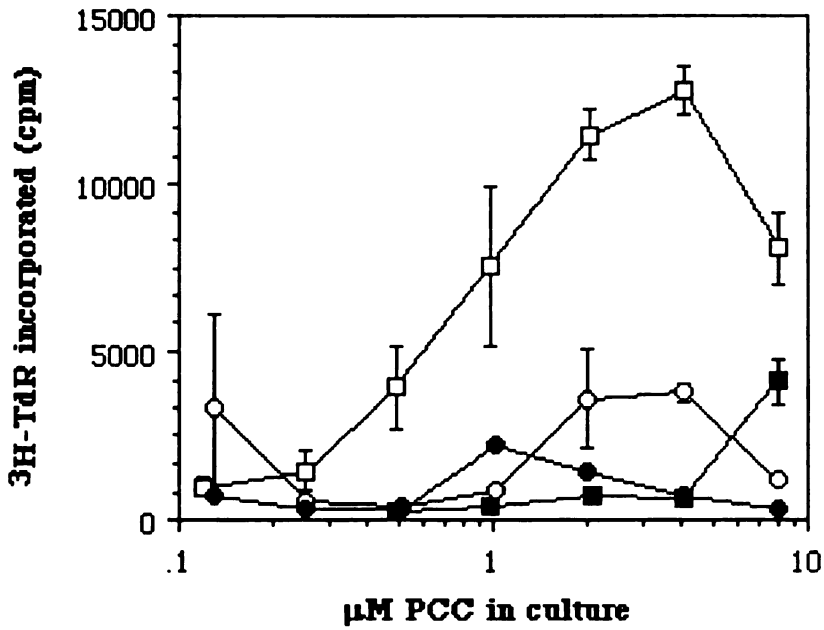


Figure V.3: Comparison in the IL-2 response when liposome-encapsulated PCC is presented by: fresh thioglycollate-elicited PEC (□), fresh Con A-stimulated PEC (■), 3° SAC (○), and 21° SAC (●).

In conclusion, freshly isolated thioglycollate-elicited PEC are the most efficient at presenting unencapsulated or liposomal PCC.

The APC function is significantly reduced or abolished after prolonged cultivation. The inhibition was not reversed with the addition of RCS. A possible reason for the loss of APC function which could be easily tested with further study comes from studies by Cowing et.al. (1978) which demonstrated the loss of Ia expression on SAC after prolonged incubation.

In the case of the 3° SAC and the Con A-stimulated PEC, the IL-2 response was lower when the antigen was encapsulated than when unencapsulated.

CHAPTER VI: ANTIGEN PRESENTATION OF CYTOCHROME C AND ITS ANTIGENIC FRAGMENT WHEN CONJUGATED TO THE SURFACE OF LIPOSOMES BY B CELLS AND MACROPHAGES

VI.1: INTRODUCTION

Phospholipid vesicles (liposomes) have been shown to enhance the antibody response to thymus dependent antigens i.e. proteins and glycoproteins when administered in vivo (Allison and Gregoriadis, 1974; Alving, 1987). These findings have generated enthusiasm for the use of liposomes as adjuvants for vaccine formulation. Although macrophages are a significant factor in the generation of this antibody response (Classen and van Rooijen, 1987; Shek and Lukovich, 1982), the exact mechanism(s) for the liposome enhancement is not completely understood.

The major role of the macrophages in the immune response toward a liposome associated antigen has been attributed to their antigen presenting function and the fact that macrophages can efficiently internalize and degrade liposomes (van Rooijen and Su, 1989). Other cell types capable of antigen presentation, such as B-cells and dendritic cells may also be able to unmask and process liposomal antigens (ibid). In either case, antigen presentation, in the appropriate histocompatibility context, results in helper T-cell activation indicated by interleukin 2 (IL-2) secretion (Unanue, 1984).

In vitro antigen presentation systems have been developed which reduce the variables in the immune response and permit initial steps in antigen processing and presentation to be studied. We have previously used an in vitro antigen presentation system to study the IL-2 response by PCC- specific helper T-cells when PCC was encapsulated in liposomes. Using this system we demonstrated that macrophages but not B-cell tumors were able to present liposome encapsulated PCC (Chapter III). Herein, we coupled PCC or a synthetic peptide representing a T-cell determinant of PCC to the liposome surface and quantitated the IL-2 response when either macrophages or B-cells were the presenting cell.

VI.2: MATERIALS AND METHODS

VI.2.1: Chemicals and solutions

Cholesterol (CH) was obtained from Sigma Chemical Co. (St. Louis, MO); egg phosphatidylcholine (EPC) from Avanti Polar Lipids, Inc.(Birmingham, Al); 2-iminothiolane and N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) from Pierce Chemical Co. (Rockford, IL); N-[4-(p-maleimidophenyl)-butyryl] phosphatidyl-ethanolamine (MPB-PE) was prepared by the method of Martin and Papahadjopoulos (1982). All other reagents are of analytical purity or better.

VI.2.2: Protein antigens

PCC from breast muscle was purchased from Sigma. The peptide, Pc81-104 was synthesized by the Merrifield procedure (Merrifield, 1969). This peptide corresponds to the 24 amino acid carboxy terminal region of native PCC (Schwartz et.al., 1985). The sequence of the peptide is H_2N-I-F-A-G-I-K-K-K-A-E-R-A-D-L-I-A-Y-L-K-Q-A-T-A-K-COOH The peptide was purified by HPLC utilizing a Dynamax modular macro HPLC column (10 mm X 25 cm) prepacked with 8 micron C_{18} capped silica (Ranin Instrument Co., Inc., Emeryville, CA). The mobile phase consisted of a linear gradient of 30 minutes from 10 to 90% CH_3CN in H_2O with 0.1% trifluoroacetic acid; flow rate of 3 ml/minute. The peak corresponding to Pc81-104 (R_t = 11 min), monitored at 220 nm, was pooled and lyophilized. An aliquot of this preparation was analyzed by HPLC using a Vydac C_{18} (10 µm particle, 0.46 X 15 cm; 1.5 ml/min; Hesperia, CA) analytical column and found to be of greater than 95% purity. The peptide amino acid composition was determined after HCl digestion. The measured and [expected] values were: A = 6.48 [6], D = 1.08 [1], E = 1.10 [1], F = 0.53 [1], G = 1.06 [1], I = 2.70 [3], K = 5.16 [5], L = 2.1 [2], Q = 1.09 [1], R = 0.89 [1], T = 0.94 [1], Y = 0.63 [1]. The peptide molecular weight was determined by liquid secondary ion mass spectrometry on a Kratos MS-50S double focusing mass spectrometer. The MH+ ion was found at 2647.55 which agrees with the predicted molecular weight.

VI.2.3: Iodination of protein antigens

Radiolabelled PCC and Pc81-104 were prepared by a modification of the method of Greenwood and colleagues (Greenwood et.al., 1963) utilizing [^{125}I]-NaI (Amersham, Arlington Heights,IL), 350-600 mCi/ml. Final specific activities for the labeled PCC was 1095 µCi/µmole and 2101 µCi/µmole for the Pc81-104 . Unbound ^{125}I was removed by gel filtration and the iodinated protein was stored at -20°C and used within 1 week of preparation.

VI.2.4: Thiolation of protein antigens

PCC and Pc81-104 were thiolated by reacting the iodinated protein with 2-iminothiolane (Jue et.al., 1978) or with SPDP (Carlsson et.al., 1978). [^{125}I]-PCC (0.59 mM) was thiolated at a 1 : 6.9 molar ratio, PCC to 2-iminothiolane in 0.1M PO_4, pH8.0 with 50 mM NaCl (PO_4/NaCl) for 90 minutes at 0°C in an N_2 atmosphere. [^{125}I]-Pc81-104 (0.49 mM) was thiolated at a 1 : 0.92 molar ratio of peptide to reagent. The thiolated proteins were immediately used for coupling to preformed liposomes. In cases where only thiolated PCC or Pc81-104 was needed, the 2-iminothiolane/protein reaction mixture was passed through a gel filtration column equilibrated with PO_4-buffered saline containing 1 X 10^{-4} M 2-mercaptoethanol to remove unreacted reagents. Thiolated PCC and Pc81-104 prepared in this manner were monomeric as determined by molecular sieve chromatography. A summary of the reaction scheme is shown in figure VI.1. Derivatization

with SPDP was done at a ratio of 10 : 1, SPDP : PCC and 1 : 1, SPDP : Pc81-104 as described by Carlsson and colleagues (ibid).

VI.2.5: Liposome coupling

Large unilamellar vesicles composed of EPC, CH and MPB-PE, molar ratio of 9:8:1, were prepared by extrusion through 0.1 μm polycarbonate membranes (Szoka et.al., 1980). Thiolated protein was immediately added to the preformed liposomes at a 16 : 100 molar ratio, PCC to lipid and 59 : 1000 molar ratio, Pc81-104 to lipid, and allowed to react for 2.5 hours at room temperature under N_2 (Martin and Papahadjopoulos, 1982). The products were separated by gel filtration on a Bio-Gel A5M (1 X 25 cm) column equilibrated with 10 mM N-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid, 150 mM NaCl, 0.1 mM diethylenetriaminepenta-acetic acid, pH 7.4 buffer. The liposome peak (excluded volume) was collected and analyzed for lipid (Bartlett, 1959) and protein content (radioassay). Liposome diameter was measured by laser light scattering on a Nanosizer model N-4 (Coulter Electronoics, Hialeah, FL.). The liposome preparation was stored under N_2 at 4°C. Sterile reagents were used in the preparations and aseptic conditions were maintained throughout the procedures.

VI.2.6: Determination of non-specific binding of PCC and Pc81-104 to MPB-PE containing liposomes

Preformed liposomes (0.4 ml; 6.15 mM lipid) were mixed with 0.4 ml of [^{125}I]-PCC (0.20 mM) or [^{125}I]-Pc 81-104 (1.1 mM) in pH 8 PO_4/NaCl buffer for 90 minutes, placed in an ice bath and flushed with N_2. The mixture was passed through a Bio-Gel A5M (1 X 10 cm) column and the radioactivity associated with the liposome peak was determined.

VI.2.7: Cells, medium and incubation conditions

The PCC-specific T-cell hybridoma, 2B4 (H-2^k) (Samelson and Schwartz, 1983) and the B-cell hybridoma, LK35.2, were generously provided by Dr. Ron Schwartz (National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). The antigen presenting B-cell hybridoma, LK35.2 (H-$2^{d/k}$), was developed and described by Kappler and colleagues (Kappler et.al., 1982). The CTLL-20 line, an interleukin-2 (IL-2) dependent cell line, was provided by Dr. Daniel Stites (University of California at San Francisco, CA). The cell lines were tested and found to be free from mycoplasma contamination. Thioglycollate-elicited peritoneal exudate cells (PEC) were used as a source of antigen presenting macrophages. These cells were harvested from male C3H/HeJ mice (5-6 weeks old; Charles River, Wilmington, MA.) pretreated with thioglycollate broth (3% in H_2O) by intraperitoneal administration 4 days prior harvesting.

Both of the antigen presenting cells (APC), the B-cell tumor, and the PEC, were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, INC. Logan,Utah), 2 mM glutamine, 25 mM N-2-hydroxy-ethylpiperazine-N'-2-ethane sulfonic acid, 1 mM pyruvate, 5 X 10^{-5} M 2-mercaptoethanol, and antibiotics (RPMI-CM). CTLL-20 were grown in Dulbecco's minimum essential medium supplemented with 5% FBS and 40% Concanavilin A-stimulated rat spleen cell supernatant as a source of IL-2 (Steeg et.al., 1980). The 2B4 cell line was cultured in MEM Eagle's with Hank's balanced salt solution and RPMI 1640 (1:1 v/v) supplemented with 10% FBS, glutamine, non-essential amino acids, and antibiotics (EHAA:RPMI). Fresh cultures of 2B4 and LK35.2 cell lines were initiated from frozen (liq. N_2) samples of early passages every 20-25 passages. Media and FBS were obtained from the Cell Culture Facility, University of California at San Francisco. All incubations, unless otherwise stated were done at 37°C, 5% CO_2, 95% relative humidity.

VI.2.8: Antigen presentation assay

PEC were seeded at 2 X 10^5 cells/well (96-well, flat bottom ; Costar, Cambridge, MA) in 0.2 ml RPMI-CM. Nonadherent T-cells were removed by washing the monolayers with ice-cold Hank's buffered salt solution (HBSS) two times. Fresh RPMI-CM (200 µl/ well) and 50 µl aliquots of graded dilutions of the appropriate antigen were added to the wells

and incubated for 24 hours. At least three cultures per condition were included in each experiment. After the initial pulse with the antigens, the cells were washed three times with ice-cold HBSS to remove any unbound antigen from the culture medium. Fresh EHAA:RPMI was added to each well and 1 X 10^5 2B4 T-cell hybridomas per well were added in 100 µl volume. The co-culture was incubated for 24 hours and 100 µl of the culture supernatant was analyzed for IL-2 using 1 X 10^4 CTLL-20 per well in a 24 hours assay (Gillis et.al., 1978). The cells were pulsed in the final 4 hours of the incubation with ^{3}H- thymidine(TdR), 1 µCi/well, and subsequently harvested on glass fiber discs and counted.

LK35.2 were incubated with antigens in suspension culture at 3.7 X 10^6 cells in 1 ml using 15 cc conical tissue culture tubes (Costar). The antigens were added in 0.25 ml volumes. The cultures were gently rocked while kept at 37°C. After 24 hours the cells were thoroughly washed by serial centrifugation (four times). Cells were plated at 1 X 10^5 cells per well in 100 µl volume (96 well plates). The 2B4 cells (1 X 10^5 per well) were immediately added to the B-cells. IL-2 assay on 24 hour supernatants was done as described above.

VI.2.9: Determination of PCC and Pc81-104 cell associated with APC

PEC, washed to remove nonadherent cells as described above, were seeded on 35 mm diameter tissue culture dishes

(Costar) at 2 X 10^6 cells per dish in 1 ml RPMI-CM. [^{125}I]-labelled antigens were added in 0.25 ml aliquots. Duplicate cultures were included for all conditions studied. The cells were incubated for 24 hours and washed 6 times with ice-cold HBSS to remove unbound antigens. The cells were lysed with 0.1 M NaOH, 0.4% sodium deoxycholate (v/v) and assayed for radioactivity. The LK35.2 were incubated with ^{125}I-labelled antigens in the same manner as described under antigen presentation assay and radioactivity was determined as described above. Control samples were included to determine nonspecific binding. The results are expressed as the range of duplicate samples in the units of picomoles of PCC or Pc81-104 equivalents associated per 1 X 10^5 APC.

VI.2.10: Glutaraldehyde-fixed APC

Antigen presentation and cell association determinations were carried out in glutaraldehyde pre-fixed APC. The cells were fixed with glutaraldehyde (grade II, 25% aqueous solution, Sigma), 0.05% v/v final concentration for 80 seconds at room temperature and blocked with 0.2 M lysine in HBSS, pH 7.4. The APC were thoroughly washed and incubated with antigens as described above.

VI.3: RESULTS

VI.3.1: Characteristics of phospholipid vesicles

The characteristics of the liposome-antigen conjugates used in these studies are listed in table VI.1.

Two procedures were used to attach antigen to the surface of liposomes. Both procedures involved the introduction of free sulfhydryl groups onto the protein and then covalent attachement, via thioether bond, to MPB-PE containing liposomes. When SPDP was used as a thiolating agent, for either PCC or Pc81-104, the modified protein underwent significant aggregation at the concentrations used for coupling and the solubility was quite low. This resulted in low yields of coupling to the preformed liposome and liposome aggregation.

The use of iminothiolane to introduce free sulfhydryl groups into the PCC was previously reported by Casten and colleagues (Casten and Pierce, 1988). Thiolation with 2-iminothiolane is very efficient and results in the retention of the surface charge in the derivatized molecule (Jue et.al., 1978). Moreover, unlike the SPDP method, reduction with dithiothreitol to generate the free thiol groups is not necessary. The overall coupling efficiency, calculated as the percentage of the added ligand, with the liposome was 3.4% for PCC and 19.3% for the peptide. With this method, the liposome diameter did not change after coupling (Table VI.1).

Table VI.1: Liposome characteristics

		PCC-liposome	Pc81-104-liposome
I. **Liposome Composition**		EPC/MPB-PE/CH	EPC/MPB-PE/CH
	mole ratio	9/ 1/ 8	9/ 1/ 8
II. **Size of liposomes**	(nm)		
i) before coupling:	Number Ave.	108 ± 26	125 ±19
	Intensity Ave.	144 ± 34	135 ± 21
ii) after coupling:	Number Ave.	128 ± 25	119 ±24
	Intensity Ave.	158 ± 43	135 ± 22
III. **Protein : Lipid**	(µmole/µmole)	5.46×10^{-3}	1.14×10^{-2}
	(µg/µmole)	66.49	30.20
IV. **Molecules protein/vesicle** (*)		1217	1715
V. **% MPB-PE sites coupled** (†)		19.7%	41.01%

*) Based on theoretical assumption: 70 $Å^2$ /molecule; bilayer thickness 40 Å

†) Assume unilamellar liposomes and equal distribution of MPB-PE molecules distributed between outer and inner leaflets of bilayer.

A reaction scheme of the coupling procedure is shown in figure VI.1.

To determine the extent of non-covalently associated antigens, the purified liposome/antigen complex was analyzed by gel filtration or by centrifugation through a metrizamide gradient. By these techniques less than 0.1% of the antigen dissociated from the liposome. This was essential to

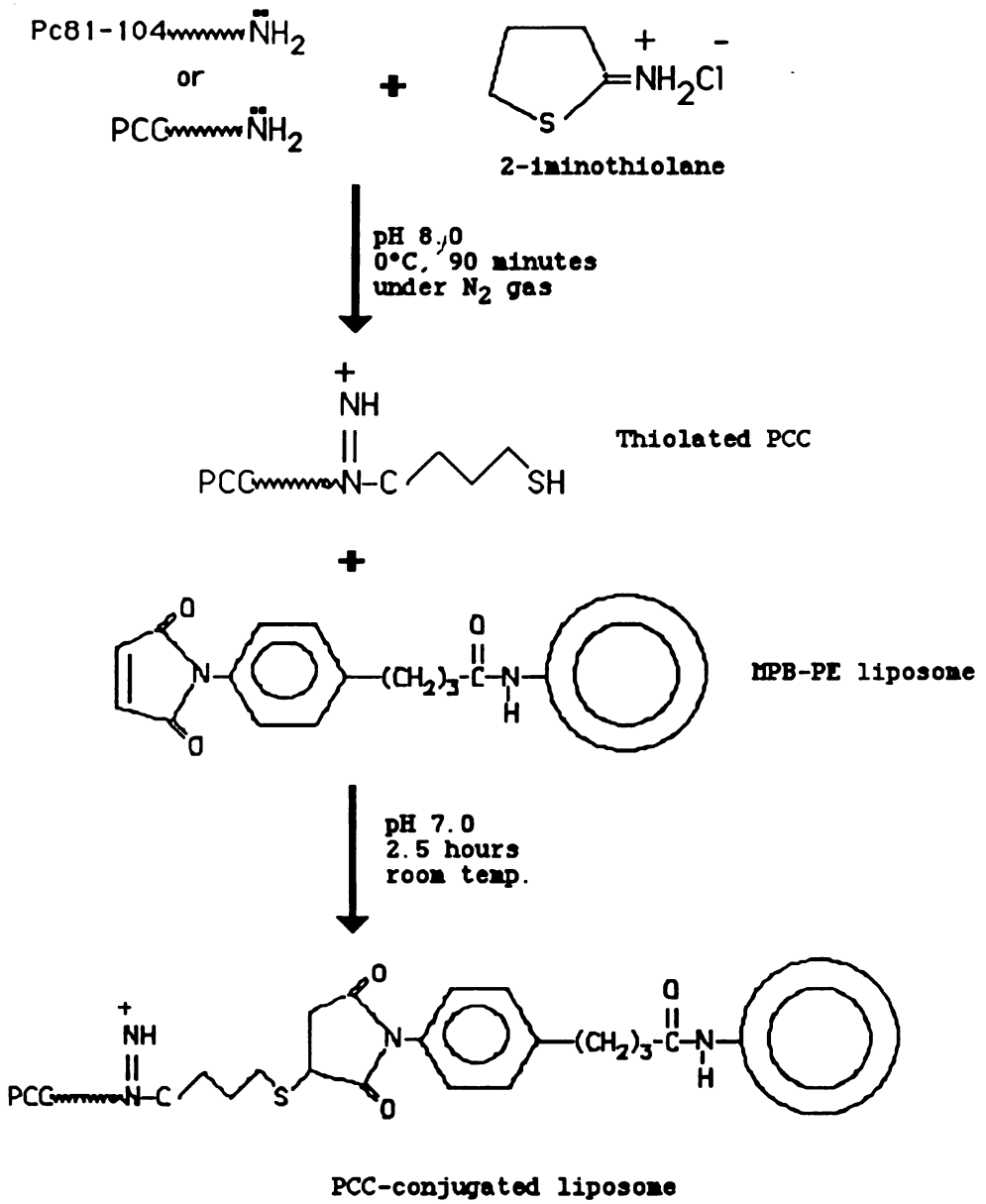


Figure VI.1: Reaction scheme for coupling PCC or Pc81-104 to the surface of preformed liposomes

determine since the unconjugated native PCC or Pc81-104 are antigenic and if present in high amounts might complicate interpretation of the results. The amount of non-specific binding of the antigens was determined by incubating unmodified, radiolabelled PCC or Pc81-104 and preformed liposomes for the same period as in the reaction and then separating. Measured in this way, non-specific binding did not exceed 0.2% of the added radioactivity.

VI.3.2: Presentation of PCC and PC81-104 liposome conjugates by PEC

Both the PCC and the Pc81-104 were efficiently presented by the PEC (figure VI.2). The ability of PCC or Pc81-104 to activate the 2B4 cells when PEC are the antigen presenting cell was not reduced when the antigens were thiolated with iminothiolane (figure VI.2, insert).

Approximately 30-40 fold less antigen is required for an equivalent IL-2 response when PCC is coupled to liposomes as compared to unconjugated PCC. This increased efficiency of presentation is due to the covalent coupling of PCC to the liposome since, as noted above, unconjugated thiolated PCC demonstrated equivalent ability to stimulate IL-2 response as did native PCC (figure VI.2).

Pc81-104 does not require processing in order to stimulate T-cells and on a molar basis is considerably more potent then the intact protein. In the case of Pc81-104, covalent coupling to the surface of liposomes results in a

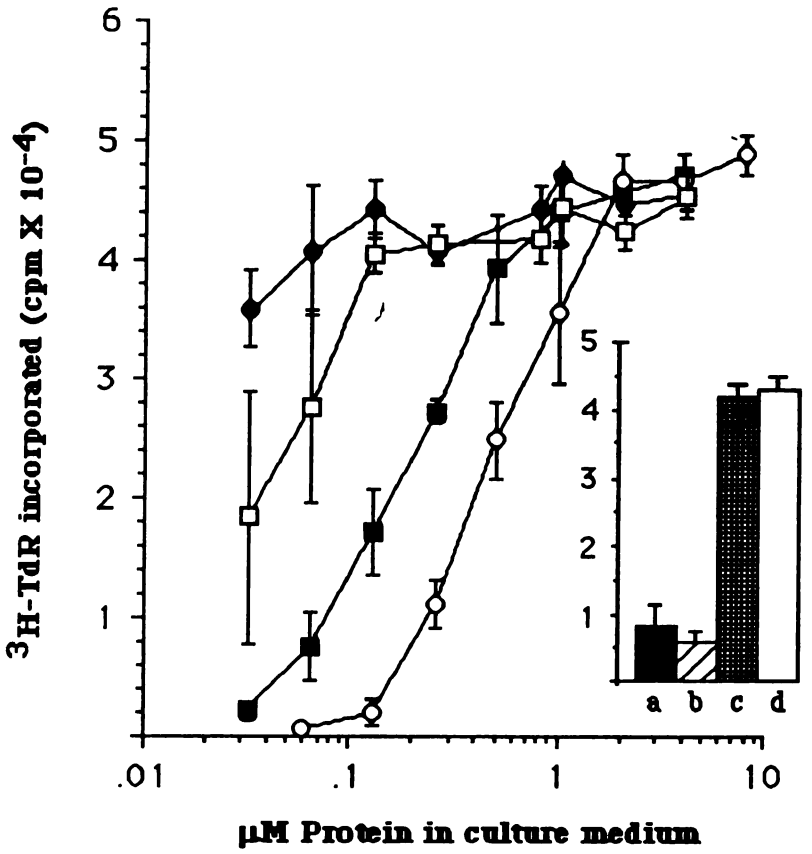


Figure VI.2: Presentation of PCC and Pc81-104 when coupled to the surface of lipid vesicles by peritoneal macrophages. PEC cells were pulsed with varying concentrations of PCC, ○ ; PCC coupled liposomes, ● ; Pc81-104, □ ; and Pc81-104 coupled liposomes ■ . Maximal levels of incorporation by CTLL-20 are 58,103 ± 1816 cpm. Background levels of incorporation are 283 ± 100 cpm. Values presented are the mean ± s. d.. Insert: Comparison of the IL-2 response by 2B4 cells to unmodified PCC (**a**), or thiolated PCC (**b**); and unmodified Pc81-104 (**c**), or thiolated Pc81-104 (**d**), for a single concentration of 0.2 µM of the respective antigen in the culture medium when presented by PEC. Similar results were observed for 0.02 µM to 20 µM antigen. When error bar is not shown, s.d. falls within symbol.

slight decrease in the IL-2 response when presented by PEC as compared to the unconjugated peptide (figure VI.2).

The inability of glutaraldehyde-fixed PEC to present native PCC or PCC coupled to the surface of liposomes is an indication that such treatment blocks the processing function of the APC (figure VI.3.A). The antigenic fragment, Pc81-104 is efficiently presented by prefixed cells , although approximately 20 fold more peptide is needed to obtain a similar IL-2 response by the fixed macrophage as for the live macrophage (figure VI.3.B).

Having demonstrated that prefixed PEC can present the peptide and its thiolated derivative, we were interested in determining if the peptide could be presented by these cells when covalently anchored on the liposome surface. Processing functions as well as endocytotic functions are blocked in prefixed cells so liposomes are not internalized. As shown in figure VI.3.B a slight but significant response is observed. The response induced by prefixed cells is not as great as that observed using live PEC. The response to liposome-Pc81-104 conjugates presented by prefixed PEC suggests that the peptide becomes available to interact with surface Ia to stimulate T-cell production of IL-2.

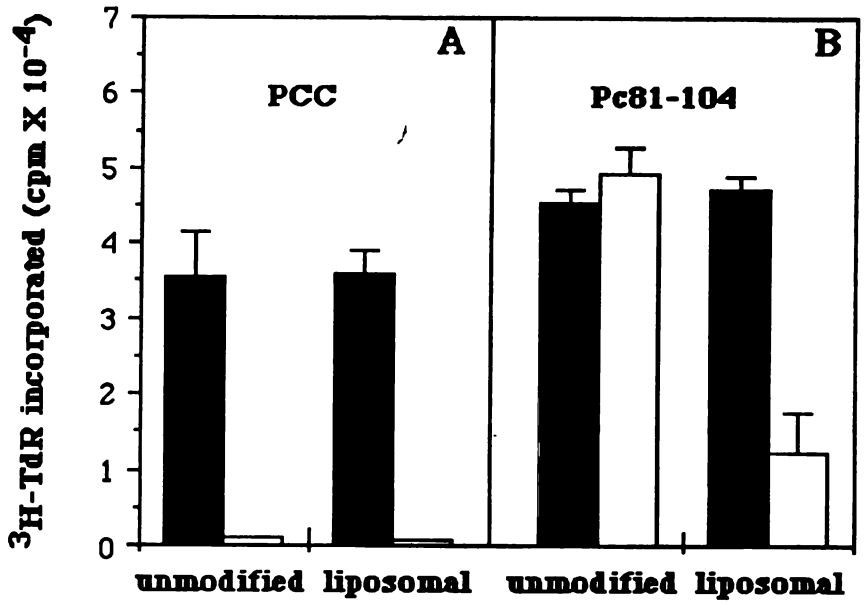


Figure VI.3: Presentation of PCC and Pc81-104 conjugates to 2B4 cells by live and glutaraldehyde fixed PEC. Where possible, culture concentrations of antigen shown represent those which yielded similar IL-2 responses; some of the data points are extracted from figure 1 and included for comparison. A. Live PEC were incubated with PCC (1 μM) or PCC coupled liposomes(0.03 μM), ■ ; Prefixed PEC were incubated with PCC or PCC coupled liposomes(4 μM), □ . B. Live PEC were incubated with Pc81-104(0.1 μM) or Pc81-104 coupled liposomes (0.5 μM), ■ ; Prefixed PEC were incubated with Pc81-104(2 μM) or Pc81-104 coupled liposomes (4 μM), □ . Maximal and background cpm are the same as those shown in figure 1. Values presented are the mean ± s. d.When error bar is not shown, s.d. falls within symbol.

VI.3.3: Cell association of liposome PCC/Pc81-104 conjugates to live and prefixed PEC

The initial step in presentation of protein antigens by APC is the binding of antigen to the surface of the cell. Cell association of PCC was enhanced approximately 6.5 fold (figure VI.4.A) as a result of coupling the PCC to the surface of the liposome when compared to native PCC.

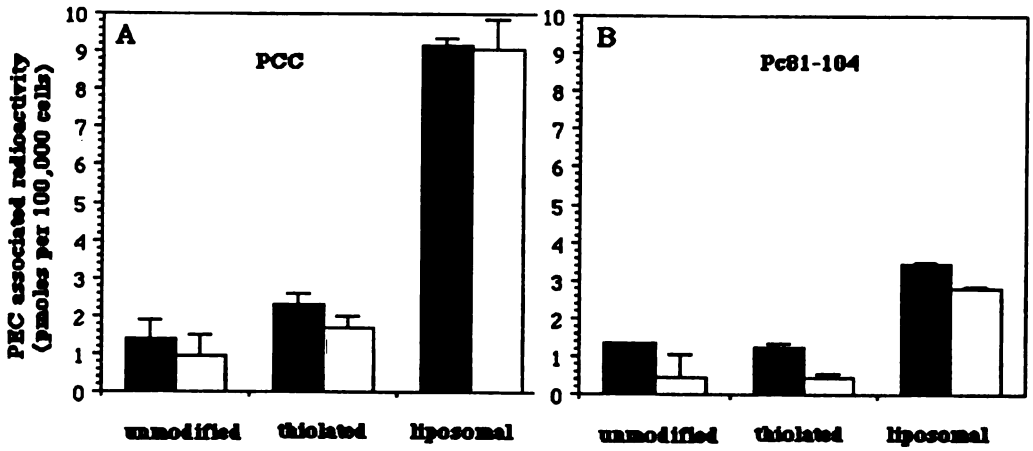


Figure VI.4: Cell association of PCC or Pc81-104 antigenic forms with PEC. Live, ■ ; or prefixed, □ ; PEC were incubated with: (A) 2 μM [^{125}I]-PCC in the unmodified(sp. act. 119 μCi/ μmole),thiolated (92.3 μCi/ μmole), or liposome (119 μCi/ μmole) forms; (B) 2 μM [^{125}I]-Pc81-104 in the unmodified, thiolated, or liposomal (126.4 μCi/ μmole) forms. The amounts bound are the range of duplicate determinations in picomoles of protein equivalents per 1 X 10^5 cells. Results are corrected for the amount of non-specific binding which was between 5-10% of the total cell associated value. When error bar is not shown, s.d. falls within symbol.

Thiolated PCC had a 1.7 fold increased amount of cell associated radioactivity compared to native PCC. It is noteworthy that the 6.5 fold increase in cell association resulted in a 30-40 fold increase in efficiency of presentation compared to the native PCC. This is in spite of the fact that the thiolated protein, by itself, had a decreased efficiency of presentation when normalized to the amount that was cell associated (figure VI.4.A).

Liposome conjugated, thiolated, and native PCC bound to prefixed macrophages to a similar extent as to live macrophages (figure VI.4.A). Thus the inability of prefixed macrophages to present PCC or PCC coupled to liposomes is not due to their inability to bind antigen but rather their inability to internalize and process the antigen.

Macrophage associated Pc81-104 was enhanced 2.6 fold when conjugated to liposomes compared to the peptide or its thiolated derivative (figure VI.4.B). In spite of the increased amount of cell associated radioactivity, liposomes did not enhance the efficiency of presentation of Pc81-104 by PEC (figures VI.2 and VI.3.B). The conjugated and free Pc81-104 have a similar pattern of cell association in the prefixed as in the live PEC. However, the amount bound tends to be slightly lower on the fixed PEC than on the live PEC.

VI.3.4: Presentation of PCC and Pc81-104 -liposomes when B-cells are the APC

B-cells have been shown in various cases to be efficient presenters of soluble protein antigens (Chesnut and Grey, 1986). Soluble PCC was efficiently presented by the B-cell hybridoma LK35.2 whereas PCC encapsulated in liposomes was not (Chapter III). Interestingly, when PCC or the antigenic peptide are attached to the liposome surface, B-cells can present them, albeit not as efficiently as soluble PCC or Pc81-104 (figure VI.5.A,B). The IL-2 response for thiolated PCC or thiolated Pc81-104 was similar to that of unmodified antigens (not shown). This suggests that the response to liposomal antigens is due to coupling and not just modification with free thiols.

Metabolically active B-cells can present both unconjugated PCC and liposomal PCC to T-cell hybridomas. Fixation of the B-cells totally eliminates their ability to present the intact PCC (figure VI.5.A). In contrast, the peptide fragment does not require internalization for its presentation and is presented by pre-fixed cells as efficiently as live cells (figure VI.5.B). When the endocytic process is blocked by fixation, the IL-2 response to liposome-surface coupled peptide is reduced by only 50% (figure VI.5.B). As in the case of the macrophage, this indicates that the peptide can still interact with the Ia on the APC.

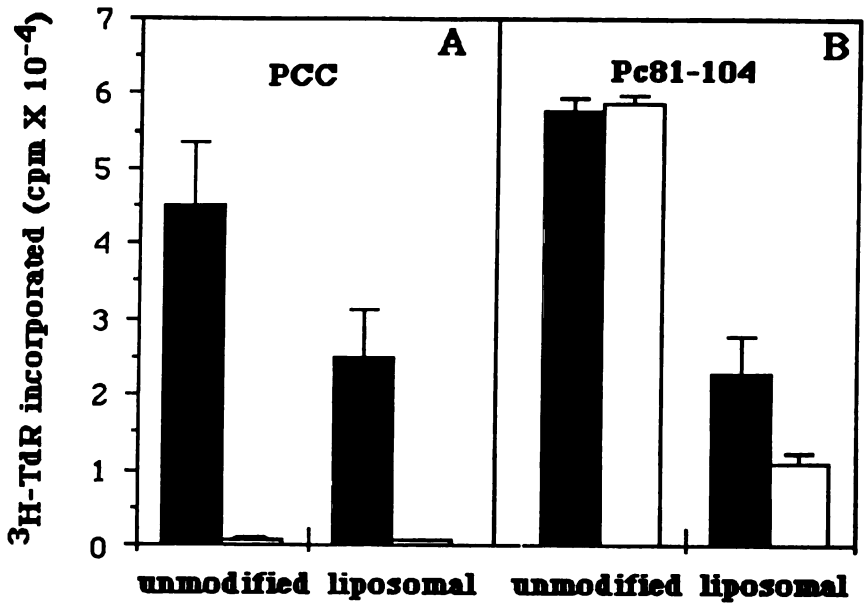


Figure VI.5: Presentation of PCC (A) and Pc81-104 (B) conjugates to 2B4 cells by live and glutaraldehyde fixed LK35.2. Live, ■ ; or prefixed, □ ; LK35.2 cells were incubated with 4 μM antigen either unmodified or coupled to the surface of liposomes. Similar effects were observed at a lower concentration of antigens. Maximal levels of incorporation by CTLL-20 are 65,000 ± 2500 cpm. Background levels of incorporation are 243 ± 141 cpm. Values presented are the mean ± s. d.When error bar is not shown, s.d. falls within symbol.

VI.3.5: Cell association of PCC/Pc81-104 conjugated and unconjugated to live and prefixed B-cells

Thiolation of PCC, without conjugation to liposomes, did not significantly increase the amount of cell associated radioactivity over unmodified PCC (figure VI.6.A). Incubation of LK35.2 with PCC coupled to the surface of liposomes resulted in a 40 fold enhancement in the amount of cell associated radioactivity over unmodified PCC at equimolar concentrations (figure VI.6.A). In spite of the large enhancement of cell associated radioactivity of the liposomal PCC, as noted above, the IL-2 response to this form was less efficient than to the unmodified PCC (figure VI.5.A).

When the LK35.2 are fixed with glutaraldehyde, cell association of unmodified, thiolated, and liposomal PCC was increased over that of live B-cells (figure VI.6.A). Prefixed cells are not able to present PCC whether in the free state or when conjugated to liposomes (figure VI.5.A) thus this enhancement in cell association is most likely due to protein adsorbed to the surface of the cells.

In the case of the Pc81-104, thiolation resulted in a 2 fold increase in B cell association (figure VI.6.B). When coupled to the surface of liposomes, cell association of the peptide was enhanced approximately 3.5 fold compared to Pc81-104 (figure VI.6.B). In spite of the greater cell association of the liposomal Pc81-104 compared to the free Pc81-104, the free peptide was a more potent inducer of IL-2 (figure VI.5.B).

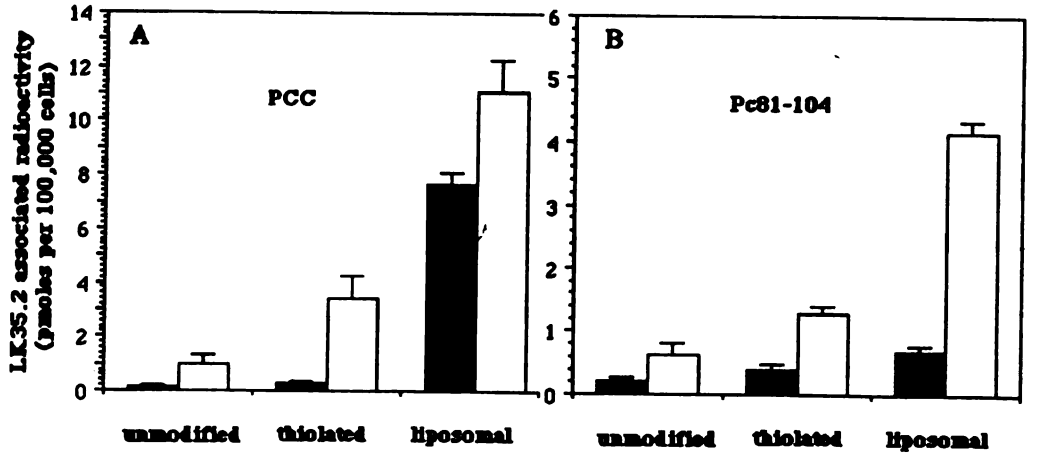


Figure VI.6: Cell association of PCC(A) or Pc81-104(B) antigenic forms with LK35.2. Live, ■ ; or prefixed, □ ; PEC were incubated with: (A) 2 μM [^{125}I]-PCC in the unmodified(sp. act. 73.9 μCi/ μmole), thiolated(92.3 μCi/ μmole), or liposomal (119 μCi/ μmole) forms; (B) 2 μM [^{125}I]-Pc81-104 in the unmodified (2091 μCi/ μmole), thiolated, or liposomal (126.4 μCi/ μmole) forms. The amounts bound are the range of duplicate determinations in picomoles of protein equivalents per 1 X 10^5 cells. Results are corrected for the amount of non-specific binding which was 5-10% of the total cell associated value.

Prefixed B-cells bound more peptide, regardless of its chemical nature, than live B-cells (figure VI.6.B). In the prefixed B-cell, association of Pc81-104 is enhanced 2 fold by thiolation and 7 fold by attaching it to the surface of liposomes, when compared to the unmodified peptide. For the underivatized peptide, the increased cell association did not

result in increased presentation by prefixed cells (figure VI.5.B). The liposomal peptide was presented less efficiently by prefixed cells than live cells in spite of the large increase in the cell associated peptide (figure VI.5.B).

VI.3.6: Stimulation of IL-2 production by antigens in the absence of antigen presenting cells

Stimulation of IL-2 production by helper-T-cells almost always requires presentation of processed antigen in conjunction with Ia glycoprotein. To test whether the requirement for Ia could be bypassed, we incubated the antigen at high concentrations with the antigen specific T-cells and tested the 24 hour supernatants for the presence of IL-2 in the absence of antigen presenting cells. Neither PCC, Pc81-104, nor the thiolated Pc81-104 stimulate IL-2 production by the T-cells. Thiolated PCC however induces a significant IL-2 response when incubated with T-cells at a high concentration (figure VI.7).

PCC and Pc81-104 conjugated to the surface of liposomes can also stimulate T-cells to secrete IL-2 independent of Ia (figure VI.7). The stimulation is significantly less than the response observed when APC are pulsed with the liposome conjugates. Nonetheless the results indicate antigens presented on the surface of a lipid vesicle, can in certain cases bypass the need for the APC. Further work is needed to establish the specificity of this type of T cell stimulation.

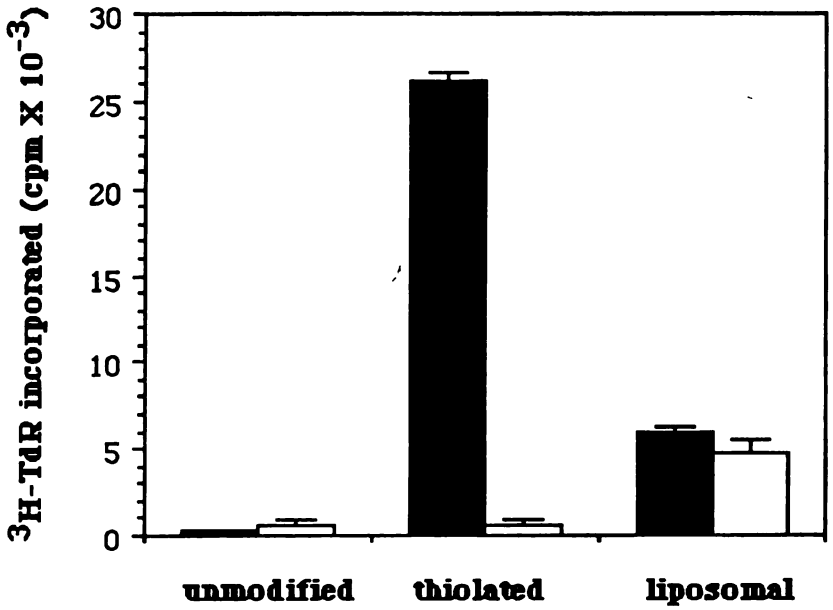


Figure VI.7: The IL-2 response of 2B4 cells in the absence of antigen presenting cells. The 2B4 (1 X 10^5) cells were incubated with PCC in the unmodified (8 μM), thiolated (28 μM), and liposomal (4 μM) forms, ■ ; or with 4 μM Pc81-104 in the unmodified, thiolated, and liposomal forms, □ . Maximal and background cpm incorporation by CTLL-20 are the same as those shown in figure 4. Values presented are the mean ± s. d. When error bar is not shown, s.d. falls within symbol.

VI.4: DISCUSSION

In the present study, we utilized an in vitro antigen presentation assay to study the adjuvant effects of liposomes with surface coupled antigen. The levels of cell associated antigen were quantitated and IL-2 measurements were used as an indicator of antigen presentation to the T-cell.

Antigen presentation to helper-T-cells is the initial event in the cascade leading to a humoral response by antibody producing B-cells. The uptake and intracellular processing of antigen by an APC which expresses the appropriate major histocompatibility complex-encoded Ia glycoprotein on its surface is the first step in the sequence. The processed peptide, containing the T-cell antigenic determinant, is transported to and held on the APC surface where it is recognized by a T-cell specific for the antigen in conjunction with the Ia (Unanue, 1984). Thus an in vitro system is ideal to evaluate how a delivery system interacts with various presenting cell types to initiate the immune response.

VI.4.1: Antigen presentation of PCC coupled liposomes by PEC and B-cells

PEC are efficient presenters of surface coupled PCC. This was demonstrated as an increase in potency of 30-40 fold when PCC was coupled on the surface of liposomes over native PCC (figure VI.2). The increase in potency cannot be attributed to the addition of sulfhydryl groups to PCC since the IL-2 response to thiolated PCC is similar to the response of the native PCC. A 6 fold increase in cell associated radioactivity for surface PCC over unmodified or thiolated PCC (figure VI.4.A), leads to the 30-40 fold enhancement in IL-2 response. We previously demonstrated that PCC entrapped in liposomes resulted in a 2 fold increase in potency in IL-2

production. This correlated with a 2-5 fold enhancement in cell association over native PCC (Chapter III).

The exact reason for the ehanced efficiency demonstrated by the liposome surface-coupled antigen over the entrapped antigen is not known. At least three possibilities come to mind: 1) PCC localized on the surface of the liposome may allow more efficient internalization and processing of the liposomes than when it is encapsulated, 2) Surface attached PCC may direct the liposome into a different internal compartment with different processing capabilities or alter the rate at which it is delivered to the processing compartments, and 3) Lipid linked antigenic fragments arising from the degradation of the lipid bound PCC may be re-expressed and maintained on the macrophage cell surface increasing the probability of interacting with the Ia molecule. These possibilities are currently under investigation.

We demonstrated that the macrophage is an efficient target for liposomal surface attached PCC. One of the advantages of the liposomal carrier is its ability to carry multiple ligands. It is possible that by adding a ligand specific for some APC surface structure we could further enhance the IL-2 response over what we observed. Indeed, Garcon and colleagues, found the adjuvant effect of liposomal antigen could be further enhanced by targeting the liposomes to the mannose receptor on PEC (Garcon et.al., 1988). The authors suggested that the augmented adjuvanticity

of the mannose coupled liposomes was related to an increase in binding to PEC over the non-mannosylated control preparations.

It has been suggested that B-cells might have the ability to process liposomal antigens and present them for helper-T-cell activation (van Rooijen and Su, 1989). However, previously we demonstrated a B cell tumor, LK35.2, was unable to present liposome entrapped PCC due to its inability to internalize and degrade the PCC (Chapter III). The results for the surface attached PCC are different. The B-cell tumor is able to present PCC coupled to the surface of liposomes, although not as efficiently as it can present the unconjugated PCC (figure VI.5.A). One factor that certainly contributes to this effect is that cell association is enhanced 40 times by surface coupling of the PCC over the unconjugated PCC (figure VI.6.A). Thus the efficiency of this process when normalized to the amount of material that becomes cell associated, is orders of magnitude less than for the soluble PCC.

The traditional view of antigen processing assumes that the processing events take place intracellularly. Soluble PCC requires partial degradation for presentation to helper T-cells, and the site of processing has been shown to occur within chloroquine sensitive intracellular organelles (Kovac and Schwartz, 1985). Glutaraldehyde fixed cells cannot present either PCC or liposome surface bound PCC (figure VI.5). It is possible that surface-coupled PCC is being

internalized by live B-cells into sites where PCC can be processed. Machy and Leserman have shown that B-cells are capable of internalizing liposomes smaller than 2000 Å in diameter (Machy and Leserman, 1983). The liposomes used in our study are from 1300-1600 Å in diameter, hence within the B-cell limits of internalization. However, for cells normally considered to be non-phagocytic, such as B-cells and dendritic cells, cell surface localized processing sites have been found and could be responsible for the presentation of liposome-surface attached PCC by B-cells (Grey et.al., 1982; Kim et.al., 1985; Lombardi et.al., 1987; Chain et.al., 1986). Whichever is correct, cell surface processing or uptake of liposomes for intracellular processing, it is still less efficient than the processing of soluble PCC.

VI.4.2: Antigen presentation of Pc81-104 coupled liposomes by PEC and LK35.2

The processing events for antigen presentation can be bypassed with fragments of antigen carrying helper-T-cell epitopes. These can bind directly to the appropriate surface Ia to form peptide-Ia complexes necessary for activation of the T-cells (Babbitt et.al., 1985; Buus et.al., 1987). In this study, we determined that a significant level of presentation by both types of APC can also be achieved by coupling Pc81-104 to the surface of liposomes. In the case of the PEC, the IL-2 induced by surface coupled peptide was not as great as that induced by PCC on the liposome surface.

Still, the surface-coupled peptide was a better inducer of IL-2 than soluble PCC. When the B cell was the APC, Pc81-104 on the surface of liposomes and PCC coupled to liposomes induced equivalent IL-2 responses. The surface attached peptide was not as effective as free antigen for induction of IL-2. In all cases, more peptide became cell associated when attached to liposomes than in the unmodified state.

At the concentration studied, the peptide fragment Pc81-104 is presented equally well by live and prefixed B-cells. This confirms that the peptide does not require further processing to be presented. In the case of the PEC, approximately 20 times more peptide is needed for an equivalent IL-2 response when fixed cells are used. The liposomal peptide could induce an IL-2 response in conjunction with either live or prefixed cells, although the response with live cells was greater.

Our results suggest that Pc81-104 on the surface of liposomes can become available for presentation via two routes: 1) an intracellular route requiring processing and 2) a surface event in which the peptide becomes available for presentation at the surface of the APC without internalization and processing. In the case of the PEC, attaching the peptide on the surface of the liposome, might increase the amount of peptide which is presented via route 1 (internalization and processing) and decrease the amount which directly binds on the surface. This could result in intracellular digestion of the peptide thereby decreasing the

IL-2 response. Minimal antigenic peptides can undergo further processing by live APC, which alter interaction of the peptide with the Ia and markedly affect its potency (Fox et.al., 1988).

Based on this working model, the level of presentation observed for liposome-surface coupled Pc81-104 by either of the prefixed APC, represents the amount of peptide which is available to be presented via route 2. How the peptide becomes available to interact with Ia is not known. The peptide-liposome complex might bind directly to Ia but the large size of the liposome should be a significant steric constraint to functional complex formation. In fact, prefixed B-cells bind considerably more liposomal peptide than free peptide, yet the IL-2 response generated to this complex is considerably less. A second possibility is that the peptide-lipid anchor dissociates from the liposome, transfers to the cell surface, and is able to productively interact with Ia and the T-cell receptor.

These results with liposomes are analogous to those of Casten and colleagues, who showed peptide-antibody conjugates directed to APC surface structures like membrane-Ig, Ia, or Class I can also induce fixed B-cells to present antigen in vitro (Casten et.al., 1988). In their studies, the efficiency of presentation of the antibody conjugates was enhanced from 100 to 1000 fold over the unmodified peptides in either live or prefixed B-cells. The higher efficiency of the antibody carrier as compared to the liposomal carrier may be due to

the large amount of liposomal peptide which becomes cell associated with the prefixed B-cell. As discussed above, liposomes with surface antigen may sterically interfere with the interactions required for activation of the T cell.

VI.4.3: Stimulation of T-cells in the absence of APC

An important confirming observation of our work is that T-cells can be stimulated by antigen independent of Ia. Walden and colleagues covalently coupled cytochrome-c to liposomes and showed the conjugate stimulated antigen specific T-cells independent of Ia (Walden et.al., 1986a & 1986b). We confirmed that T- cells pulsed with liposome conjugates with either PCC or Pc81-104 on their surface, secrete IL-2. We also found that T-cells could be stimulated to produce IL-2 in the presence of a high concentration of thiolated PCC but not unmodified PCC, Pc81-104, or thiolated Pc81-104. The concentrations of antigens in these experiments are 100-200 fold greater than the amount of APC-associated material which is available to stimulate T-cells in the antigen presentation assay. This is because unbound antigen is removed from the APC by the washing step prior to the addition of the T-cells. The mechanism and extent of specificity of these processes are unknown but worthy of further study.

VI.4.4: Liposomes as adjuvants for vaccines

From the results presented here, surface localized peptides may be able to stimulate T-cells through a broader repertoire of cell types than when the antigen is encapsulated (Chapter III). Thus, attachment of the antigen on the surface of the liposome may allow cells, such as B-cells and dendritic cells, which are not capable of phagocytosis, to contribute to the immune response to liposomal antigens. Synthetic peptides from immunogenic proteins representing determinant regions for both B-cells and for T-cells coupled to liposomes may offer an alternative approach for the safe and effective modulation of the immune response.

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GLOSSARY

ADJUVANT: any agent that acts nonspecifically to increase an immune response to a specific antigen.

ANTIGEN PRESENTING CELLS (APC): Cells of the immune system which express MHC-encoded glycoproteins on their surface and present foreign antigen to T cells. Cells such as: marginal zone macrophages, follicular dendritic cells, dendritic cells,monocytes/macrophages, Langerhans cell, and some T cells are all examples of antigen presenting cells. APC process and present the foreign antigen on their surface in conjunction with the MHC glycoprotein. In this manner, antigen presenting cells interact with different popularions of lymphocytes.

ANTIGEN PROCESSING: Antigen processing can be defined as the conversion of an antigen by an APC which expresses Ia, from a native to a non-native form.

CELL MEDIATED IMMUNITY: The arm of the immune response which involves the production of specialized cells that react mainly with foreign antigens on the surface of host cells, either killing the host cell in the case of a virally infected cell, or inducing other cells, such as macrophages, to destroy antigen.

GLOSSARY (continued)

CLASS I-MHC GLYCOPROTEIN: A glycoprotein encoded by at least three separate genetic loci of the MHC: H-2K, H-2D, and H-2L in the mouse. These glycoproteins are integral membrane proteins found on the surface of all nucleated cells and platelets. They are classical transplantation antigens involved in graft rejection. Class I MHC molecules are recognized on the surface of antigen presenting cells by cytotoxic T cells.

CLASS II-MHC GLYCOPROTEIN: Highly polymorphic glycoproteins encoded by the I-region of the MHC. Also known as I-region associated (Ia) antigens. Class II antigens differ from Class I in having a much narrower tissue distribution: they are expressed on B cells, macrophages, monocytes, dendritic cells, Langerhans cells, and some T cells. Class II molecules are required to present antigen to T helper cells, in T-B cooperation in the production of antibody, and in regulatory interactions between T cells.

EPITOPE: Epitopes, paratopes, agretopes, desetope, and histotope are part of a nomenclature used to describe the interaction between processed antigen on the surface of an APC and T cell receptor. An **epitope** is a

GLOSSARY (continued)

determinant on an antigen whereas the **paratope,** formed by hypervariable domains is the part of the T cell receptor which binds to the epitope. The **agretope** is the part of the antigen which associates with the MHC molecule; **desetope** is the part of the MHC molecule which binds to the antigen; the **histotope** is the part of the MHC which is recognized by the T cell. Epitope and paratope are used analogously to describe antibody-antigen interactions.

HAPLOTYPE: A set of genes located on a single chromosome. An individual has two haplotypes of each set of genes.

HUMORAL IMMUNITY: The arm of the immune response involving the production of antibodies, which circulate in the blood stream and bind specifically to the foreign antigen that induced them. Binding of antibody to the antigen makes it easier for phagocytic cells to take up antigen.

MAJOR HISTOCOMPATIBILITY COMPLEX (MHC): A cluster of genes encoding a large number of different proteins important in immue recognition and signalling between cells of the immune system. MHC antigens are expressed on the cells of higher vertebrates. First demonstrated in mice (H-2

GLOSSARY (continued)

antigens) on chromosome 17. In man they are known as HLA and located on the short arm of chromosome 6. MHC encoded molecules have an enormous amount of structural polymorphism and the rate of gene alteration within this locus is very high.

MHC RESTRICTION: Interactions between cells of the immune system are controlled by the MHC. The cells must share an MHC region (or subregion) to cooperate optimally.

PHASE TRANSITION TEMPERATURE (Tc): A temperature-dependent reversible phase transition, where the hydrocarbon chains of the phospholipids undergo a transformation from ordered (gel) state to a more disordered (liquid crystalline) state. These changes can be easily detected by differential scanning calorimetry (DSC). The physical state of the bilayer is a function of the phospholipid content of the bilayer and affects the permeability, leakage, and overall stability of the liposomes.

PRIMARY IMMUNITY: Following primary antigenic challenge with an antigen, the immune response, whether humoral or cell-mediated will appear after an initial lag period of several days, followed by a rapid logarithmic rise to a

GLOSSARY (continued)

plateau which eventually declines. Antibodies of the IgM type form a major proportion of the primary response

SECONDARY IMMUNITY: The secondary immune response has a shorter lag phase, an extended plateau, and a more gradual decline. The plateau levels are much greater in the secondary response than in the primary response.Secondary response consists almost entirely of IgG.

T-DEPENDENT ANTIGENS: Antigens which need to be recognized by both T cells and B cells to elicit antibody responses. Most protein antigens fall in this category

T-INDEPENDENT ANTIGENS: Antigens which are capable of stimulating B cells to produce antibody without T cell help. Most are large polymeric molecules with repeated antigenic determinants, which are only slowly degraded.