USE OF LIPOSOMES AND CYTOKINES AS IMMUNOLOGICAL ADJUVANTS IN VACCINES

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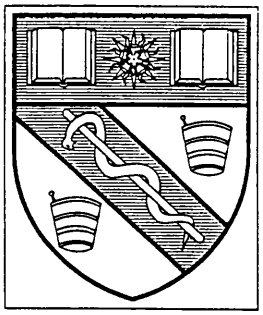

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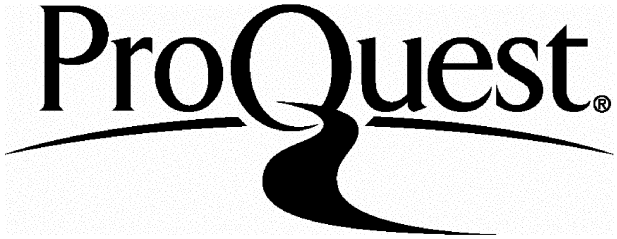

To my parents Altan and Irina

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ABSTRACT

The potential of liposomes and some of the cytokines as immunological adjuvants was investigated throughout this thesis. Preliminary experiments involved dose response studies with free or liposomally associated tetanus toxoid. Two methods of antigen association with liposomes were used: encapsulation or covalent coupling to the surface of the vesicles. The toxoid was also mannosylated in order to augment targeting of the antigen to macrophages. A low mannose:protein containing product and another one with higher mannose content were used in immunization studies in free, entrapped or surface-linked forms. Next, the potential of giant vesicles as multiple vaccine carriers for particulate and/or soluble antigens was analyzed and compared to results obtained with dehydration-rehydration vesicles. The ability of both carriers to protect their contents from binding of antibodies was also assessed in experiments where the the liposomes as such or after treatment with Triton X-100 were incubated with FITC-labelled anti-spore IgG that was raised in rabbit, washed and observed under fluorescence microscope for the presence of antigen-antibody interaction. In the other half of the thesis, the ability of four different cytokines in various liposomal formulations to potentiate the immune responses to the model antigen tetanus toxoid was assessed. IL-1β (163-171) peptide was entrapped or covalently coupled to liposomes. IL-2, IL-12 and IL-15 were also used in various liposomal preparations at different dosages. IL-2 was also used as a co-adjuvant with a synthetic lipidic peptide carrier systen derived from the variable outer membrane protein region of *Chlamydia trachomatis* as a different antigen.

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ABBREVIATIONS

APSA	N-(p-aminophenyl)stearylamide
BisHOP	1,2-Bis (Hexadecylcycloxy)-3-trimethyl aminopropane
BSA	Bovine serum albumin
DMSO	Dimethylsulphoxide
DOPE	Dioleoyl phosphatidylethanolamine
DRV	Dehydration-rehydration vesicles
DSPC	Distearoyl phosphatidylcholine
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
FCA	Freund's complete adjuvant
FCS	Fetal calf serum
FIA	Freund's incomplete adjuvant
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte macrophage colony-stimulating factor
GV	Giant vesicles
HDL	High density lipoproteins
IFN-γ	Interferon-γ
IL	Interleukin
ISCOM	Immune stimulating complex
LCPH1	Lipid core carrier peptide
LPS	Lipopolysaccharide
LUV	Large unilamellar vesicles

MDP	Muramyldipeptide
MHC	Major histocompatibility complex
MLV	Multilamellar vesicles
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NAGO	Neurominidase-galactose oxidase
PA	Phosphatidic acid
PALS	Periarteriolar lymphocyte sheaths
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PG	Phosphatidylglycerol
PHA	Phytohaemagglutinin
PPD	p-phenylenediamine
PS	Phosphatidylserine
SPDP	N-succinimidyl pyridyl dithiopropionate
SUV	Small unilamellar vesicles
Tc	Gel-to-liquid crystalline transition temperature
TCA	Trichloroacetic acid
TEA	Triethylamine
TGF-β	Transforming growth factor-β
TO	Triolein

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CHAPTER 1

GENERAL INTRODUCTION

1. 1. General structure and properties of liposomes

Hydration of dry phospholipids in aqueous media results in the arrangement of phospholipid molecules in bilayers to form closed spherules known as liposomes (Bangham et al, 1965). During this process, liposomes entrap hydrophilic solutes that are present in the hydration medium. Lipophilic molecules can also be incorporated into liposomal membranes by dissolving these molecules together with the lipids. Liposomes can be prepared from a variety of lipids (usually phospholipids) and lipid mixtures. Most commonly used phospholipid molecules and their gel to liquid crystalline transition temperatures (Tc) are presented in Table 1.1. Vesicles composed of phospholipids that are at temperatures below the phospholipid Tc are known as "solid" and those at temperature above their Tc, as "fluid" liposomes. The Tc is a function of acyl chain length; in phospholipids composed of the same acyl chain in both positions, Tc increases by about 14-17° with every 2-methylene unit increase in chain length. The presence of unsaturated acyl chains, branched chains, or those carrying bulky side groups leads to decreased transition temperatures (Szoka and Papahadjopoulos, 1980). Besides this property of phospholipids, the surface charge of liposomes can be varied from neutral (with phospholipids such as PC and PE) to negative (with acidic phospholipids such as PA, PS or PG) or positive (by the use of lipids such as BisHOP, DOTMA or stearylamine) in physiological pH ranges.

Table 1.1. Most commonly used phospholipids in liposome preparations and their gel to liquid crystalline transition temperatures (adapted from Weiner, 1990).

Phospholipid	Abbreviation	Transition temperature (°C)
Egg phosphatidylcholine	EPC	-15 to -7
Dilauryloyl phosphatidylcholine	DLPC	-1.8
Dimyristoyl phosphatidylcholine	DMPC	23
Dipalmitoyl phosphatidylcholine	DPPC	41
Distearoyl phosphatidylcholine	DSPC	55
Dioleoyl phosphatidylcholine	DOPC	-22
Dilauryloyl phosphatidylglycerol	DLPG	4
Dimyristoyl phosphatidylglycerol	DMPG	23
Dipalmitoyl phosphatidylglycerol	DPPG	41
Distearoyl phosphatidylglycerol	DSPG	55
Dioleoyl phosphatidylglycerol	DOPG	-18
Dimyristoyl phosphatidylethanolamine	DMPE	50
Dipalmitoylphosphatidylethanolamine	DPPE	66
Phosphatidylserine	PS	7
Dimyristoyl phosphatidylserine	DMPS	38
Dipalmitoyl phosphatidylserine	DPPS	51
Dimyristoyl phosphatidic acid	DMPA	51 (pH 6.0)
Dipalmitoyl phosphatidic acid	DPPA	67 (pH6.5)
Sphingomyelin	SPH	32
Dipalmitoyl sphingomyelin	DPSPH	41
Distearoyl sphingomyelin	DSSPH	57

1.2. Liposome preparation techniques

There are several methods of liposome preparation giving rise to vesicles of different sizes ranging from 25 nm (smallest possible) to several microns in diameter and composed of one or more bilayers. These were discussed extensively by Gregoriadis (1984; 1993a) and New (1990). Table 1.2 shows the physical properties and entrapment efficiencies of various liposome formulations prepared by different methods.

Table 1.2. Physical properties of liposome formulations (adapted from Mayer et al, 1986).

Vesicle type	Preparation procedure	Vesicle diameter (μm)	Entrapped agent	Entrapment efficiency (%)
SUV	Sonication	0.02-0.04	Cytosine arabinoside, methotrexate, CF	1-5
SUV	French press	0.02-0.05	CF, inulin, trypsin, BSA	5-25
SUV	Detergent removal	0.036-0.05	CF, inulin	12
LUV	Detergent removal	0.1-10.0	Inulin, cytochrome c, CF	12-42
LUV	Reverse phase evaporation	0.2-1.0	CF, cytosine, arabinoside, 25 s RNA, DNA, insulin, albumin	28-45
LUV	Solvent vaporization	0.1-0.5	Chromate, glucose, trypsin inhibitor, DNA	2-45
LUV	Extrusion	0.056-0.2	^{22}Na inulin, methotrexate, cytosine arabinoside, DNA	15-60
MLV	Mechanical mixing	0.4-3.5	^{22}Na CF, glucose, albumin, DNA	1-8.5
MLV	Sonicate-freeze-thaw	0.17-0.26	Asparaginase	50-56
MLV	Freeze-thaw	0.5-5.0	^{22}Na, inulin	35-88
MLV	Dehydration-rehydration	0.3-2.0	CF, sucrose, albumin, Factor VIII, ATP, vincristine, melphalan	27-54
MLV	Solvent evaporation-sonication	0.3-2.0	Inulin, streptomycin sulfate, chloramphenicol, oxytetracycline, sulfamerazine	6.3-38

SUV: small unilamellar vesicles; LUV: large unilamellar vesicles, MLV: multilamellar vesicles; CF: carboxyfluorescein.

The examples given in Table 1.2, correspond to passive entrapment techniques, while for a number of amphiphilic drugs like doxorubicin, active entrapment (or loading) can also be achieved by the use of pH gradients (Zhou et al, 1991). However, most of the techniques established require the use of organic solvents or sonication in the presence of the molecules to be entrapped which can be detrimental to labile molecules such as enzymes or cytokines. Kirby and Gregoriadis (1984) developed a mild procedure for the entrapment of solutes in liposomes. The procedure, called dehydration-rehydration (DRV) method, is based on the induction of fusion of preformed empty small unilamellar vesicles (SUV) by means of dehydration and controlled rehydration eventually yielding vesicles of multilamellar type. The technique is described in detail in Materials and Methods section of the thesis.

1.3. Covalent coupling of proteins to the liposomal surface

Besides entrapment, liposomes also allow covalent attachment of cell-specific or other ligands on their surface. Such liposomes are particularly relevant for targeted drug delivery. There are various methods of covalent coupling which make use of glutaraldehyde, diethyl suberimidate or other homobifunctional reagents (New, 1990) which are not very efficient and may also lead to protein aggregation (for a comparison of most commonly used conjugation methods, see Table 1.3.). Protein conjugation has been largely improved by the use of heterobifunctional cross linking reagents, for instance, N-succinimidyl pyridyl dithiopropionate (SPDP) (Heath, 1987). With this method, a sulphydryl-(SH-) residue is introduced to the phospholipid phosphatidyl ethanolamine (PE) and also to the protein to be conjugated by reacting the two components individually with SPDP. In order to link the protein to liposomes,

liposomes incorporating the lipid derivative (PDP-PE) are incubated with the derivatized protein previously treated with dithiothreitol (DTT), which reduces its disulfide bonds. During the reaction, the less stable aromatic pyridyl sulphydryl group (-S-Pyr) on the lipid is displaced from the disulfide bond by the aliphatic -SH group of the reduced protein, resulting in the linkage of the protein to liposomes via a disulfide bridge. However, this method requires a large number of purification steps which leads to a considerable loss of protein.

Table 1.3. Summary of conjugation procedures (adapted from New, 1990)

Lipid-reactive agent	Lipid moiety	Linkage	Protein functional group	Protein reactive agent
SPDP	PE-NH_2	-S-S-	NH_2-protein	SPDP+DTT
Activated dicarboxylic acids	PE-NH_2	-CO-$(CH_2)_n$-CO-	NH_2-protein	-
-	PE-NH_2	Peptide	HOOC-protein	NH_2 block, then carbodiimide
-	PE-NH_2	secondary amine	Glycoprotein	Periodate/borohydride
Nitrite	APSA	-N=N-	Aromatic side groups	-

One of the recent methods reported (Snyder and Vannier, 1984) involves the linkage of proteins to a synthetic lipid in the liposomal membrane via a diazotization reaction. The aromatic groups of the protein react with the sodium nitrite-activated lipid (N-(p-

aminophenyl)stearylamide; APSA) to give a diazo linkage. Besides the ease of preparation of the conjugates with this procedure, another advantage is that the success of the final conjugation step can be observed visually by a colour change which takes place as a result of diazo bond formation, to give liposomes with a distinctive light brown colour. This method was modified by Senior and Gregoriadis (1989) for DRV and by Garcon et al (1986) so that ligands could be coupled to liposomes (SUV) before the entrapment of agents sensitive to coupling procedures.

1.4. Behaviour of liposomes in vivo

One of the factors affecting the behaviour of a carrier when administered intravenously is its stability in blood. Upon i.v. administration, liposomes encounter a variety of plasma proteins (Gregoriadis, 1988b). Of these, opsonins adsorbing on liposomes, promote phagocytosis primarily by affecting the critical first step of attachment, in which opsonins form a bridge between the liposome and the phagocyte (Patel, 1992). However, phagocytic ingestion of particles can also occur in the absence of opsonins through nonspecific recognition of particles with surface properties such as hydrophobicity, charge and chemical composition, playing an important role in determining the nature of particle-cell interaction. Another class of proteins, namely plasma high density lipoproteins (HDL) remove phospholipids from the liposomal bilayers and thus destabilize them (Kirby and Gregoriadis, 1981). This HDL attack and destabilization can be avoided by the incorporation of excess amounts of cholesterol and/or by using phospholipids with a high Tc in the liposome structure (Kirby and Gregoriadis, 1980; 1983). Sphingomyelin also renders liposomes resistant to HDL attack, but it is known to be toxic in vivo (Weereratne et al , 1983). Stable liposomes

containing high amounts of cholesterol or composed of high melting phospholipids have longer circulation half-lives in the blood stream due to reduced opsonin adsorption on such vesicles (Patel, 1992). Besides lipid composition, two other major factors that influence the blood clearance rates are vesicle charge and size. Half-life is extended with reduced vesicle size and a positive surface charge (Gregoriadis, 1983; 1985). The stable large liposomes end up in the macrophages of the liver and spleen, while small vesicles also show increased accumulation in the phagocytic cells of the bone marrow (Senior et al, 1985). Although the presence of negative surface charge is generally associated with accelerated clearance rates, some negatively charged lipid molecules can actually cause the opposite effect. For example PS accelerates liposome clearance but the presence of asialoganglioside in the bilayers reverses this phenomenon by a steric conformation effect preventing interaction of plasma proteins (opsonins) with the carboxyl group of the sialic acid moiety (Gabizon and Papahadjopoulos, 1992). Three necessary conditions seem to be required for liposomes to exhibit long half-lives: partially shielded (or absent) surface negative charge, rigid bilayer structure and a small size (<100 nm). Inclusion of certain synthetic diacyl lipids with bulky polyethylene glycol (PEG) headgroups also results in the further prolongation of blood circulation times (Lasic et al, 1991). Such liposomes, known as Stealth® liposomes exhibit, in addition to long half-lives, dramatically altered pharmacokinetics and biodistribution due to the presence of the surface-attached polymer chains. These prevent vesicle-plasma protein interactions and reduce adsorption of the liposomes to cells and blood vessel walls, thus enhancing transcapillary passage of the vesicles (Working et al, 1994; Maruyama et al, 1994; Torchillin and Papisov, 1994; Allen et al, 1994).

When liposomes come into contact with cells, three different phenomena can take place depending on the characteristics of the liposomes and the cell type: endocytosis, fusion and/or adsorption (Gregoriadis et al 1981). With phagocytic cells, liposomes are endocytosed in coated vesicles encountering a low-pH compartment inside the cell and then delivered to lysosomes and degraded by phospholipases there (Harashima et al, 1995). Certain types of liposomes such as pH-sensitive ones can (at least in vitro) become destabilized in the endosomes due to the low pH and fuse with the endosomal membrane to release their contents intact into the cytoplasm (Litzinger and Huang, 1992). Fusion of liposomes with the plasma membrane of cells requires a less stable liposome structure with PC and cholesterol inhibiting such interactions (Bentz and Ellens, 1988).

1.5 Immunological Adjuvants

Immunological adjuvants are highly heterogeneous groups of compounds that are able to augment or modulate antigen-specific immune responses. In general, most of the newly developed recombinant or synthetic subunit vaccines are poor immunogens and require adjuvants to evoke an immune response. Adjuvants are thought to exert they effect by one or more of the following mechanisms (Gupta et al, 1993):

i) formation of an antigen depot at the site of injection while allowing slow antigen release

ii) presentation of antigen to immunocompetent cells

iii) induction via cytokine production

The sources of adjuvants vary enormously and include vegetables and bacteria. Other adjuvants are made synthetically. Some major groups of adjuvants are discussed briefly below.

Freund's complete adjuvant (FCA) is one of the most known adjuvants and is composed of mineral oil (paraffin) mixed with killed mycobacteria. It was shown to enhance both humoral and cell mediated immune responses to several antigens, probably by creating a depot for the antigen, enhancing uptake by macrophages and inducing costimulants (Gupta et al, 1993). However, despite its potent adjuvant effects, FCA causes abscess formation and inflammation at the site of injection, severe pain, fever and may induce permanent organ injury by generalized granulamatous proliferation. The water-in-oil emulsion without mycobacteria, known as Freund's incomplete adjuvant (FIA), enhances only humoral responses via delayed antigen release and enhanced uptake by macrophages but it may still cause some side (eg. granulomas and inflammation at the injection site) effects although less severe than with FCA. Use of vitamin E in conjunction with FIA was shown to improve antibody titers probably due to its scavenger action of reactive oxygen radicals (at the site of injection), generated during antigen processing and presentation (Hogan et al, 1993).

Mineral gel adjuvants among which aluminium phosphates or hydroxides are the most common, are the only licenced adjuvants for use in humans (Bomford, 1989a). Generally, a solution of antigen is mixed with a preformed $Al(OH)_3$ or $AlPO_4$ gel at a specific pH depending on the pI of the protein used. The antigen thus adsorbs on the gel. These mineral gel adjuvants are effective especially in augmenting primary immune

responses but are very poor in terms of cell mediated immunity. They act mainly by depot formation allowing the release of antigen slowly and thus prolonging the time for interaction between antigen and antigen presenting cells (APC).

Nonionic block copolymers are composed of hydrophilic blocks or chains of polyoxyethylene and hydrophobic blocks of polyoxypropylene (Hunter et al, 1989). Their adjuvant activity varies with the chain length of the hydrophobic or hydrophilic blocks. Although non-biodegradable, these copolymers can be excreted intact in the urine. Adhesive nonionic block copolymer surfactants can bind protein to the surface of oil droplets and these complexes act on macrophages to enhance MHC class II molecule expression on the cell surface. They are thought to be Th1-clone stimulants due to a shift in IgG subclasses from IgG_1 to IgG_{2a} in mouse (Hadden, 1993).

Saponins are mainly plant extracted triterpene glycosides with Quil A being the most known. It is composed of several different types of triterpene glycosides (Bomford, 1989b). Quil A has been shown to be a potent adjuvant at low doses and to enhance antibody dependent cell mediated immune reactions (Tiong et al, 1993).

Lipopolysaccharide (LPS), is an amphipathic molecule with three different covalently linked regions (Gupta et al, 1993): O-specific polysaccharide, core oligosaccharide and lipid A, which accounts for most of the biological activity. Both LPS and lipid A are potent adjuvants for humoral and cell-mediated immunity, however are too toxic to be used as such. Monophosphoryl lipid A which is relatively less toxic can increase primary and secondary antibody responses. Reduction of lipid A toxicity was also

achieved by entrapping in liposomes (Alving et al, 1992). Lipid A adjuvant activity is though to be mediated via macrophage activation and increased antigen presentation by macrophages.

N-acetyl muramyl-L-alanyl-D-isoglutamine (MDP) is the smallest subunit of the mycobacterial cell wall with adjuvant activity. MDP alone increases humoral responses to an antigen (Gupta et al, 1993) but also enhances cell mediated immune responses when incorporated in an oil emulsion. MDP activates several cell types including macrophages, polymorphonuclear leukocytes, mast cells and platelets and induces the secretion of various cytokines. It is also a B-cell mitogen, however is not considered to be safe due to its toxicity and pyrogenicity. Another derivative of MDP (Threonyl-MDP), is safe to use and was shown to be a very effective adjuvant in boosting humoral and especially cell mediated immune responses when used in a squalene-Pluronic polymer emulsion known as Syntex adjuvant formulation-1 (SAF-1) (Allison and Byars, 1986).

Immune stimulating complexes (ISCOMs) are non-covalently bound complexes (30-40 nm in diameter) of Quil A, cholesterol and an amphipathic antigen in a molar ratio of 1:1:1. These were shown to be very effective in enhancing both humoral and cellular immune reactions to several amphipathic antigens (mainly of viral origin) and is comparable to SAF-1 in its action (Morein et al, 1989).

Neuraminidase-galactose oxidase (NAGO), one of the new adjuvants tested, enzymatically oxidizes the cell surface galactose residues to increase the number of

amine reactive carbonyl groups on lymphocytes and antigen presenting cells. NAGO was shown to enhance T cell priming and was effective in the induction of secondary antibody responses to antigens (Zheng et al, 1992).

PICKCa, is a modified polyinosinic:polycytodylic complex (double stranded complexes of synthetic polyribonucleotides) derivative containing kanamycin and $CaCl_2$. Kanamycin, which is positively charged, neutralizes the negatively charged Poly I:C and thus stabilizes their structure, while $CaCl_2$ enhances the penetration of the complex into the cells. PICKCa was shown to enhance antibody production and cell mediated immunity and is thought to increase IL-2 production from T-lymphocytes (Lin et al, 1993).

Since this study mainly focuses on the use of liposomes and certain cytokines as immunological adjuvants, these two classes will be discussed in the following sections separately from the other adjuvants.

1.6. Liposomes as immunological adjuvants

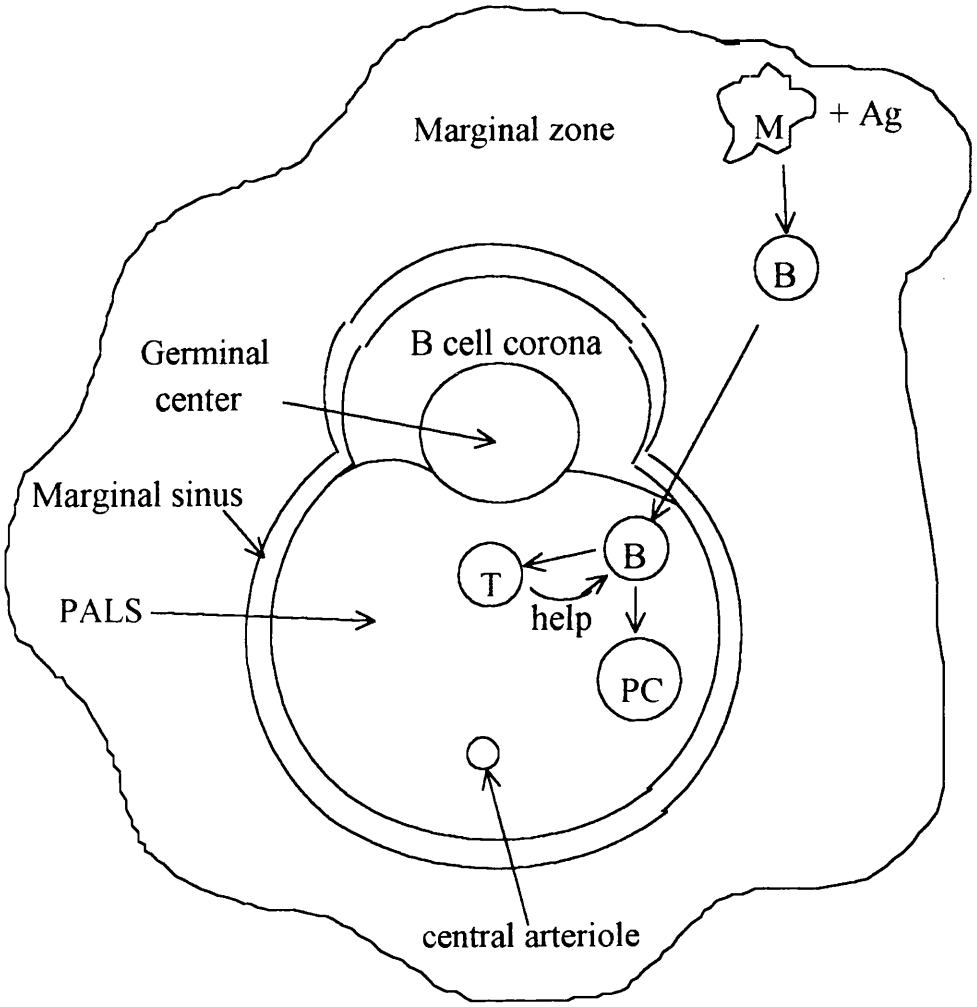
Since the first demonstration of the use of liposomes as immunological adjuvants (Allison and Gregoriadis, 1974), numerous studies were carried out demonstrating the ability of these carriers to enhance both humoral and cell mediated immune responses (reviewed by Gregoriadis, 1990). It appears that there are no specific liposome formulations that are superior in adjuvanticity. Table 1.4. presents results obtained with antigens in different types of liposomes. The only prerequisite for liposomal adjuvanticity is a physical association of the antigen with liposomes in the context of

Table 1.4. Immunization studies with liposomal antigens: examples of experimental protocols and observations (adapted from Gregoriadis, 1990).

Antigen	Liposome composition	Co-adjuvants	Animal species	Route of administration	Observations
Tetanus toxoid	Various phospholipids, CHOL; PC, CHOL, lipid-bound mannosylated ligand	IL-2, (Thr1)MDP, IFN-γ	Mouse	i.m.	Adjuvant effect dependent on liposomal structural characteristics; co-adjuvant action dependent on source, amount and formulation of co-adjuvant; receptor-mediated targeted adjuvanticity
S.sobrinus ribosomal protein	DPPC, CHOL, DCP		Rat	Oral	Adjuvant effect, induction of specific salivary IgA; fewer carious lesions on challenge
P.falciparum synthetic peptide	DMPC, CHOL, DCP	Lipid A, alum	Rabbit, monkey	i.m.	Adjuvant effect enhanced by alum and lipid A; protective antibody levels
Influenza virus glycoproteins	Viral lipid extract		Mouse	i.n., i.p., i.m.	Superior systemic and local response, protection from virus infection

entrapment, passive adsorption or covalent linkage. Liposomes do not alter the antibody subclasses with respect to the free antigen. Although a depot effect can sufficiently explain the augmented humoral responses with liposomes this can not account for the increased cell mediated immune responses. In the case of entrapment, the only appropriate type of antigen presenting cells for the processing of liposomal antigens appears to be macrophages since entrapped antigens are masked and thus prevented from recognition by surface receptors on lymphoid cells. However, whether macrophages process liposomal antigens and present the fragments directly to T cells or if macrophages cooperate with other antigen presenting cells in the microenvironment so that these accomplish the presentation task is not clear (Szoka, 1992). A model has been proposed for cooperation among B cells, T cells, dendritic cells and macrophages for the processing and presentation of liposomal antigens (van Rooijen, 1994). In this model, marginal zone macrophages at the end of the white pulp capillaries in the spleen pre-process the particulate antigens. The processed antigenic fragments are then transferred to B cells in the marginal zone followed by their migration into the outer part of the periarteriolar lymphocyte sheaths (PALS). Here, the B cells present the processed antigens to T cells of appropriate specificity and receive T cell help to be differentiated into antibody forming plasma cells. This process is shown schematically in Figure 1.1. In the inner part of the PALS, dendritic cell-T cell clusters are activated by antigen, resulting in the proliferation of antigen-specific T cells, an event which might explain the increased cell mediated responses obtained with liposomal antigens.

Figure 1.1. Proposed model of cooperation among B-cells, T-cells and macrophages in the humoral response against liposomal antigen



The transverse section of the white pulp in spleen is demonstrated in the figure. M: macrophage; B: B lymphocyte; T: T lymphocyte; PC: plasma cell; Ag: antigen. The macrophage-processed antigenic fragments are transferred to B-cells which migrate to PALS, present the processed antigens to T cells and receive T cell help and some differentiate into antibody secreting plasma cells (see text for more details).

Since liposomal lipid composition is known to influence the in vivo behaviour of liposomes, it may also influence their adjuvant action. Bilayer fluidity, number of lamellae, vesicle size and mode of antigen localization within liposomes as well as lipid to antigen mass ratio seems to influence liposomal adjuvanticity (Gregoriadis, 1990). For example, the presence of phospholipids with high Tc values (above 37 °C) improved antibody responses to hydrophobic antigens but not to soluble antigens. It was suggested (Gregoriadis, 1990) that membrane antigens in solid liposomes were transferred to the plasma membranes of antigen presenting cells for association with MHC molecules there without a need for intracellular processing, while soluble antigens require intracellular processing prior to exposure on the surface of antigen presenting cells. The latter process could be inhibited by high melting lipids at any of the stages between liposome internalization by cells and peptide migration to the plasma membrane.

The phospholipid to antigen mass ratio seems to affect adjuvanticity of liposomes drastically (Davis and Gregoriadis, 1987; 1989, Gregoriadis, 1990). A low (ca. 50) phospholipid to antigen mass ratio was less effective in improving primary and secondary antibody responses than those obtained with higher phospholipid to antigen mass ratios (ca. 2×10^3). Interestingly, with a high ratio, even DSPC liposomes proved to be as effective as the fluid liposomes for the soluble antigen. However, with very high (ca. 10^4) phospholipid to antigen mass ratios the adjuvanticity was abolished, probably due to very low amounts of antigen per vesicle being non-immunogenic.

Liposomes also proved to be valuable in maintaining immunological memory to peptides containing T and B cell epitopes (such as hepatitis B virus pre-S and foot-and-mouth disease virus VP1 peptides). This approach might avoid the neccessity to use an irrelevant carrier protein to achieve responses for such peptides.

There are conflicting reports on the effect of vesicle surface charge on liposomal adjuvanticity. In some cases, negatively charged and neutral vesicles were shown to be equally effective while a positive charge led to reduced responses. In other studies however, positively charged liposomes performed as well as negatively charged or neutral ones (Alving, 1991). For example, incorporation of a positively charged lipid (BisHOP) into the bilayers of PC and DSPC liposomes was shown to enhance IgG_1 responses to tetanus toxoid (Tan and Gregoriadis, 1991). A 20 % positively charged lipid ratio was superior to a 10 % one. On the other hand, by the intraperitoneal route, negatively charged liposomes were more effective probably due to their preferential uptake by peritoneal macrophages (van Rooijen, 1994).

Another attractive application of liposomes as adjuvants is their ability to induce cytotoxic T lymphocytes. Antigen presenting cells contain two segregated pathways to process and present endogenous and exogenous antigens (Raychaudhuri and Morrow, 1993). Humoral responses are regulated by the MHC class II pathway. Antigen is internalized via receptor-mediated endocytosis (for example Fc-mediated uptake) and, following processing in the endosomes, fragmented antigens associate with MHC class II molecules, to be subsequently expressed on the cell surface. Once on the cell surface, antigen fragments are presented to $CD4^+$ T cells which then promote an

appropriate B-cell response. On the other hand, MHC class I molecules associate with endogenously derived, processed antigen in the lumen of the endosplasmic reticulum. Peptide antigen generated in the cytoplasm is imported into the endoplasmic reticulum from where complexes of MHC class I molecules and peptide antigen are transported to the cell surface for presentation to $CD8^+$ cells. Thus, an exogeneous antigen has to penetrate the endosomal membrane and translocate into the cytosol in order to be presented by the MHC class I molecules. pH-Sensitive liposomes can accomplish this delivery of foreign molecules to the cytosolic compartments in vitro from within the endosomes (Zhou et al, 1994). Such liposomes are mainly composed of DOPE and an acidic amphiphile. DOPE alone cannot form bilayers and has a high tendency to form reverse hexagonal structures rather than a bilayer configuration but can be forced to form bilayers in the presence of an amphiphile with a bulky or charged headgroup. The amphipathic stabilizer serves as a pH sensor for the liposomes. Liposomes destabilize when the amphiphile is protonated at mildly acidic pH such as that in endosomes, leading to the release of entrapped contents and vesicle to vesicle fusion as well as fusion with the endosomal membrane. Thus, such liposomes can very efficiently induce cytotoxic T cells.

The advantages of using liposomes as immunological adjuvants and vaccine carriers are summarized below:

1. Ability to convert a non-immunogenic molecule to an immunogenic one.
2. Ability to accomodate both hydrophilic and hydrophobic antigens.
3. Can improve antibody responses even with lower doses than the free antigen.
4. Can accomodate multiple antigens in the same vesicle.

5. Allows the entrapment of antigen together with a co-adjuvant.
6. Augments humoral responses to several antigens.
7. Can induce cell-mediated immunity to antigens.
8. Ability to induce cytotoxic T cells with certain liposome formulations.
9. Reduces toxicity of an antigen or a co-adjuvant, increases their half-lives in blood and alters the biodistribution.
10. Allows ligand mediated targeting to antigen presenting cells
11. Allows choice of spatial distribution of the antigen or the co-adjuvant within the same vesicle (i.e. adsorption, covalent coupling to the vesicle surface or entrapment).
12. Liposomally associated antigens and co-adjuvants can be kept in freeze-dried form without substantial loss of activity after reconstitution.

1.7. Cytokines as immunological adjuvants

Cytokines can be defined as polypeptide hormones that act locally and modify cellular processes in the vicinity. Table 1.5 presents the known members of the cytokine family.

A successful immune response to an infectious agent depends on the activation of an appropriate set of immune effector functions i.e., cell-mediated or humoral (or both in some cases). These two arms of the immune response are regulated by distinct $CD4^+$ T cells designated as Th(helper)1 and Th2 cells that secrete different patterns of cytokines (Powrie et al, 1993). Th1 clones produce mainly IL-2, IFN-γ and TNF-β, which are factors responsible for the promotion of delayed type hypersensitivity (DTH) reactions being a characteristic of cell mediated immune responses (O'Garra and

Table 1.5. The cytokine family and their major biological actions (Adapted from Hopkins and Rothwell, 1995).

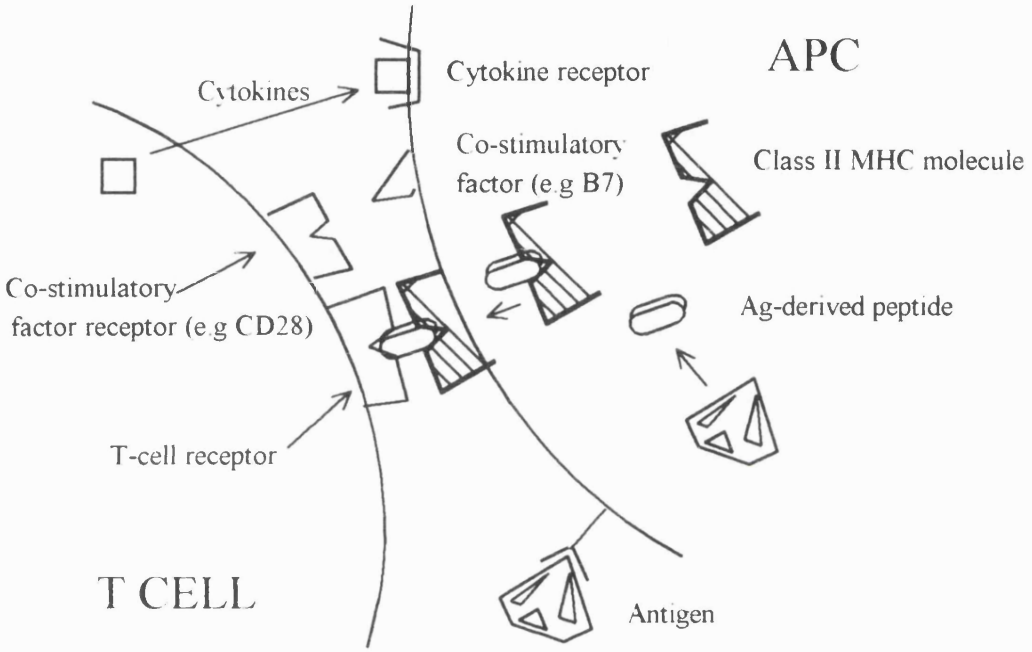
Family	Members	Major activities and features
Interleukins	IL-1α, IL-1β, IL1ra, IL-2-IL-15	Multiple tissue and immunoregulatory activities
Chemokines	IL-8/NAP-1, NAP-2, MIP-1α and β, MCAF/MCP-1, MGSA and RANTES	Leukocyte chemotaxis and cellular activation
Tumour necrosis factors	TNF-α and TNF-β	Similar to IL-1 in addition to tumour cytotoxicity
Interferons	IFN-α, β and γ	Inhibition of intracellular viral replication and cell growth regulation; IFN-γ is primarily immunoregulatory
Colony stimulating factors	G-CSF, M-CSF, GM-CSF, IL-3, and some of the other ILs	Colony cell formation in the bone marrow and activation of mature leukocyte functions
Growth factors	EGF, FGF, PDGF, TGF-α, TGF-β and ECGF	Cell growth and differentiation
Neurotrophins	BDNF, NGF, NT-3-NT-6 and GDNF	Growth and differentiation of neurons
Neuropoietins	LIF, CNTF, OM and IL-6	Cytokines acting on the nervous system, and acting via a related receptor complex

BDNF, brain derived neurotrophic factor; CNTF, cilliary neurotrophic factor; EGF, epidermal growth factor; ECGF, endothelial cell growth factor; FGF, fibroblast growth factor; GDNF, glial derived neurotrophic factor; G, M and GM-CSF, granulocyte, macrophage and granulocyte/macrophage colony stimulating factor(s); IFN, interferon; IL, interleukin; IL-1ra, interleukin-1 receptor antagonist; MCAF, monocyte chemotactic and activating factor; MCP, monocyte chemotactic protein; MGSA, melanoma growth stimulatory activity; MIP, macrophage inflammatory protein; NGF, nerve growth factor; NT, neurotrophin; NAP, neutrophil activating protein; OM, oncostatin M; PDGF, platelet derived growth factor; RANTES, regulated upon activation normal expressed and secreted; TGF, transforming growth factor; TNF, tumour necrosis factor.

Murphy, 1994). Th2 clones on the other hand produce IL-4, IL-6, IL-10 and IL-13 and provide help for humoral immunity via these mediators (Seder and Paul, 1994). Cell populations capable of producing a combination of Th1 and Th2 cytokines are referred to as Th0 cells. Naive $CD4^+$ T cells have to undergo a priming step in order to develop into effector cells (summarized in Figure 1.2). Growth of Th2 cells was shown to be strictly dependent on the expression of IL-1 by the antigen presenting cells (Weaverat al, 1988) while Th1 clone development seems to be regulated by IFN-γ, IL-12 and partly by TGF-β (O'Garra and Murphy, 1994). In general, the cytokines secreted by Th1 and Th2 clones tend to oppose each other's actions. Three cytokines (IL-4, IFN-γ and TGF-β) play a pivotal role in regulating immunoglobulin isotype switching in the mouse (Snapper and Mond, 1993). It is believed that IL-4 stimulates IgG and IgE production while IFN-γ favouring IgG_{2a} and TGF-β enhances IgG_{2b} and IgA production.

Thus, since cytokines can modulate both cellular and humoral immune responses, their use as adjuvants in vaccines can be very promising. There are several reports on the adjuvant actions of several cytokines and some of these are reviewed in detail by Heath and Playfair(1992). For example, IFN-γ was shown to be an effective adjuvant when mixed with vaccines prior to injection. It boosted the immune responses by increasing lymphocyte homing to the injection site and enhancing MHC class II expression on antigen presenting cells, enabling them to present antigens more efficiently (Heath et al, 1991). However, with some strains of mice in which the cytokine was not as effective, lymphocyte homing was very strong. It was thus assumed that the adjuvant

Figure 1.2. Factors involved in differentiation of $CD4^+$ T cells (adapted from Seder and Paul)



Different cytokines such as IL-12 and IFN-γ modulate the development and differentiation of naive T cells. Although other factors may play a role in the determination of lymphokine-producing phenotype (eg. antigen dose, type of antigen presenting cell, and expression of accessory molecules and hormones), these effects appear to be secondary to the dominant role of cytokines.

effect is mediated mainly by the former mechanism. The same authors also emphasized the importance of simultaneous administration of the antigen and cytokine together at the same site and showed that an antigen-cytokine conjugate had better adjuvant properties than the unconjugated mixture suggesting a need for both components to contact the same antigen presenting cell simultaneously in order to be effective (Heath and Playfair, 1990). Among the interleukins, the first one shown to have an adjuvant effect was IL-1 (Staruch and Wood, 1983). This cytokine enhances both primary and secondary antibody responses probably by its ability to enhance IL-2 production and antigen specific T helper cell activity, as well as B-cell proliferation and differentiation directly or indirectly via IL-2 (Heath and Playfair, 1992). IL-2 is one of the most tested cytokine among others. It was shown to overcome genetic non-responsiveness to a peptide antigen, (Good et al, 1988),and to boost antibody titers when used in a bovine respiratory disease virus vaccine (Reddy et al, 1993). However, despite these data showing that IL-2 augments humoral responses to the antigen, in some cases only cell mediated immune reactions were shown to be enhanced rather than antibody responses (Hazama et al, 1993). The mechanism of action of IL-2 is thought to be via the induction of cytotoxic and helper T cells (Utermohlen et al, 1994) , induction of IFN-γ expression (Murray et al, 1993) and probably by stimulation of B cell proliferation and differentiation (Nakagawa et al, 1986). However, use of IL-2 in its free form cannot be considered safe due to the need to administer large amounts in order to overcome the short-half life of the cytokine. This can lead to increased vascular permeability causing vascular leak syndrome (Puri et al, 1989). Therefore, entrapment of IL-2 in liposomes can be promising as it was shown that liposomally entrapped IL-2 exhibited a modified biodistribution, prolonged blood clearance and reduced toxicity in vivo (Anderson et

al, 1992a). This approach also can eliminate the need for multiple injections and large dose administrations (Anderson et al, 1992b). There are several reports on the adjuvant action of IL-2 in liposomes as mentioned in more detail in the introductory part of Chapter 6. However, with most of these studies, IL-2 was entrapped alone in liposomes. Work by Tan and Gregoriadis (1989) suggests a neccessity for the cytokine and the antigen to be entrapped in the same liposomes for the optimal adjuvant effect to be observed, supporting the observations by Heath and Playfair (1990). IL-6 is another cytokine that was shown to enhance mucosal IgA (Ramsay et al, 1994) and primary and secondary antibody responses in vivo (Takatsuki et al, 1988). The adjuvant activity of IL-6 was significantly enhanced when the cytokine was entrapped in liposomes and co-injected with the liposomal antigen (Duits et al, 1993). There are other reports on liposomal cytokines such as IL-7, where co-administration together with the antigen reduced the severity and course of the primary HSV-2 infection after challenge with the organism (Bui et al, 1994). Table 1.6 summarizes the results obtained with some liposomal cytokines.

It was suggested that the adjuvant action of cytokines can be enhanced by the use of a combination of cytokines that can synergize one another's action, by increasing their serum half-lives in the circulation or by physical association of the cytokine with the antigen in order to ensure targeting to the same antigen presenting cells (Heath and Playfair, 1992). The latter can be achieved either by co-entrapment in a suitable carrier system or by the construction of antigen-cytokine fusion proteins. For example, Tao and Levy (1993) demonstrated that a fusion protein comprising the variable region of the immunoglobulin molecules expressed on malignant B cells and GM-CSF was

Table 1.6. Liposomal cytokines and results obtained in vivo and in vitro (Adapted from 8th Liposome Workshop handout, Oberjoch, Allgau, 1993)

Cytokine	Lipid composition	Liposome type	Route of administration	Results
IL-2/IL-4	PC/chol/PS	LUV	intranasal	Reduced mortality from *P. Aeruginosa*; increased titers of bacterial polysaccharide specific IgA
IL-2	PC/chol/DOPG	SUV	i.m.	Enhancement of HSV-rec. glycoprotein D- mediated immunotherapy
IL-2	DMPC	MLV	i.p.	Effective as an adjuvant for influenza virus vaccines in the aged
IL-2	PC/chol	LUV	in vitro, CTLL cells	Increased proliferation of CTLL cells due to an interaction between IL-2 and liposomes
IL-1	PC/PS	MLV	in vitro, blood monocytes	Stimulation of monocytes only with surface bound IL-1
IL-1	DPPC/chol	MLV	in vitro	Antigen-induced stimulation of T cells by liposomes bearing membrane-IL-1
TGF-β1	PC or PC/PS	SUV	i.v.	TGF associated with negatively charged liposomes inhibited immune responses in vivo

capable of inducing idiotype-specific antibodies (without other carrier proteins or adjuvants) and protecting recipient animals from challenge with an otherwise lethal dose of tumour cells. Although this appears to be a very promising approach there is always the possibility for the final product to be unable to bind to its receptor and can not improve clearance rates that much. On the other hand, use of carriers such as liposomes can enhance clearance rates significantly while allowing the antigen and cytokine transportation simultaneously in the case of co-entrapment. One drawback of this approach is the lack of control over the doses of antigen and cytokine administered simultaneously. Either the antigen dose or the cytokine dose must be kept fixed while the remainder is left somewhat uncontrollable. However, this still would not be problematic if the preparation method is well standardized and especially when suitable methodology such as the DRV method (shown to yield reproducible entrapment values; Kirby and Gregoriadis, 1984) is used.

Since liposomes fullfill the two general mechanisms of adjuvant action (natural targeting to antigen presenting cells, and ability to create a depot of antigen) incorporation of cytokines as co-adjuvants can modify responses by their ability to act on various cells of the immune system. In this thesis, we have investigated the importance of spatial distribution of an antigen as well as of certain co-adjuvants within liposomes (i.e. entrapment versus covalent coupling). Attempts to improve adjuvanticity with mannose-mediated targeting to macrophages and immunization studies with a new liposome type and some novel cytokines are also discussed.

2. MATERIALS AND METHODS

2.1. MATERIALS

The following chemicals were purchased from Sigma Chemical Co., Poole, Dorset, UK: p-nitrophenyl stearate, p-phenylenediamine, triethylamine, thiophosgene, p-aminophenyl α-D-mannopyranoside, coomassie brilliant blue G-250, mannose, chloramine-T, bovine serum albumin, fluorescamine, tetrazolium dye MTT, tissue culture grade penicillin-streptomycin, Protease (Type XIV from *Streptomyces griseus*, 4 units/mg) triolein (TO), fluorescein isothiocyanate (FITC), trypan blue, o-phenylenediamine and cholesterol (CHOL).

Dimethyl sulfoxide, sodium hydroxide, ethanol, chloroform, phenol, sodium metabisulphite, potassium iodide, trichloroacetic acid, Triton X-100, sodium nitrate, sucrose, glucose, ammonium sulphate, Tween-20 and acetonitrile were purchased from British Drug Houses (BDH), Lutterworth, Leicester, UK. Horse-radish peroxidase conjugated goat anti mouse IgG_1, IgG_{2a}, and IgG_{2b}, foetal calf serum and RPMI 1640 tissue culture medium were from Sera-Lab Ltd., Crawley Down, Sussex, UK. Ficoll-Hypaque density gradient solution (density 1.114 g/mL) under its brand name of Mono-poly resolving medium and 96-well sterile tissue culture plates with lids were ordered from ICN Biomedicals Inc., Aurora, Ohio, USA. Human interleukin-2 ELISA kits were purchased from Serotech, Kiddlington, Oxford, UK. Ninety six well flat-bottom microtiter plates (Immunolon I) for ELISA and sterile flat-bottom 96-well tissue culture plates were from Dynatech Labs., Billingshurst, W. Sussex, UK.

All the column materials used including Sephadex G-25, Sepharose CL 4B and DEAE cellulose ion-exchange gels were from Pharmacia Biotech., St. Albans, Herts, UK. Egg phosphatidylcholine (PC) and phosphatidylglycerol (PG) were from Lipid products, Redhill, Surrey, UK. Carrier-free ^{125}I (24 Mbq) was from Amersham International, Amersham, Bucks, UK. Bicinchoninic acid was purchased from German Sci. Ltd., Northampton, UK. Drierite (calcium sulfate) appropriate for reagent drying (about 6 mesh) was from Hopkin and Williams Ltd., Chadwell Heath, Essex, UK. Human recombinant interleukin-2 (des-Ala_1 -Ser_{125} mutein, 18 x 10^6 IU/mg) was purchased from EuroCetus UK Ltd., Harefield, UK. Interleukin-1β (163-171) (human).2HCl was from Calbiochem-Novabiochem (UK) Ltd., Beeston, Nottingham, UK. Two miligrams of this peptide was a gift of the same company. Murine recombinant interleukin-12 (0.112 mg/mL; 4.6 x 10^6 U/mg) was a gift by Genetics Institute Inc., Cambridge, Massachusetts, USA. Simian recombinant interleukin-15 (1.72 mg/mL; 5.8 x 10^5 U/μg specific activity; 9.98 x 10^8 U/mL CTLL activity) was a gift from Immunex Corporation, Seattle, Washington, USA. Killed and live *B. subtilis* were gifts from Dr. Bruce Jones (Public Health Laboratories Service, Porton Down, Salisbury, Wilts, UK.). Immunopurified tetanus toxoid (2.38 mg/mL), was a gift of Dr. Phil Brookes, Pitman Moore, Harefield, Herts, UK. The lipidic amino acid based synthetic peptide (LCPH1) was synthesized by Dr. I. Toth, The School of Pharmacy, University of London. This product was composed of sequences from variable domains IV and I of the major outer membrane proteins of *Chlamidia trachomatis* in a lipid-core peptide system. CTTL-2 cell line was a gift of Dr. Benny Chain, Middlesex Hospital, Department of Immunology.All the other reagents used were of analytical grade.

2.2. METHODS

2.2.1. Preparation of N-(p-aminophenyl)stearylamide (APSA)

APSA was synthesized according to the procedure of Snyder and Vannier (1984). Two grams of p-nitrophenyl stearate (NPS) were dissolved in 30 mL of dimethyl sulfoxide (DMSO) at 50°C and 0.8 g of p-phenylenediamine (PPD) was added to the solution above. The colour of the solution was light brown at this stage. One drop of triethylamine (TEA) was added and the solution was incubated at 50°C for two h. A colour change to dark brown was observed at the end of the incubation period. This solution was poured into 200 mL of ice-cold distilled water that was made basic by the addition of 2mL 6M NaOH. A yellow precipitate that formed was collected by filtration and was transfered to a beaker. Into this, 100 mL absolute ethanol was added and the mixture was heated to 70°C untill complete dissolution. The solution was slowly cooled and crystallization was seen. The precipitate was collected by filtration and the recyrstallization process was repeated twice. The final product (white in colour) collected on a filter paper was dried. The identity of the product was assessed (after dissolving a small portion in chloroform) using its λmax (385 nm). The product was weighed and dissolved into warm chloroform. The procedure is shown schematically in Figure 2.1.

2.2.2. Preparation of mannose-linked tetanus toxoid

The method used for the synthesis of neoglycoproteins was essentially as described by Kataoka and Tavassoli (1984). Eighty μL thiophosgene, was added to a solution of p-aminophenyl α-D-mannopyranoside in ethanol (10 mL, 80%). The concentration of the

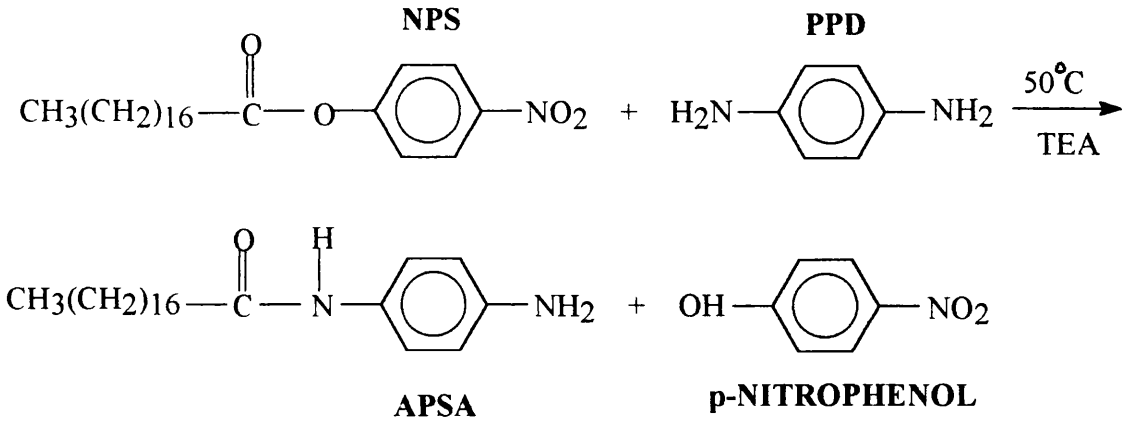


Figure 2.1. Scheme of p-aminophenylstearylamide synthesis

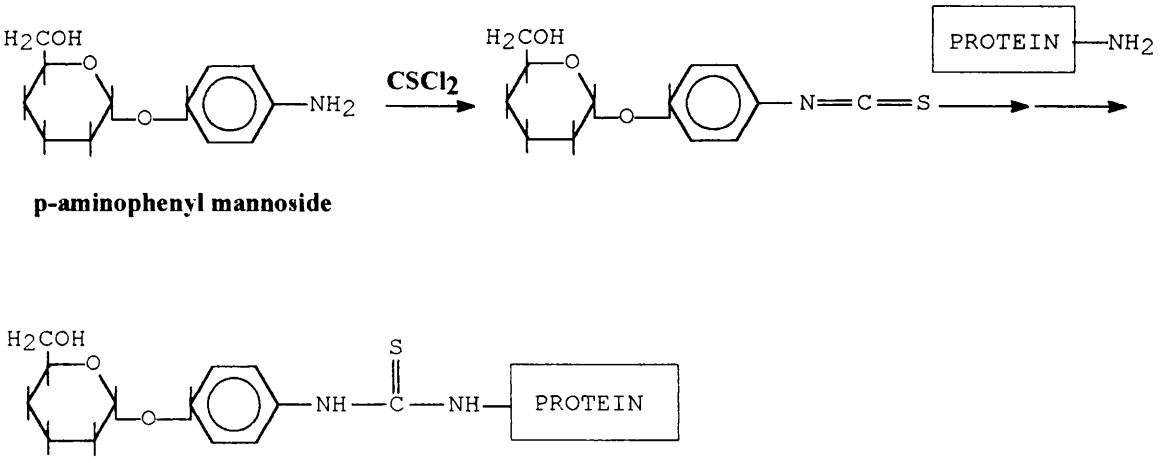


Figure 2.2. Scheme of mannosylation of tetanus toxoid

sugar in two different experiments was 0.185 mmol and 0.0184 mmol, respectively. The mixture was stirred for 1.5 h at 20°C. At the end of this period, N_2 gas was bubbled through the solution until most of the odour was removed. The pH was adjusted to 6 using 2 M NaOH and the solution was completely dried using rotary evaporation. The sugar derivative was dissolved in distilled water (5 mL) and this was slowly added to a solution of protein in 0.01 M borate buffer (15 mL, pH 9.0) while stirring. The concentration of protein in two different experiments was 47.6 μmol and 41.1 μmol, respectively. The pH of the solution was adjusted to 9 using 1 M NaOH and the mixture was stirred at 20°C for 18 h. The final concentrations of sugar and protein in the reaction mixture were calculated to be 9.25 mM sugar, 2.38 μM tetanus toxoid for experiment I and 0.92 mM sugar and 2.055 μM toxoid in experiment II, respectively. The sugar to protein ratio in these two experiments was calculated to be 3886 and 448 for I and II, respectively. The bound sugar was separated from the free sugar by extensive dialysis for 2 days at 4°C against several changes of 1 L PBS (experiment 1) or, after being concentrated in a dialysis bag using polyethyleneglycol as the drying agent, using a Sephadex G-25 column equilibrated with 0.1 M, pH 7.4 PBS containing 0.9% NaCl (experiment 2). Fractions (1.5 mL each) collected were analyzed for their protein and sugar contents and the fraction corresponding to the protein peak was pooled. Protein and sugar determinations were carried out using Bradford and phenol-sulphuric acid methods, respectively in order to determine the sugar to protein ratio of the final product. Figure 2.2 describes the mechanism of sugar coupling to the protein.

2.2.2.1. Determination of mannose

Determination of sugar was carried out as described by Weissig et al (1989). Mannose standards ranging between 25-125 μg/mL were prepared in water and 200 μL of these and the sample to be analyzed at various dilutions were placed into pyrex tubes. An aqueous solution of phenol in distilled water (400 μL, 2%) was added to each tube and vortexed. After the addition of concentrated sulphuric acid (1 mL), the tubes were incubated at 20°C for 30 min and the colour developed was quantitated at 485 nm using a Wallac CompuSpec UV-visible spectrophotometer. The amount of sugar present in the sample was quantitated using the calibration curve.

2.2.2.2. Determination of protein content

Protein determination was carried out as described by Bradford (1976). Briefly, coomassie Brilliant Blue G-250 (100 mg) was dissolved in absolute ethanol (50 mL) and phosphoric acid (85 % w/v, 100 mL) was added to the solution. This mixture was diluted to 1 L with distilled water. Standards (10-100 μg/mL, 0.1 mL) and samples were mixed with 1 mL of the reagent described above and absorbances were recorded at 595 nm.

2.2.3. Radiolabelling of proteins

In alkaline conditions, chloramine-T is slowly converted to hypochlorous acid which acts as an oxidising agent. At pH <8.0, the oxidation results in iodine incorporation into tyrosine residues of proteins (McConahey and Dixon, 1066).

Carrier free ^{125}I (2 μCi, 5 μL) was mixed with phosphate buffer (10 μL, 0.25 M, pH 7.5), and a solution of protein (100 μL, 0.5 mg/mL) was added to this mixture. In order to incorporate iodine into tyrosine residues of the protein, chloramine-T (10 μL, 5 mg/mL in 0.05 M phosphate buffer of pH 7.5) was added and the mixture was incubated at 20°C for 10 min. The reaction was stopped by the addition of sodium metabisulphite (20 μL, 1.2 mg/mL in 0.05 M, phosphate buffer pH 7.5) and the overall volume of the solution was made up to 0.5 mL using potassium iodide (1 mg/mL in 0.05 M, phosphate buffer pH 7.5). The labelled protein was applied on a Sephadex G-25 column (1x10 cm) equilibrated with phosphate buffer (0.05 M, pH 7.5), and fractions (1 mL each) were collected. From each fraction, 5 μL was transferred to gamma vials for counting (60 sec) in a 1275 Minigamma γ-counter (Wallac). The fractions corresponding to the maximum protein radioactivity peak were pooled and then dialyzed against four changes of sodium phosphate buffer (0.05 M, pH 7.5) to remove the unbound ^{125}I. The amount of protein-bound radioactivity was assessed with trichloroacetic acid (TCA) precipitation as follows: labelled protein (5 μL) was mixed with BSA (100 μL, 10%) and TCA (1.25 mL, 20%) was added to this mixture. After incubation at 4°C for 1 h, centrifugation was carried out at 3,000 rpm for 10 min. The supernatant and the pellet were transferred separately to gamma vials and their radioactivity content was determined. The ratio of counts in the pellet to that of the total radioactivity, multiplied by 100 gives the percent radioactivity in the protein pellet. Radiolabelled proteins giving a percentage of less than 90, were subjected to dialysis again until this ratio was reached. TCA precipitation was repeated regularly for all iodinated proteins.

2.2.4. Preparation of liposomes

2.2.4.1 Preparation of multilamellar liposomes (MLV)

MLV were prepared from egg phosphatidylcholine (PC) and cholesterol, 16 μmoles each and 0.75 μmoles APSA in the experiments where covalent coupling of a protein on the liposomal surface was required. The lipid mixture in chloroform was dried to a thin film at 20°C by evaporation of the solvent at a low speed in a rotary evaporator (Buchi) connected to a running tap water pump. In order to ensure removal of residual organic solvent, the lipid film obtained was left under a stream of nitrogen for 5 min. Rehydration and formation of MLV was effected by the addition of 2 mL distilled water and disruption of the lipid film with glass beads. Liposomes were allowed to anneal at 20°C for about 1 h.

2.2.4.2. Preparation of small unilamellar vesicles (SUV)

MLV prepared as described above were sonicated using a MSE sonicator (probe of 19 mm diameter) in an ice bath for 10 one-minute cycles alternating with 30 sec intervals to obtain a clear suspension. SUV produced were then centrifuged at 1000xg for 5 min to spin down titanium particles shed from the probe of the sonicator.

2.2.4.3. Preparation of dehydration-rehydration vesicles (DRV)

DRV preparation was carried out according to the method of Kirby and Gregoriadis (1984). SUV prepared as above in the presence of distilled water were mixed with 1 mL of the substance(s) to be encapsulated together with corresponding radiolabelled tracers (10^4 cpm) when available, frozen at -20°C and freeze-dried overnight in a

Christ freeze-drier untill a powder was obtained. The powder was rehydrated with 0.1 mL of distilled water, and incubated at 20°C for 30 min. At the end of this period, 0.1 mL of 0.1 M sodium phosphate buffer, pH 7.4 containing 0.9 % NaCl (PBS) was added and the mixture incubated for another 30 min. After the addition of 0.8 mL PBS, the entrapped drug was separated by ultracentrifugation at 27,300xg for 20 min using a Sorvall Combi Plus Du Pont ultracentrifuge. The liposome pellet was washed 2 more times with 6 mL PBS and the final pellet was suspended in 1 mL PBS for further use. In most cases percent entrapment calculations were based on ^{125}I radioactivity. For this, the radioactivity in the wash solutions and the liposomal pellet was used. In some cases where a radioactive marker was unavailable, 0.1 mL of washed liposomes (suspended in 1 mL of PBS) were lysed by the addition of an equal volume Triton X-100 (10 % (v/v)) and the amount of drug in the solution was quantitated using an appropriate method depending on the properties of the drug used. The amount of drug present in liposomes was used to derive values of percent encapsulation.

2.2.5. Determination of protein with fluorescamine

The method used was essentially as described by Anderson and Desnick (1979). Acetonitrile (300 mL) was dried by the addition of non-indicating Drierite (100 g, 6 mesh, $CaSO_4$) followed by filtration through a cone containing 100 g Drierite. A stock of fluorescamine (30 mg in 100 mL dry acetonitrile) was prepared. Standards of peptide LCPH1 (5-25 μg/mL) and samples with various dilutions were prepared in borate buffer (0.2 M, pH 9) and 2 mL of these were pipetted into glass tubes. Fluorescamine (0.7 mL) was added to the tubes rapidly while vortexing. Fluorescence values were determined in a spectrofluorimeter with excitation and emission

wavelengths at 360 and 465 nm, respectively. The concentration of protein in the sample was determined using a calibration curve.

2.2.6. Determination of protein with bicinchoninic acid

Determination of protein was carried out using the micro-method described by Smith et al (1985). A solution of 8 % $Na_2CO_3.2H_2O$, 1.6 % NaOH and 1.6 % Na-K tartrate was prepared and the pH was adjusted to 11.25 using powder of $NaHCO_3$ (Reagent A). Reagent B which was prepared on the day of the experiment was 4% bicinchoninic acid (sodium salt) in distilled water. A 4 % solution of aqueous copper sulfate was reagent C. The working reagent (WR) was prepared by mixing initially 4 volumes of reagent C with 100 volumes of reagent B and adding this to an equal volume of reagent A. Standards of protein or peptide (2-12 μg/mL) and samples in PBS were mixed with an equal volume of WR and incubated at 60°C for 1 h. Color development was determined at 562 nm after samples were cooled.

2.2.7. Covalent coupling of proteins to the liposomal surface and their characterization

2.2.7.1. Covalent coupling of proteins to the surface of SUV

The method used was described previously by Garcon et al (1986). SUV composed of 16 μmoles PC, equimolar CHOL and 0.75 μmoles APSA were prepared as above except that evaporation of lipids in chloroform was carried out at 37°C in order to prevent APSA coming out of solution. The vesicles were activated by the addition of

ice-cold $NaNO_2$ (0.16 mL, 1 M) followed with HCl/NaCl (0.16 mL, 1M). The mixture incubated in ice for 5 min and the SUV were rapidly separated from the reaction mixture by minicolumn centrifugation (Fry et al, 1978) on Sephadex G-25 gels: plastic syringes (5 mL) containing glass wool at the bottom were filled with Sephadex G-25 and centrifuged at 2500 rpm for 5 min after being placed in glass centrifuge tubes. Buffer was expelled from the gel leaving a semi-dry gel. Activated liposomes were applied dropwise on top of the gel and the syringes were spun at the same speed as before. Vesicles collected from centrifuge tubes were mixed with a protein solution (0.75 mg/mL in 0.05 M pH 10 borate buffer) also containing ^{125}I labelled protein as a tracer. The mixture was placed in an ice bath and slowly allowed to reach 20°C overnight. The surface linked protein was separated from the free protein by chromatography on Sepharose CL-4B that was equilibrated with PBS (0.1 M, pH 7.4 containing 0.9% NaCl). Fractions collected (1 mL each) were analyzed for ^{125}I radioactivity in a γ-counter. Percent surface linkage was calculated using the total radioactivity in the liposome peak and that of the free protein peak. To generate DRV as above, the pooled SUV fractions were freeze-dried either alone or, in cases where entrapment of another drug was required, after the addition of the drug (1 mL). The percent of covalently linked protein that was exposed on the surface was determined after treatment with protease.

2.2.7.2. Covalent coupling of proteins to the surface of DRV

In experiments where proteins were coupled directly onto preformed DRV, DRV activation was carried out (Snyder and Vannier, 1984) using 2 mL of 0.2 M $NaNO_2$ and 0.2 M NaCl/HCl each. Activated liposomes were separated from the solutions

used by centrifugation at 27,300xg for 20 min and the pellet obtained was suspended in protein solution in borate buffer. Separation of the linked materials was achieved by ultracentrifugation as above. Figure 2.3. shows the mechanism of binding of protein to the liposome surface.

2.2.7.3. Incubation with protease

In order to determine the amount of radiolabelled protein exposed on the surface of the vesicles prepared according to Section 2.2.7.1., liposomes (0.5 mL) were incubated with protease (1 mL, 4 U/mL; Type XIV from *Streptomyces griseus*, Sigma) at 37°C for 2 h (Gregoriadis et al, 1993). After centrifugation and washing (2 times) at 27,300xg for 20 min, wash solutions and the pellet were placed in γ-vials for the determination of radioactivity. The percent of surface-exposed protein was deduced using the total radioactivity in the wash solutions and the liposomal pellet.

2.2.8. Preparation of giant vesicles (GV)

Giant vesicles were prepared according to the procedure of Kim and Martin (1984) as modified by Antimisiaris et al (1993): A chloroform solution of lipids (9 μmol total lipids in 1 mL) were prepared using PC:CHOL:PG:TO in molar ratios of 4:4:2:1. Another organic solution in diethyl ether of the same lipids, molar ratios and volume was prepared. Two samples (0.5 mL each) of the ether solution were introduced into two glass vials (1.4 x 4.5 cm, with a screw cap lined with aluminium foil) containing 2.5 mL 0.2 M sucrose. The vials were vortexed for 15 s to form an oil-in-water (o/w) emulsion (mother liquid). Subsequently, 1 mL of 0.15 M sucrose was added in 3 equal

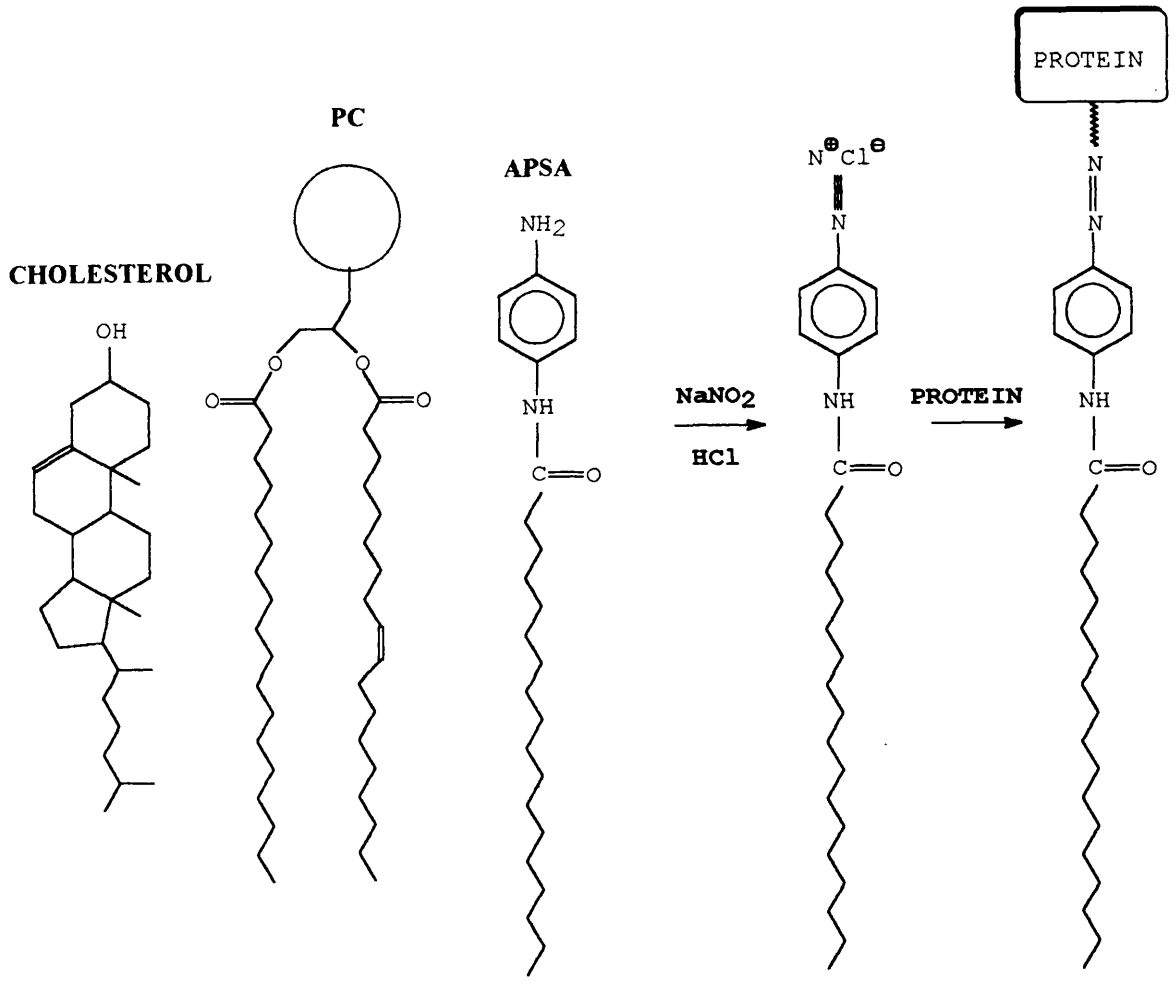


Figure 2.3. Scheme of covalent coupling of a protein to liposome surface

portions to the lipid mixture in chloroform (see above) with manual swirling between additions to prevent the formation of chloroform-in-water emulsion. The mixture was immediately vortexed for 45 s to form a water-in-oil (w/o) emulsion. One mL of this emulsion was added to the vials containing the mother liquid and the vials were immediately vortexed for another 10 s and the double emulsion was poured into a 250 mL conical flask. The flask was placed in a shaking-water bath (37°C) and a stream of nitrogen (1 L/min) was introduced 10 cm above the emulsion. The flask was shaken gently for one hour with occasional swirling to keep the chloroform droplets dispersed. At the end of the incubation period, there was a marked decrease in the turbidity of the mixture. To ensure the removal of residual organic solvents, the liposomes were placed briefly in a boiling water bath. The suspension was then mixed with an equal volume of glucose (5%), and then centrifuged at 1000xg for 20 min. The supernatant containing non-liposomal lipid debris were discarded and the giant vesicles in the pellet were resuspended in PBS (1 mL, 0.1 M, pH 7.4, containing 0.9% NaCl). The empty giant veicles prepared in this manner wc. .nixed with the substance to be encapsulated (1 mL) and freeze-dried overnight. The powder was rehydrated in a controlled fashion with distilled water (0.1 mL), followed by the addition of PBS (0.1 mL) 30 min later. Thirty min later, the volume of the liposome suspension was adjusted to 1 mL. The entrapped material was separated from the unentrapped material using two different methods depending on the properties of the material. For water-soluble molecules (eg. tetanus toxoid), separation was carried out by ultracentrifugation as above. For particulate materials (eg. spores), ultracentrifugation was inefficient since the free material also sedimented with the liposomes. In such cases, separation of entrapped spores was achieved by sucrose density gradient centrifugation.

2.2.8.1. Sucrose gradient fractionation

Ten one mL sucrose solutions (ranging between 2.84-0.284 M, with 0.284 M increments) were layered carefully in a 17 mL plastic ultracentrifuge tube and the liposome suspension was placed on the top sucrose layer. After centrifugation in a swing-out rotor at 90,000xg for 1.5 h, fractions (1 mL) were removed (starting from the top) and measured for I^{125} radioactivity. As expected (Antimisiaris et al, 1993), liposomes were recovered in the uppermost four fractions, whereas free spores were found in the bottom fraction.

2.2.9. Production and purification of anti-spore IgG from the rabbit

2.2.9.1. Immunization with spores

A male New Zeland rabbit (2 kg body weight) was injected subcutaneously three times with 1 mL killed *B.subtilis* spores (10^7) in PBS on days zero, 28 and 56. On day 70, blood (10 mL) was collected from the ear vein into heparinized tubes. A second rabbit used as a control was injected with PBS and bled 28 days later.

2.2.9.2. Purification of plasma IgG

Purification of IgG from the plasma of the control and the immunized rabbits was carried out using a two-step procedure (Duffy et al, 1989). Briefly, plasma (2 mL) was mixed with saturated ammonium sulphate (1 mL) in a dropwise fashion and stirred for 30 min at 20°C. The solution was centrifuged at 1000xg for 15 min and the precipitate was redissolved in 40% saturated ammonium sulphate (2 mL). This was stirred further

for 10 min and centrifuged as above. The precipitate was dissolved in 0.02 M sodium phosphate buffer (pH 7.2, 1 mL).

2.2.9.3. Ion-exchange chromatography of fractionated plasma

DEAE-cellulose ion-exchange material (10 g) was activated by washing the powder with 200 mL each of HCL (0.1 N) and NaOH (0.1 N), with extensive washing using distilled water after each treatment. The gel material was finally washed with 500 mL of potassium phosphate buffer (pH 7.2). A column (15 x 1.2 cm) was packed initially with 3 g (wet weigt) of DEAE-cellulose followed by Sephadex G-25. This gel filtration-ion exchange column was first washed with potassium-phosphate buffer (0.02 M, pH 7.2 containing 1 M KCl) followed by washing and equilibration with the same buffer devoid of KCl. The ammonium sulphate fractionated plasma from the control and immunized rabbits were then applied to these columns and fractions (3 mL) were collected. The fractions were analyzed for their protein content at 280 nm and those containing protein were pooled. IgG content of the pooled fractions was estimated (ε=14.3%; IgG concentration in rabbit blood was assumed as 12 mg/mL) to be 71.7 % and 88.8 % for the control and immunized IgG, respectively.

2.2.9.4. Interaction of *B. subtilis* spores with anti-spore-IgG

In order to show that liposomes can protect the antigens they entrap from binding to their relevant antibodies, a series of experiments were designed where IgG from the control and the immunized rabbits was incubated with free spores or with spores entrapped in liposomes (in the presence or absence of Triton X-100). Briefly, anti-

spore IgG (0.5 mL, 1.4 mg/mL) was mixed with FITC (0.5 mL, 2 mg/mL in 0.1 M sodium carbonate buffer, pH 9.0) overnight at 4°C. The FITC-labelled proteins were separated from the free FITC by size exclusion chromatography on Sephadex G-25. Live free spores (100 μL, $1x10^8$ cfu/mL) with or without Triton X-100 and spores entrapped in giant vesicles or DRV (both stained with oil-red-O; ca. $1x10^7$ spores per mL) were incubated with the control or immunized FITC-labeled IgG (10 μL) in the presence of BSA (1%, 100 μL) at 4°C overnight. The samples were then washed several times with PBS by centrifugation (10,000xg, 10 min) to separate the unbound IgG. The final pellets were suspended in fresh PBS (100-200 μL) and the samples were observed by fluorescence microscopy for the existence of fluorescence, an indication of spore antigen-antibody complex formation.

2.2.10. Immunization protocols

Male CD-1 mice (20-25 g) were used (in groups of four or five) in all immunization experiments. Immunization was carried out by intramuscular injection into the left hind leg using a 25 gauge needle and 0.1 mL volumes of samples on days zero and 29. Blood samples from the tail vein were collected on day 28 (primary response) and 10 days after the booster injection (secondary response). In some cases where long term antibody response was monitored, blood samples were taken three months after the booster injection. Blood samples (50μL) in heparinized capillaries were transferred into eppendorf tubes containing PBS (0.45 mL) which were then centrifuged at 1000xg for 10 min to obtain the plasma. All plasma were stored at -20°C untill further use.

2.2.11. Enzyme-linked immunosorbant assay (ELISA)

96 well flat-bottom ELISA plates were coated with the antigen (2 μg/mL tetanus toxoid or the peptide LCPH1; 60 μL/well) in 0.05 M sodium carbonate buffer of pH 9.6. The plates were incubated at 20°C for 1 h (or overnight at 4°C) and washed three times with 1/10 diluted washing buffer composed of 200 g NaCl, 36.25 g $Na_2HPO_4.2H_2O$, 5 g KH_2PO_4, 5 g KCl, 12.5 mL Tween-20 in 2.5 L total volume for a 10 x buffer. After the plates were dried, BSA (1% in washing buffer, 60 μL/well) was added to block the nonspecific binding sites and plasma (60 μL) diluted according to the strength of antibody response assessed in preliminary work were added to the top well of each column. Contents of the top wells were mixed and 60 μL samples were transferred to the second row of wells and mixed. This procedure was continued to the eighth row of wells and the final 60 μL samples from these wells were discarded. The plates were incubated at 20°C for 2-3 h (or overnight at 4°C) and after washing three times as above, plates were dried and peroxidase-conjugated goat anti mouse IgG (one of the subclasses) was added to the wells (50 μL/well; 1/2000 diluted in wash buffer containing 1% BSA and 5% FCS). The plates were incubated at 20°C for 2-3 h (or overnight at 4°C) followed by three times washing and drying. Substrate (200 μL/well) composed of 24.3 mL 0.1 M citric acid, 25.3 mL 0.2 M $Na_2HPO_4.2H_2O$, 50 mL distilled water, 40 mg o-phenylenediamine, 40 μL 30% hydrogen peroxide) was added to the wells and incubation was carried out at 20°C for 30 min. The reaction was stopped by the addition of H_2SO_4 (25 μL/well, 1.5 M). The colour developed was measured at 492 nm using a Titertek multiscan MCC/340 ELISA reader. The antibody

responses were expressed in terms of serial dilutions. The maximum dilution giving an OD value of about 0.2 was taken as the final dilution for a given plasma sample.

2.2.12. Determination of interleukin-2

The Seroscreen human IL-2 kit used is a solid phase sandwich ELISA. The wells of microtitre strips were precoated with an antibody specific for IL-2. Standards (200 μL) of known human IL-2 content provided by the manufacturer and also another set prepared in our laboratory using recombinant IL-2 were pipetted into the top wells in duplicate either alone or in combination with triton X-100 (10%); empty DRV or detergent lysed DRV as a control medium. The remaining wells were filled with 100 μL of standard diluent buffer (PBS containing 1% BSA and sodium azide as a preservative). 100 μL aliquot from the top wells were transferred to the second row of wells, mixed and serially diluted as in 2.2.11. Into these, a biotinylated monoclonal antibody to IL-2 (50 μL) in PBS containing 1% BSA was added and the plates were incubated at 37° for 2 h. This biotinylated second anti-IL-2 antibody specifically binds to a different site on the same IL-2 molecule. After removal of excess second antibody by washing with PBS-detergent buffer, 100 μL of a streptavidin-peroxidase conjugate was added. This binds to the biotinylated antibody to complete the four layer sandwich. A 45 min incubation at 20°C was followed by the washing and drying steps as in 2.2.11. Addition of 100 μL o-phenylenediamine substrate solution in citrate buffer resulted in color formation in 25 min. The reaction was stopped using 100 μL 0.5 M H_2SO_4 and the absorbance of each well was recorded at 492 nm having blanked the plate reader against a chromogen blank composed of 100 μL of each of substrate

buffer and stop solutions. The absorbance of standards were plotted against standard concentration on graph paper.

2.2.13. Bioassay of interleukin-15 and interleukin-12

For the bioassay of these two cytokines, two different cell types were used. IL-15 quantitation was carried out using the mouse T cell line (CTLL-2) and IL-12 bioassay was done with PHA-stimulated human peripheral blood mononuclear cells.

2.2.13.1. Maintenance of CTLL-2 cells

CTTL-2 cells (1 x 10^5 cells/mL, 10 mL) were maintained in RPMI 1640 medium supplemented with 5 % FCS and 30 U/mL IL-2 (complete medium). After 3 days of culturing at 37°C in a humidified chamber supplemented with 5 % CO_2, the cells were centrifuged in plastic sterile tubes at 250xg for 10 min and washed twice with the medium described above but devoid of IL-2 (incomplete medium) at the same speed in order to remove IL-2 from the medium.. A viable cell count was then carried out.

2.2.13.2. Trypan blue viable cell counting

After distributing the cells gently in a total volume of 5 mL incomplete medium, cell counting was done as follows: 100 µL of cells were mixed with an equal volume of trypan blue (0.2 % in PBS) in an eppendorf tube and allowed to stand for 5 min. A small amount of this suspension was applied in a haemocytometer under a cover slip with capillary action and a one min waiting period was allowed for the cells to settle in the chamber. Cells (both stained and unstained) were counted in the four upper and bottom side squares and the central one. Numbers of cells/mL were calculated using the equation shown below:

$$\text{cells/mL} = \frac{\text{number of lymphocytes counted}}{\text{number of squares used in counting}} \times 25 \times 10^4 \times \text{dilution factor}$$

Percent cell viability was calculated by dividing the number of unstained cells with the number of total cells and then by multiplying by 100. The cell number in the original preparation was adjusted to 5 x 10^5/mL (CTLL-2 cells) and 1 x 10^6/mL (human T cell blasts).

2.2.13.3. Quantitation of interleukin-15

All liposome samples with entrapped or surface-linked cytokines were initially lysed with an equal volume of Triton X-100 (10 %) at 37°C for an hour, the temperature for vesicle disruption being reduced in order to avoid inactivation of the cytokines. These samples and corresponding non-entrapped IL-15 samples recovered from the wash solutions as well as an IL-15 standard were then diluted with incomplete medium to a concentration of 500 U/mL and sterilized by passage through 0.2 μm sterile filters. Since entrapment and linkage values for IL-15 were not known, values of 30 % (entrapment) and 20 % (linkage) were assumed for liposomal samples as starting points for the dilutions. The standards and samples (100 μL) were distributed into the top row of wells of 96 well tissue culture plates in triplicates and 50 μL of incomplete medium was distributed to all the remaining wells. Two-fold serial dilutions were then carried out as above. Finally a 50 μL of cell suspension was added to all plates and these were incubated for 48 h in a humidified CO_2 chamber at 37°C.

2.2.13.4. Preparation of PHA-activated human lymphoblasts

Twenty-five mL of blood from a healthy human donor were collected and immediately mixed with 100 μL heparin (10 U/mL). Seven mL aliquots of this was carefully layered on top of 6 mL of Ficoll-Hypaque columns which were then centrifuged at 300xg for 30 min in a swinging bucket rotor at 20°C. The differential migration during centrifugation results in the formation of two cell bands and a red blood cell pellet as shown in Figure 2.4.

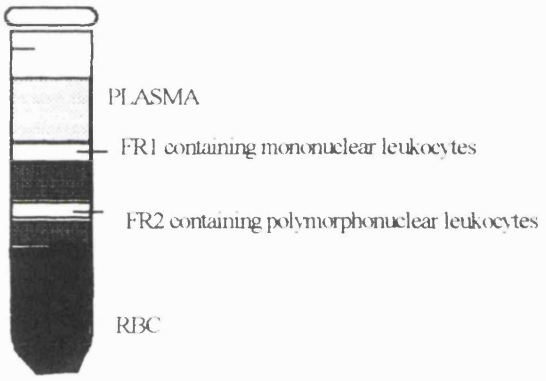


Figure 2.4. Appearance of blood after centrifugation on Ficoll-Hypaque

Fraction 1 containing the mononuclear leukocytes was aspirated into a clean tube and the cells were washed three times in RPMI 1640 at 250xg for 10 min. The collected peripheral blood cells were then suspended in 5 mL RPMI 1640 medium and a viable count was carried out as described above. After adjustment of the cell number to 1 x 10^6/mL in RPMI 1640 supplemented with 10 % FCS, 0.1 mL PHA was added (9 μg/mL) and the cells were cultured for 72 h in a CO_2 incubator. At the end of this incubation period, the lymphoblasts

generated were pelleted and washed three times with fresh medium at 250xg for 10 min. The cell density was adjusted to 1 x 10^6/mL for plate assay as before.

2.2.13.5. Quantitation of interleukin-12

Samples and IL-12 standard were prepared as described for IL-15 except that the IL-12 standard was adjusted to 40 U/mL. The plate assay was carried out as with the IL-15 samples for a period of 48 h.

2.2.13.6. Assessment of cell viability by the tetrazolium dye assay

The growth of cells were quantitated by following the formation of formazan dye (Mossman et al, 1987) from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as follows: MTT (5 mg/mL in PBS, 20 μL/well) was added to the cells at the end of their 48 h of incubation and the plates were incubated for another 4 h. The basis of this method is the cleavage of the tetrazolium ring of the soluble dye MTT by the mitochondrial dehydrogenases of cells into the purple insoluble formazan dye. The dye crystals generated at the end of incubation were solubilized by the addition of 100 μL of isopropanol (acidified in 0.04 N HCl) to each well. The HCl converts the phenol red of the medium to a yellow color which does not interfere with the quantitation of the color development at the wavelength used. Solubilisation was ensured by shaking the plates for 15 min. Color development was detected at 540 nm using an ELISA reader. Each plate contained a blank with the medium only. Negative controls were prepared using cells in the absence of stimulant.

2.2.14. Preparation of solutions

Phosphate buffered saline (PBS): 9 g NaCl and 6.8 g KH_2PO_4 were dissolved in approximately 700 mL distilled water and the pH was adjusted to 7.4 with 5 M NaOH. The volume was made upto 1 L with distilled water.

ELISA coating buffer: 0.318 g Na_2CO_3 and 0.586 g $NaHCO_3$ were dissolved in distilled water and the volume was made upto 200 mL after adjusting the pH to 9.6 with 1 M NaOH.

ELISA wash buffer (10 x): 200 g NaCl, 36.5 g $Na_2HPO_4.2H_2O$, 5 g KH_2PO_4, 5 g KCl and 12.5 mL Tween 20 were dissolved in distilled water, the pH was adjusted to 7.2 using 7 M NaOH and the volume made upto 2.5 L with distilled water. The solution was diluted with dH_2O 10 times before use.

Horse radish peroxidase-anti-immunoglobulin conjugate: The peroxidase-antibody conjugate was diluted 1/2000 x in wash buffer supplemented with 1 % BSA and 5 % FCS.

Enzyme substrate solution: 40 mg o-phenylenediamine was dissolved in 100 mL 0.18 M sodium phosphate-sodium citrate buffer, pH 5 containing 40 µL 30 % H_2O_2. This solution was prepared immediately before its use.

CHAPTER 3

IMMUNE RESPONSES TO FREE, LIPOSOMAL TETANUS AND MANNOSYLATED TETANUS TOXOID

3.1. INTRODUCTION

Since the first demonstration of the immunological action of liposomes (Allison and Gregoriadis, 1974), these had been used extensively with a wide variety of viral, protozoan and bacterial antigens to enhance humoral and cell mediated immunity (reviewed by Gregoriadis, 1990). Adjuvants are thought to act by a combination of mechanisms (Gupta et al, 1993) such as, formation of an antigen depot at the site of injection; presentation of antigens to immunocompetent cells and production of different lymphokines. Liposomes appear to fulfil the first two requirements since they form a depot for the antigen and they are also known (Gregoriadis, 1990) to be naturally targeted to macrophages which serve as the predominant antigen presenting cell for the processing and presentation of liposomal antigens (Buiting, 1992; Szoka, 1992; Alving, 1991). Although macrophages are the principal antigen-presenting cell for liposome-encapsulated antigens, it was proposed that antigens linked to the surface of liposomes might favour a more direct interaction with a specific membrane receptor on B-cells, thus using these cells for antigen presentation besides the normal macrophage processing route (Shahum and Therien, 1988; Therien et al, 1990; Shahum and Therien, 1994). In order to augment the natural targeting of liposomes to macrophages, Garcon et al (1988) covalently coupled a mannosylated ligand to the surface of antigen-containing liposomes and observed an improvement in the

immunoadjuvant action of such liposomes. The idea behind this approach was to target the mannose part of the carrier to its corresponding receptor found on macrophages (Gregoriadis, 1992).

Taking into account information given above, we have investigated immune responses to free, entrapped and surface-linked tetanus toxoid and established a dose-response curve with the antigen. As an alternative to the ligand-mediated targeting approach undertaken by Garcon et al (1988) where presence of the mannosylated protein besides the antigen would also result in antibody production against the protein, we have mannosylated the antigen (tetanus toxoid) and thus eliminated the necessity to use a foreign protein. The immune responses obtained against a low and a high mannose-containing toxoid in free, entrapped and surface-linked forms is compared to their mannose-free counterparts.

3.2. METHODS

Mannosylation of tetanus toxoid was carried out in two independent experiments as described in detail in Section 2.2.2. The sugar to protein molar ratio in the reaction mixture was calculated to be 3886 and 448 for experiments 1 and 2, respectively. The conjugate was separated from the unreacted free sugar by extensive dialysis in experiment 1 (the recommended separation method in the original paper due to its simplicity) and by gel-filtration in experiment 2 (so as to quantify the protein and bound or free sugar in the eluted fractions). Assay of the mannose and protein content of the conjugates was carried out according to Sections 2.2.2.1. and 2.2.2.2., respectively. In preliminary work, protein was assayed using the Lowry method but the

presence of sugar in the conjugate interfered with colour formations, observed as a green precipitate. Thus, the relatively sugar-insensitive Bradford assay was used in the assessment of the protein content thereafter.

Native or mannosylated toxoid (50 μg/mL PBS each) were mixed with empty SUV (Section 2.2.4.2.) in the presence of their radiolabelled tracers (10^4 cpm) and DRV generation was carried out as in Section 2.2.4.3. Covalent coupling of the toxoid (0.75 mg/mL in 0.05 M pH 10 borate buffer) to the surface of SUV is described in Section 2.2.7.1 and protease treatment (Section 2.2.7.3) was used to assess the amount of protein exposed on the surface of liposomes. The native or modified toxoid preparations in free or liposomal forms were then injected into animals. A dose of 0.1 and 1 μg/animal was used for each formulation in order to prepare a dose-response curve. Primary and secondary anti-toxoid responses were determined by the ELISA method.

3.3. RESULTS

3.3.1. Characterization of mannosylated toxoid

Table 3.1. presents both sugar and protein concentrations and their ratios in the initial reaction mixture as well as the sugar/protein ratios in the final product.

Kataoka and Tavassoli (1984) found 50 mol mannose/mol of protein (bovine serum albumin) when the sugar/protein ratio in the reaction mixture was 840, and a value of 20-27 when the ratio was 420. Although tetanus toxoid is much larger (150 kD) when

compared to bovine serum albumin (67 kD), our final sugar/protein molar ratio was similar to their findings. Because of the higher sugar to protein ratio in the conjugate obtained in experiment 1 (70.23) when compared to that of experiment 2 (27.32), the former will be referred to thereafter as mannosylated tetanus toxoid-high (MTT-high) and the latter as mannosylated tetanus toxoid-low (MTT-low).

Table 3.1. Sugar content of the neoglycoproteins

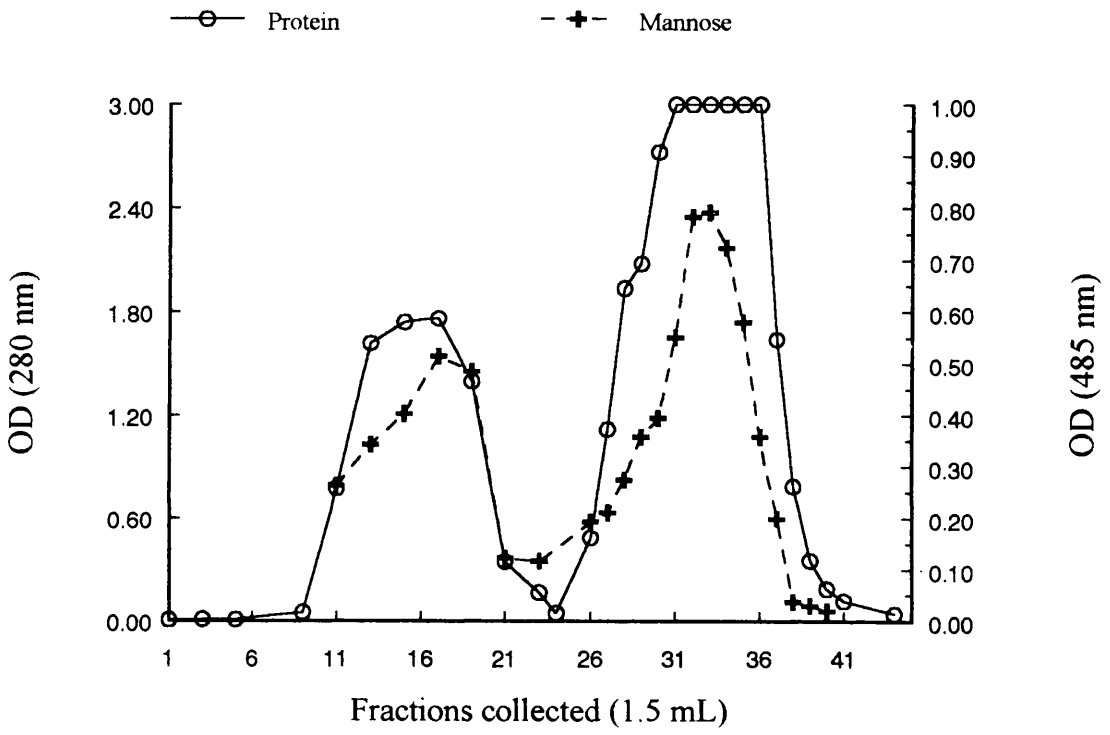
	Concentration of sugar in the reaction mixture[a] (mM)	Concentration of the toxoid in the reaction mixture (μM)	Sugar/protein molar ratio in the reaction mixture	Sugar/protein[b] ratio in the final product (mol/mol)
Experiment 1	9.25	2.380	3886	70.23
Experiment 2	0.92	2.055	448	27.32

[a] The final volume of the reaction mixture was 20 mL

[b] Mannose was determined by the phenol-sulphuric acid method and protein using the Bradford assay

Figure 3.1. shows the protein (OD 280 nm) and mannose (OD 485 nm) content of each fraction. The very high reading at 280 nm after the protein peak is due to the free sugar which, owing to its phenyl group, also absorbs at this wavelength. The overlap of the protein and the sugar peaks can be considered as direct evidence for the conjugation of mannose to the toxoid.

Figure 3.1. Elution profile of mannosylated tetanus toxoid



The mannosylated protein was applied on a Sephadex G-25 column (1 cm x 25 cm) equilibrated with 0.1 M, pH 7.4 PBS containing 0.9 % NaCl. Fractions collected (1.5 mL) were analyzed for protein content at 280 nm and for mannose using the phenol-sulphuric acid method (485 nm).

3.3.2. Toxoid (native and modified) content of liposomes

Values of toxoid (native and mannosylated) entrapment or coupling for each of the liposome formulations prepared, as well as the extent of coupled protein exposure on the vesicle surface are shown in Table 3.2.

Table 3.2. Entrapment, covalent coupling and surface exposure values for the native and mannosylated tetanus toxoid

Antigen	Entrapment (% of used)	Covalent coupling (% of used)	Surface exposure (% of coupled)
TT	30.77	14.48	34.23
MTT-low	28.93	16.35	32.85
MTT-high	33.42	18.22	34.71

Liposomes were composed of equimolar PC and cholesterol (for entrapment) and of PC, cholesterol and APSA (for covalent coupling) in molar ratios of 1:1:0.05.

The entrapment or coupling values for the three formulations of the antigen appear quite similar although a slight increase in surface linkage was observed when the amount of sugar was increased (from 14.48 to 18.22 %). It is conceivable that the presence of mannose increases the hydrophilicity of the protein and thus might improve the linkage values. With all formulations, a considerable proportion of the coupled antigen (32.85-34.71 %) was exposed on the surface of the liposome. It has been suggested (Gregoriadis et al, 1993) that the appearance of over one third of the

amount of toxoid originally coupled to the surface of SUV, on the surface of multilamellar DRV could be explained by the preferential utilization of membrane fragments with coupled toxoid (formed on dehydration of SUV) towards the formation of the outer DRV bilayer during the process of rehydration.

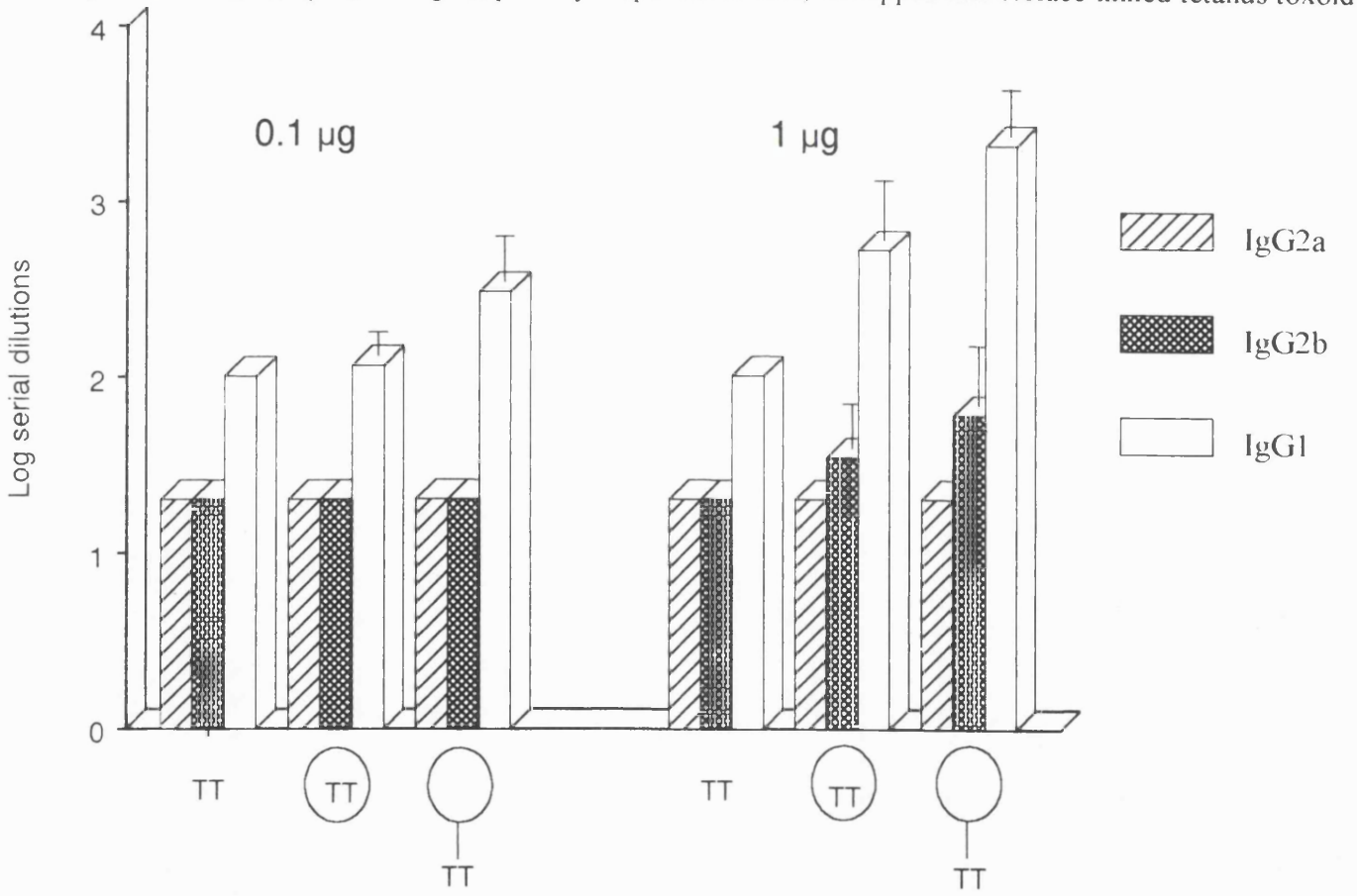
3.3.3. Immunization studies

Male CD-1 mice (in groups of four) were injected intramuscularly (0.1 mL) with free, entrapped or surface-linked antigen at two different doses (0.1 and 1 μg/mouse) and the anti-toxoid primary and secondary IgG_1, IgG_{2a} and IgG_{2b} titers were assessed by ELISA. The choice of an outbred mouse strain (CD-1) rather than the well-established inbred strains like Balb/c was based on the assumption that this would be a more accurate representation of the outbred human population.

3.3.3.1. Primary and secondary immune responses to free, entrapped and surface linked tetanus toxoid

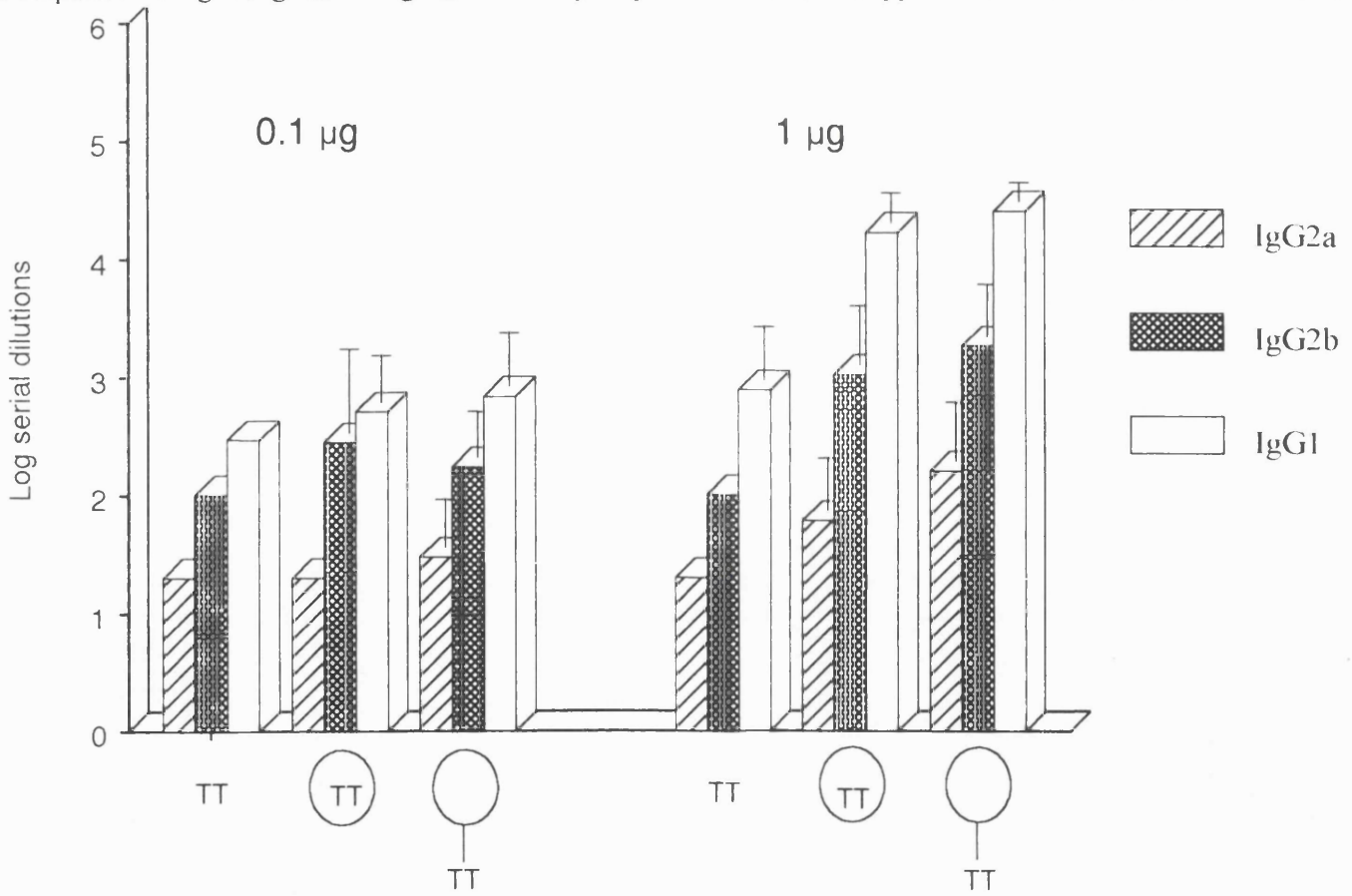
Figures 3.2. and 3.3. present the primary and secondary anti-toxoid IgG_1, IgG_{2a} and IgG_{2b} responses to 0.1 and 1 μg of free or liposomal tetanus toxoid. Injection of entrapped or surface-linked toxoid at a dose of 1 μg/mouse, boosted the primary and secondary IgG_1 responses to significantly higher levels than those obtained with the same dose of free antigen. Although there were no significant differences between the two liposomal formulations, augmentation of the IgG_1 responses with respect to free toxoid was more pronounced with the surface-linked antigen ($P<0.01$, primary; $P<0.02$, secondary) than the entrapped toxoid ($P<0.05$, primary and secondary responses). None of the groups differed significantly when the primary IgG_{2a} and

Figure 3.2. Comparison of IgG_1, IgG_{2a} and IgG_{2b} primary responses to free, entrapped and surface-linked tetanus toxoid



Tetanus toxoid (TT) was either entrapped (within circles) or covalently coupled (attached to circles) to the surface of liposomes. (For other details, see Section 3.2). Values are expressed as mean of logarithm serum dilutions ± S.D.

Figure 3.3. Comparison of IgG_1, IgG_{2a} and IgG_{2b} secondary responses to free, entrapped and surface-linked tetanus toxoid



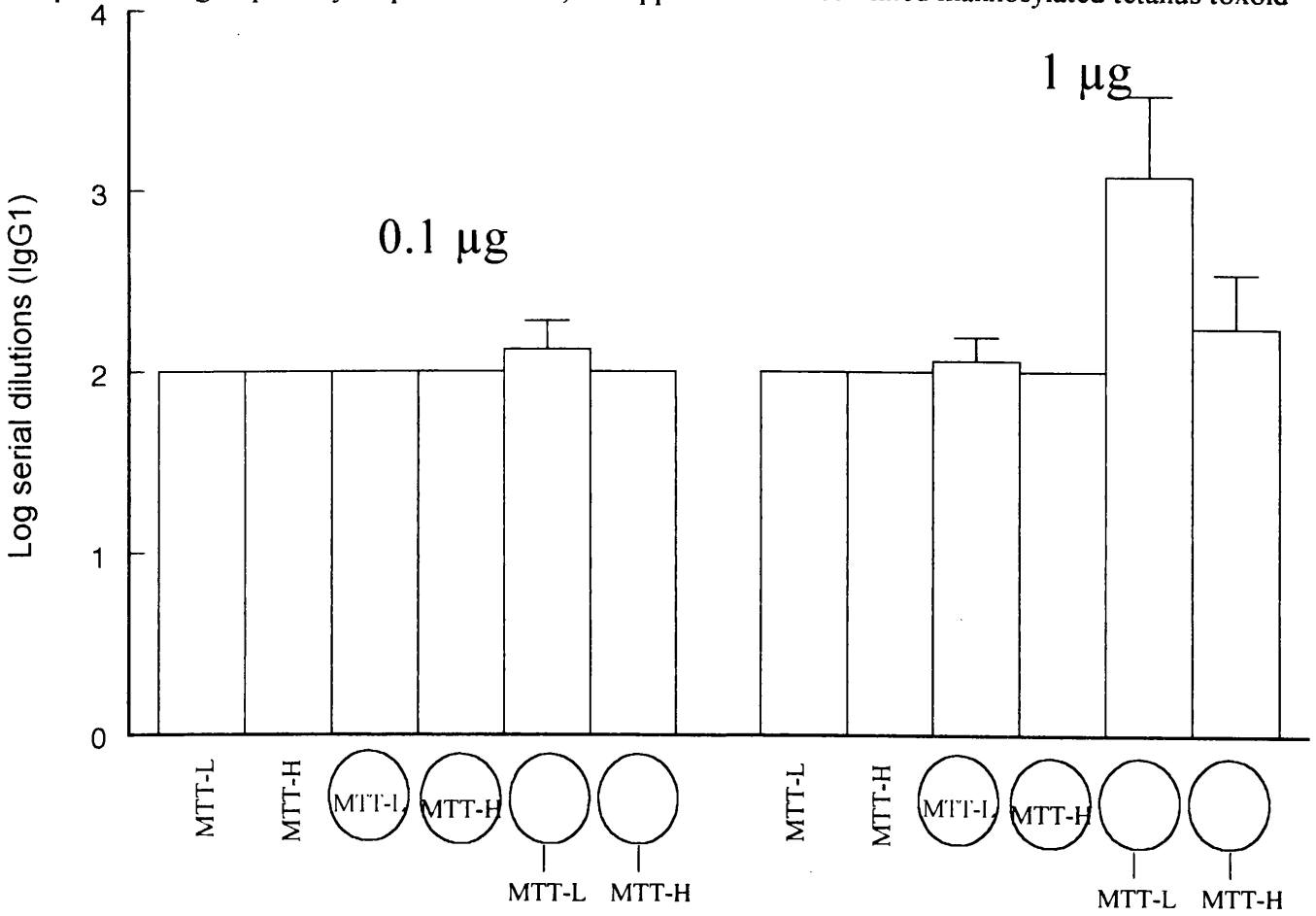
Tetanus toxoid (TT) was either entrapped (within circles) or covalently coupled (attached to circles) to the surface of liposomes. (For other details, see Section 3.2). Values are expressed as mean of logarithm serum dilutions ± S.D.

IgG_{2b}, and secondary IgG_{2a} responses were analyzed. On the other hand, surface-linked toxoid was found to be more potent in boosting the IgG_{2b} secondary responses ($P<0.02$) than the entrapped preparation ($P<0.05$) when compared to the free antigen. When the above experiment was repeated with a dose of 0.1 μg/mouse, the differences between the free and liposomal toxoid was abolished.

3.3.3.2. Primary and secondary immune responses to free, entrapped and surface linked mannosylated tetanus toxoid

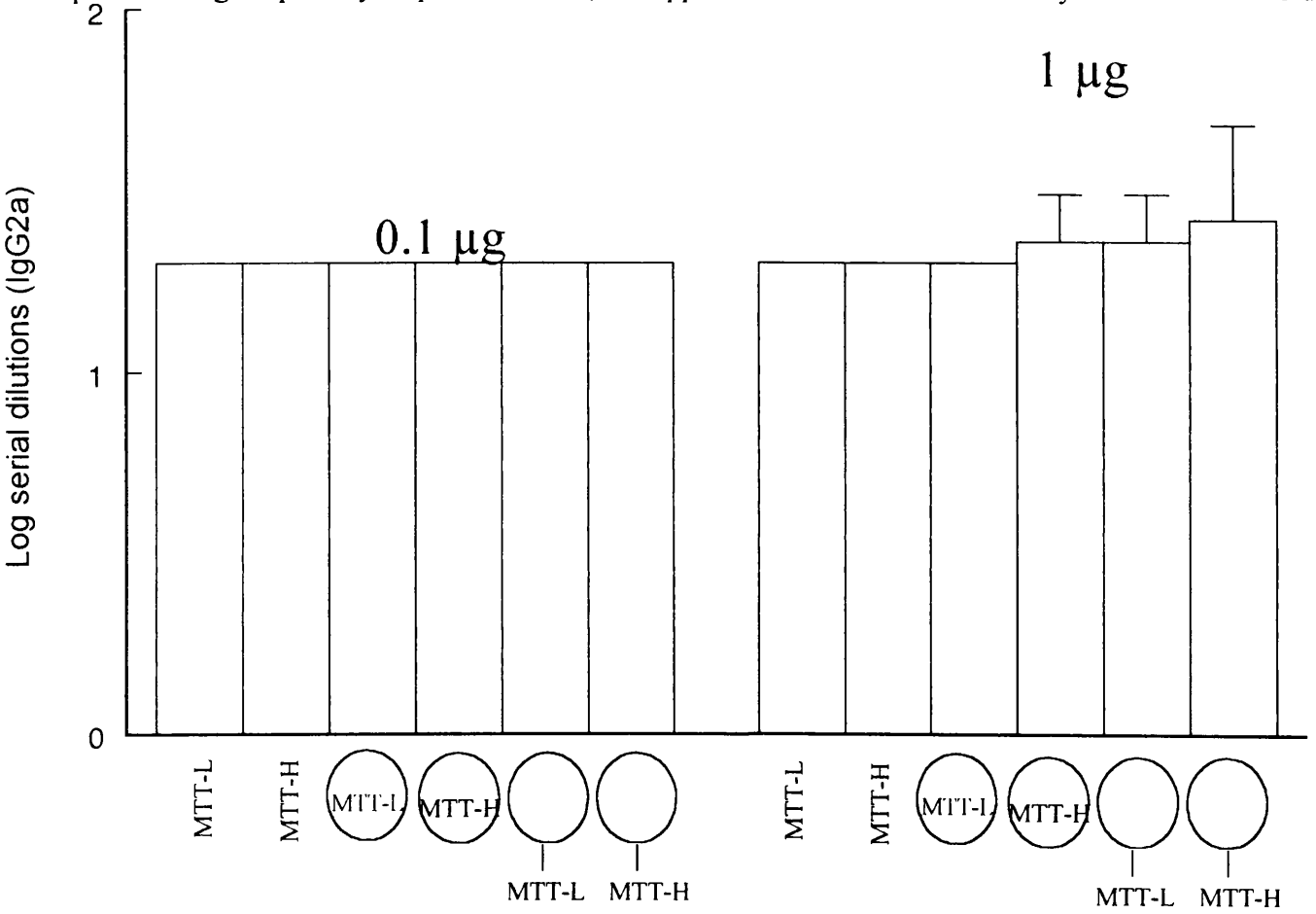
Figures 3.4 to 3.9 show the primary and secondary anti-toxoid IgG_1, IgG_{2a} and IgG_{2b} responses to 0.1 and 1 μg of free or liposomal mannosylated tetanus toxoid (MTT-low and MTT-high). Injection of entrapped (MTT-low or MTT-high) or covalently coupled (MTT-high) antigen at a dose of 1μg/mouse did not change the IgG_1 primary and secondary titers significantly when compared to the free antigens. However, MTT-low when linked to the vesicle surface boosted the IgG_1 primary and secondary titers significantly ($P<0.05$ and $P<0.001$, respectively). The same formulation was also superior to its entrapped counterpart ($P<0.05$). None of the groups differed significantly when the primary IgG_{2a} and IgG_{2b}, and secondary IgG_{2a} responses were analyzed. On the other hand, while only the surface-linked formulation augmented IgG_{2b} secondary titers with MTT-low ($P<0.05$), both of the liposomal MTT-high preparations were effective in doing so in comparison to their free counterparts ($P<0.05$ and $P<0.02$, respectively). None of the groups differed significantly when the same formulations were injected at a dose of 0.1 μg/mouse.

Figure 3.4. Comparison of IgG_1 primary responses to free, entrapped and surface-linked mannosylated tetanus toxoid



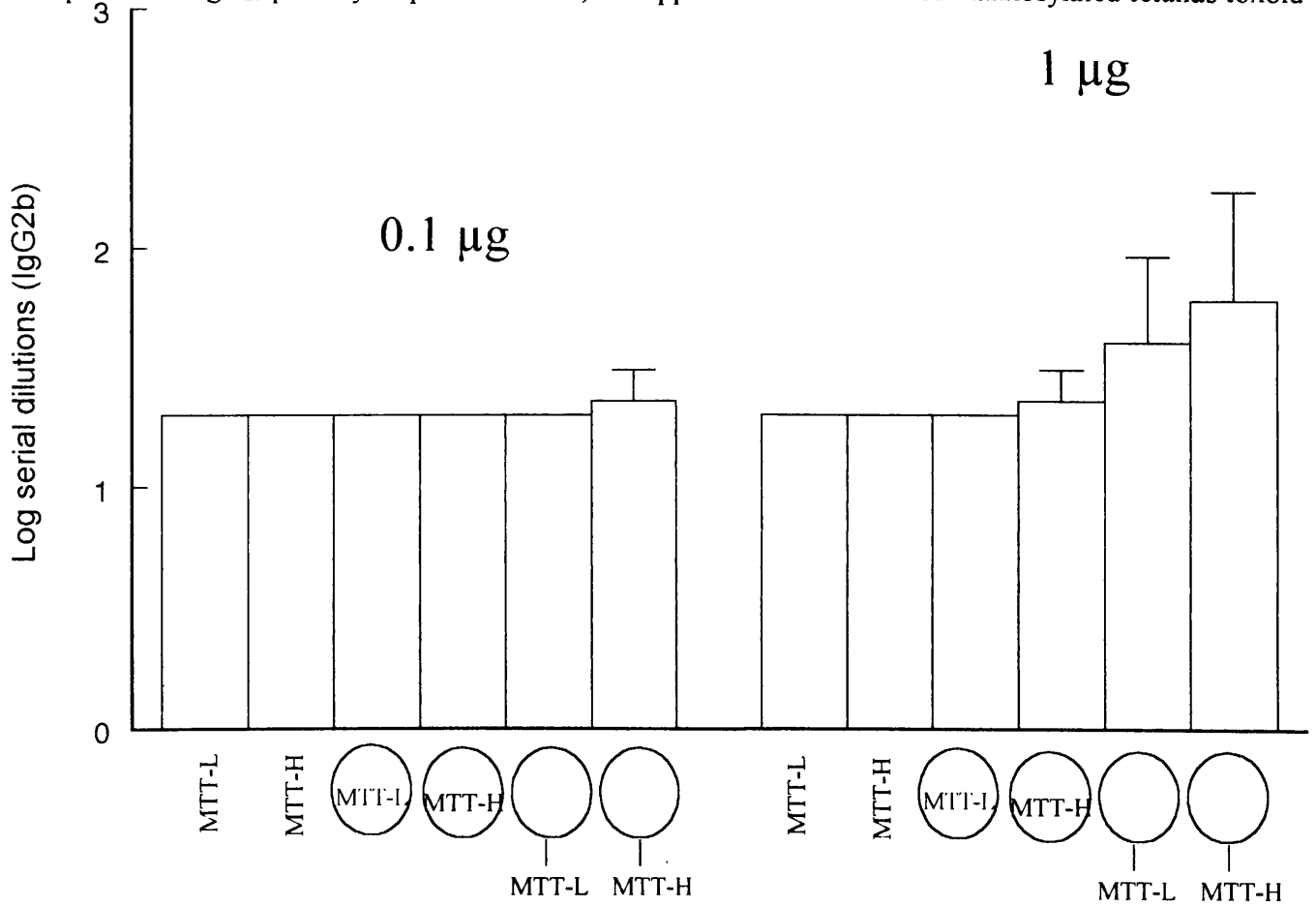
The modified proteins (MTT-low and MTT-high) were either entrapped (within circles) or covalently coupled (attached to circles) to the surface of liposomes. (For other details, see Section 3.2). Values are expressed as mean of logarithm serum dilutions ± S.D.

Figure 3.5. Comparison of IgG_{2a} primary responses to free, entrapped and surface-linked mannosylated tetanus toxoid



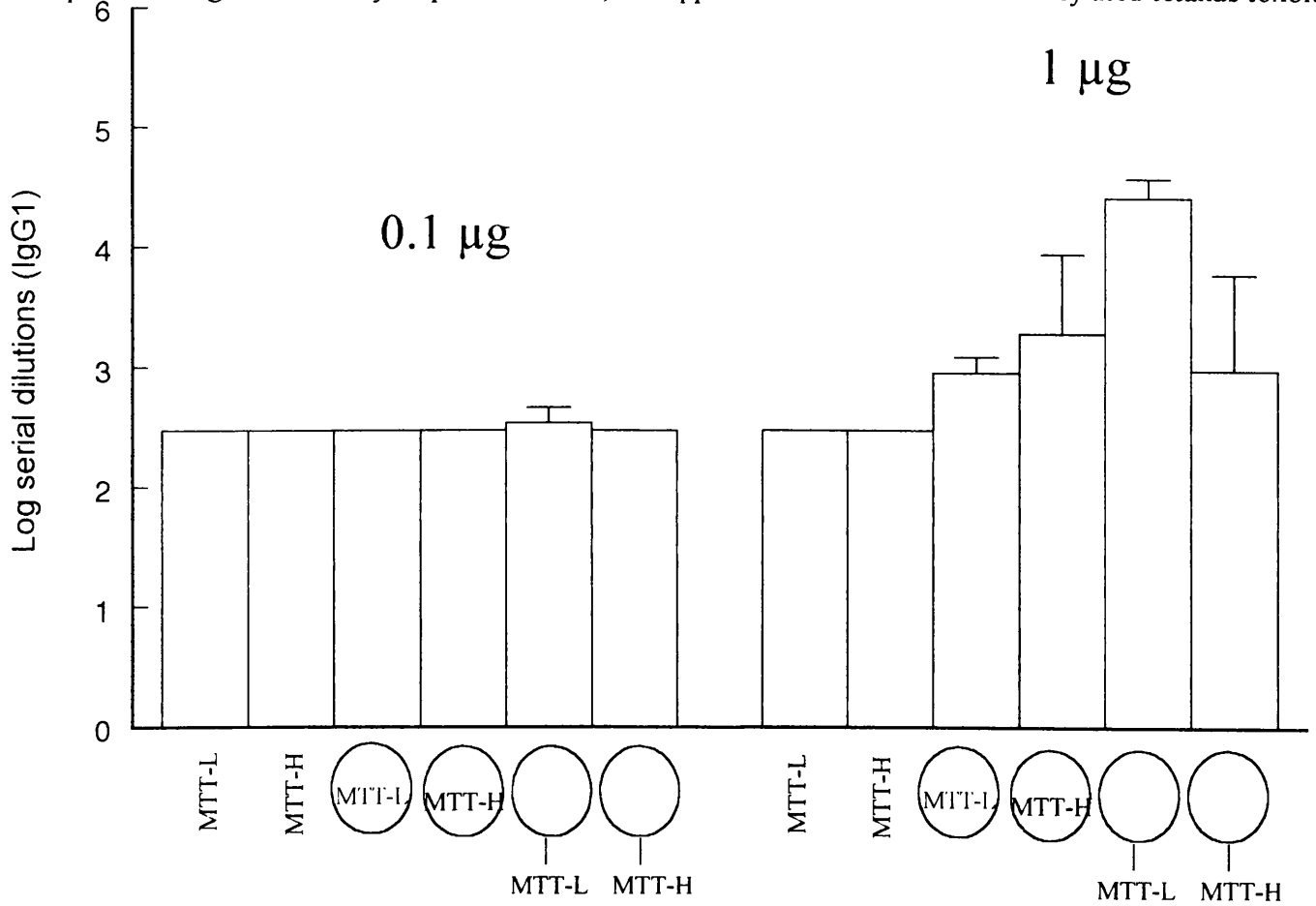
The modified proteins (MTT-low and MTT-high) were either entrapped (within circles) or covalently coupled (attached to circles) to the surface of liposomes. (For other details, see Section 3.2). Values are expressed as mean of logarithm serum dilutions ± S.D.

Figure 3.6. Comparison of IgG_{2b} primary responses to free, entrapped and surface-linked mannosylated tetanus toxoid



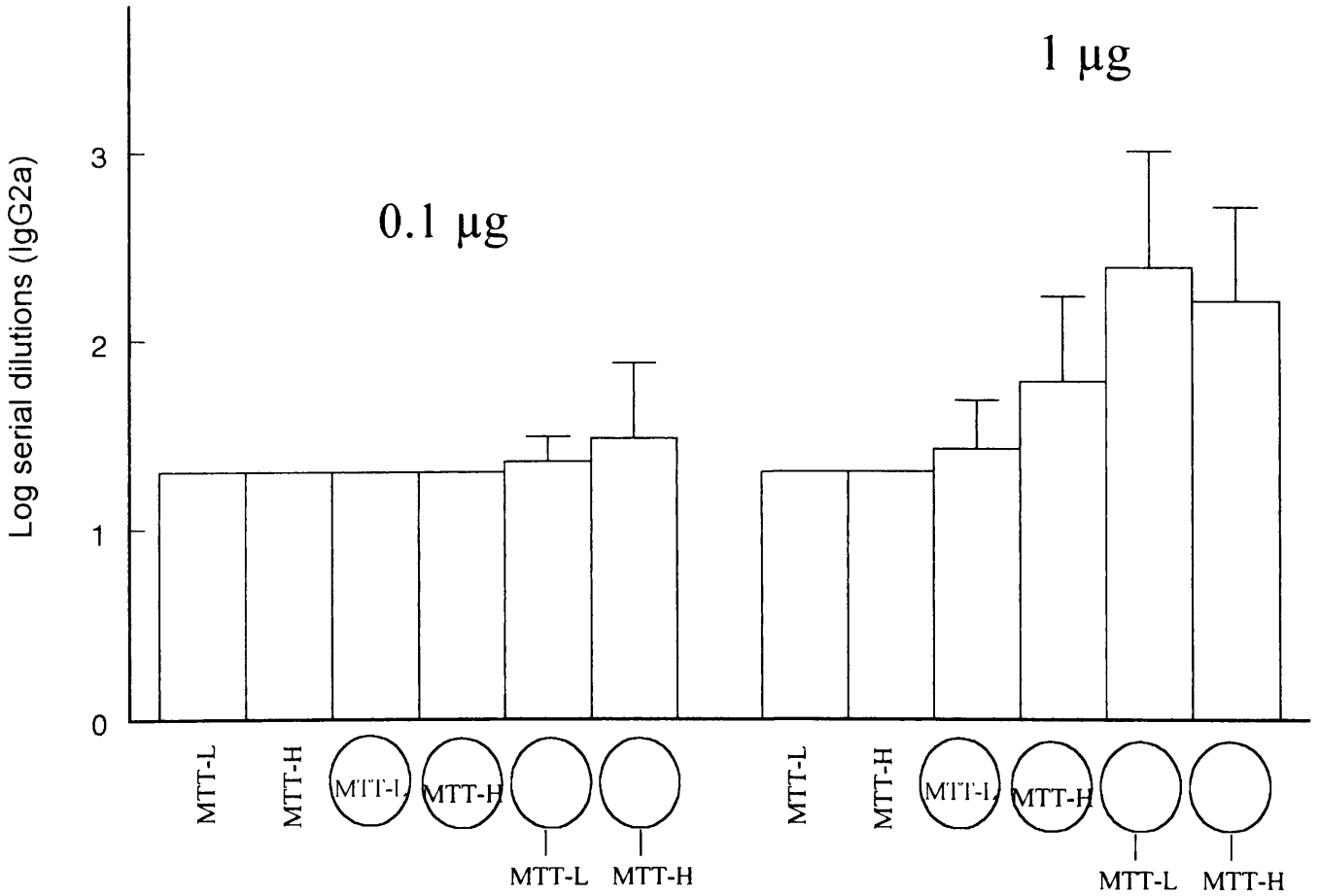
The modified proteins (MTT-low and MTT-high) were either entrapped (within circles) or covalently coupled (attached to circles) to the surface of liposomes. (For other details, see Section 3.2). Values are expressed as mean of logarithm serum dilutions ± S.D.

Figure 3.7. Comparison of IgG_1 secondary responses to free, entrapped and surface-linked mannosylated tetanus toxoid



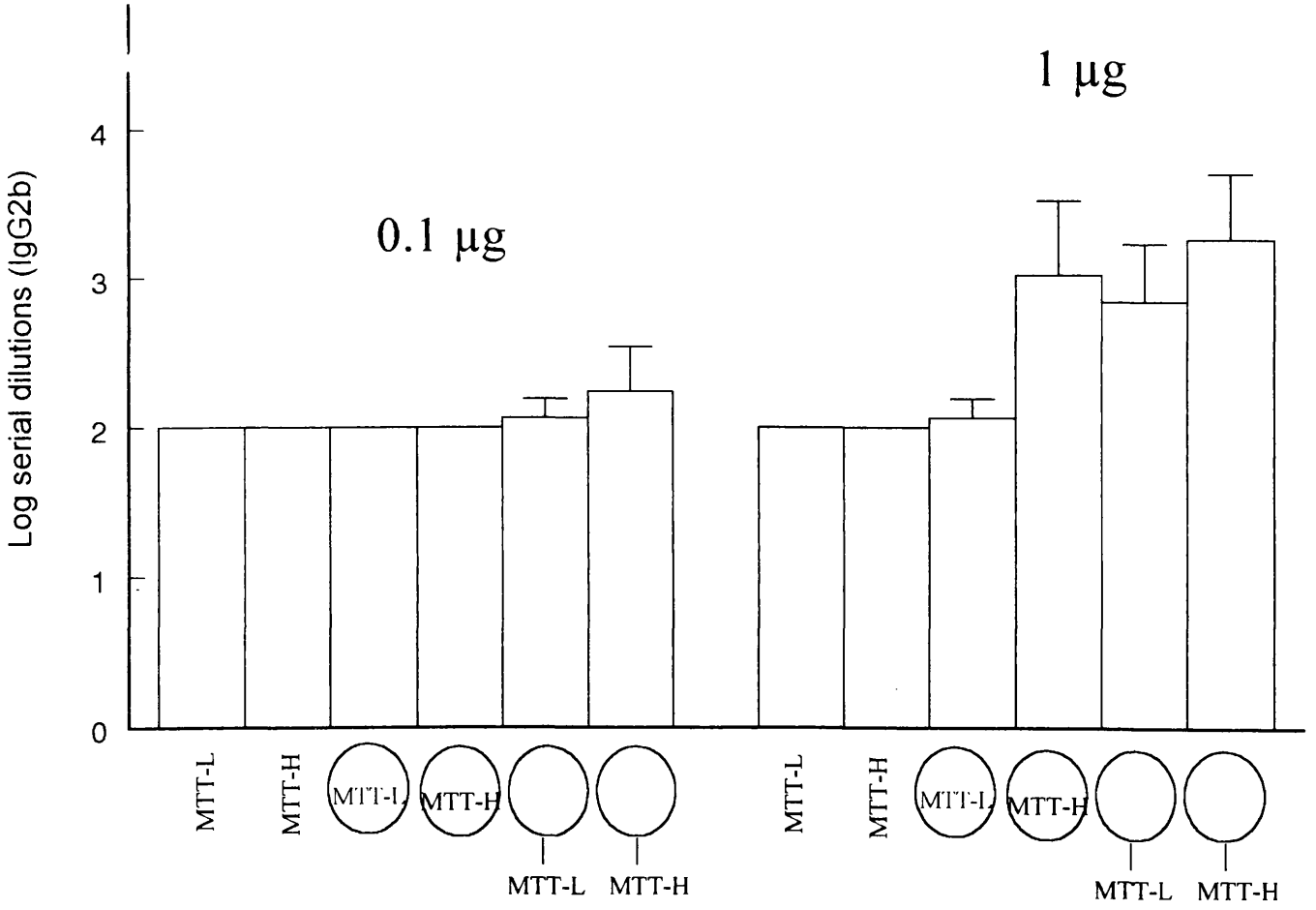
The modified proteins (MTT-low and MTT-high) were either entrapped (within circles) or covalently coupled (attached to circles) to the surface of liposomes. (For other details, see Section 3.2). Values are expressed as mean of logarithm serum dilutions ± S.D.

Figure 3.8. Comparison of IgG_{2a} secondary responses to free, entrapped and surface-linked mannosylated tetanus toxoid



The modified proteins (MTT-low and MTT-high) were either entrapped (within circles) or covalently coupled (attached to circles) to the surface of liposomes. (For other details, see Section 3.2). Values are expressed as mean of logarithm serum dilutions ± S.D.

Figure 3.9. Comparison of IgG_{2b} secondary responses to free, entrapped and surface-linked mannosylated tetanus toxoid



The modified proteins (MTT-low and MTT-high) were either entrapped (within circles) or covalently coupled (attached to circles) to the surface of liposomes. (For other details, see Section 3.2). Values are expressed as mean of logarithm serum dilutions ± S.D.

3.3.3.3. Comparison of immune responses to native and modified tetanus toxoid

Tables 3.3. and 3.4. summarize the comparisons among primary and secondary immune responses to the native and modified antigens. Contrary to our expectations, the modified toxoids were ineffective in improving the antibody titers. Native toxoid in the liposomal form was superior to the modified antigens in most of the subclasses tested. For example, covalently coupled toxoid was superior to its corresponding formulation of modified toxoids when primary ($P<0.01$ for MTT-low and $P<0.05$ for MTT-high) and secondary ($P<0.05$ for MTT-high) IgG_1 responses were analyzed. However, none of the MTT-high preparations differed significantly when compared to their native toxoid containing counterparts.

3.4. CONCLUSION

Results obtained with liposomal tetanus toxoid show that liposomes act as adjuvants for the entrapped or surface-linked antigen. The two liposomal formulations tested here did not differ significantly. There are two recent reports comparing the actions of entrapped and surface-linked antigens in liposomes. The work by Aramaki et al (1994) suggests that surface-linked antigen is superior to the entrapped antigen in terms of mucosal immunity after intranasal immunization. Shahum and Therien (1994) have shown that the encapsulated antigen induces a short-lasting response dominated by IgG_1 while an increased and long-lasting production of IgM, IgG_3, IgG_{2a} and IgG_1 was observed with covalently coupled antigen. The authors suggest that for an entrapped antigen, the processing can only be done by macrophages while for the surface-linked antigen, B cells might also contribute to the processing and presentation. Although in our study we also observed a greater response for the coupled antigen compared to

Table 3.3. Comparison of primary responses

	IgG subclass (IgG_1 in this case only)		
Preparation	Ent. TT	SL TT	SL MTT-low
TT	*	***	*
MTT-low	*	***	*
MTT-high	*	***	*
Ent. TT			
Ent. MTT-low			
Ent. MTT-high	*	***	*
SL TT		***	*
SL MTT-low			
SL MTT-high		*	

Tetanus toxoid (TT) or the mannosylated toxoids (MTT-low or MTT-high) were either entrapped (Ent.) or linked to the surface of liposomes (SL) composed of egg PC, cholesterol and APSA in molar ratios of 1:1:0.05. Only the formulations that improved the antibody titers are given in the Table. Since results from the other IgG subclasses were non-significant, only IgG_1 results are presented. Stars represent the significance level (*, $P<0.05$; **, $P<0.02$; ***, $P<0.01$).

Table 3.4. Comparison of secondary responses

Preparations	Ent. TT		Ent. MTT-high		SL TT		SL MTT-low		SL MTT-high
	IgG_1	IgG_{2b}	IgG_{2b}	IgG_1	IgG_{2b}	IgG_1	IgG_{2b}	IgG_{2b}
TT	*	*	*	*	**	*	*	**
MTT-low	***	*	*	****	**	****	*	**
MTT-high	***	*	*	****	**	****	*	**
Ent. TT								
Ent. MTT-low	*	*	*	*	*	*	*	*
Ent. MTT-high								
SL TT								
SL MTT-low								
SL MTT-high				*		*		

Tetanus toxoid (TT) or the mannosylated toxoids (MTT-low or MTT-high) were either entrapped (Ent.) or linked to the surface of liposomes (SL) composed of egg PC, cholesterol and APSA in molar ratios of 1:1:0.05. Only the formulations that improved the antibody titers are given in the Table. Stars represent the significance level (*, $P<0.05$; **, $P<0.02$; ***, $P<0.01$; ****,$P<0.001$).

that seen with the free antigen, difference between the two forms of liposomal toxoid was not significant. We are aware that the liposomes used in the studies cited above and ours were of different compositions and types besides the use of different antigens. However, we feel that another factor, namely the mode of surface-linkage might be of great importance. Figure 3.10 presents a schematic representation of a surface linked protein using APSA and the heterobifunctional cross-linking reagent SPDP. Clearly, SPDP provides a linker which is much longer than that obtained with APSA. This in turn affects the availability of the antigen on the vesicle surface. A longer anchor can facilitate the interaction with B cells as well as B-cell surface immunoglobulin cross-linking with the antigenic epitopes as suggested by Shahum and Therien (1994) which can influence immune activation. A comparison of different anchors in this respect might provide additional information on the discrepancies observed in the literature between entrapped and surface-linked antigens.

The inability of mannosylated toxoids to improve antibody titers in comparison to native toxoid might be due to a combination of different factors. For example, Murray et al (1991) demonstrated a lack of antibody response in patients with Gaucher's disease following intravenous administration of mannose-terminated glucocerebrosidase. They suggested that this might facilitate the uptake of the enzyme by the Kupffer cells in the liver and that any protein processed through these cells in the liver loses its antigenic potential. On the other hand, Funato et al (1994) showed that mannosylated liposomes were approximately 3.5 times more leaky to the drugs they entrapped than conventional liposomes in plasma and their blood clearance rates, accumulation in the liver were enhanced and was due to increased vesicle

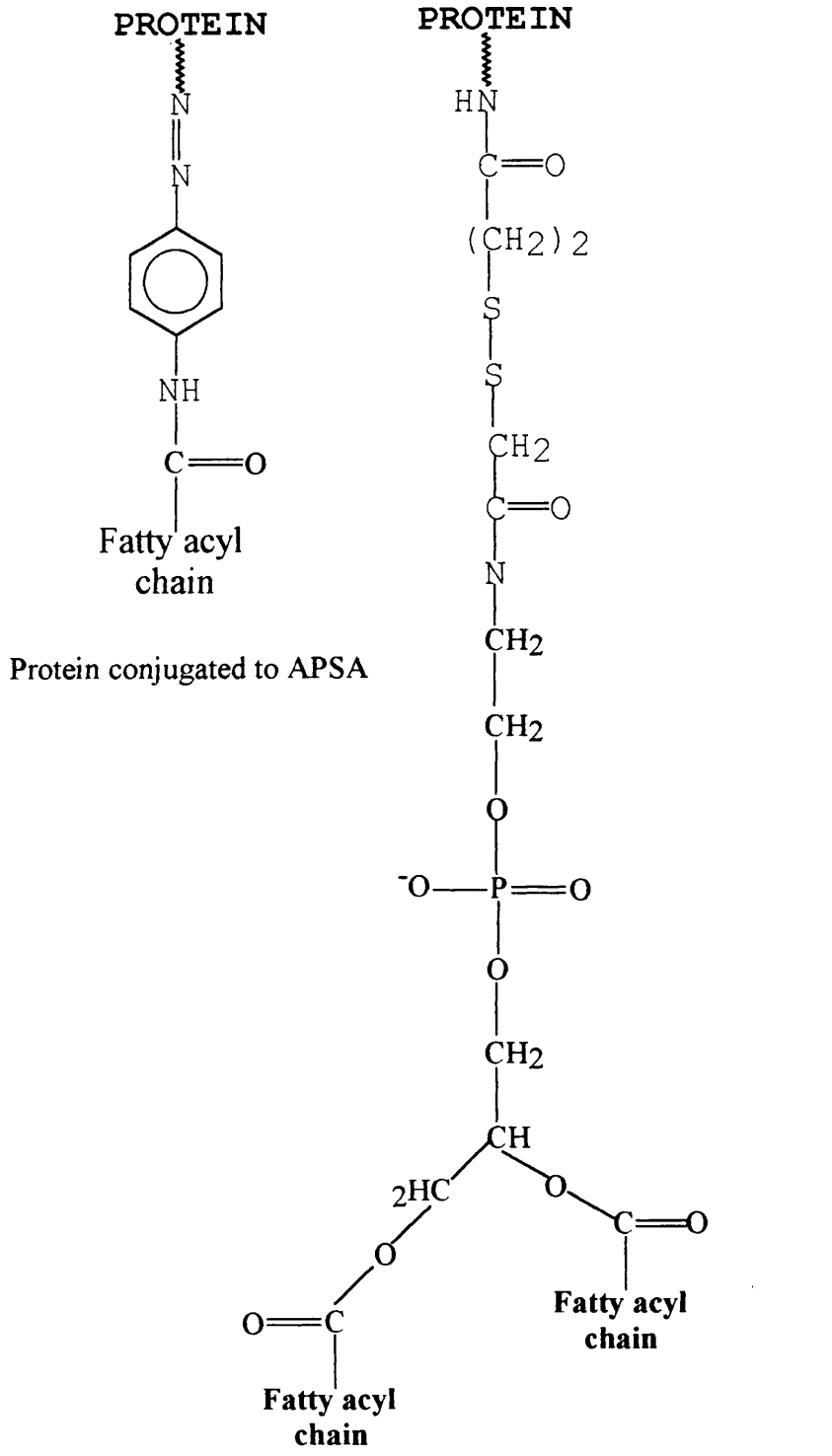


Figure 3.10. Comparison of the length of linkers employed in two different methods of covalent coupling of a protein to a lipid moiety

destabilization. Vesicle destabilization in plasma was shown to be complement mediated. Malhotra et al (1995) have recently discussed the role of serum mannose-binding protein (MBP). Upon binding to a terminal mannose residue, MBP mediates antibody-independent recognition of pathogens by phagocytic cells and this recognition is followed by either direct opsonization or complement fixation. In the light of all the information give above, the presence of mannose in liposomes used here is likely to increase their accumulation in the liver and also contribute to a more rapid vesicle destabilization, thus resulting in less favorable antibody responses than mannose lacking vesicles. Another factor which could be the most important of all is that the mannosylation of the toxoid may have altered its antigenic epitopes. The work by Abraham et al (1995) indeed shows that modification of diphteria toxoid (maleylation in order to target the protein to macrophage-specific scavenger receptor) can alter its B cell epitopes. According to the authors, maleylated toxoid did not generate a significant antibody response but led to a better T cell proliferative response in vitro. In order to decide whether this is the case with the mannosylated tetanus toxoid, T cell responses must also be analyzed in detail. However, our preliminary results indicate that this system is not favorable, at least in terms of B cell responses.

CHAPTER 4

COMPARISON OF GIANT VESICLES AND DRV AS POTENTIAL VACCINE CARRIERS FOR SOLUBLE AND PARTICULATE ANTIGENS

4.1. INTRODUCTION

Recently a mild procedure for the successful entrapment of model particulate antigens in giant vesicles (GV) was developed by Antimisiaris et al (1993). However, up to date, no immunization studies have been carried out with these vesicles.

Generally, vaccination of individuals with attenuated or avirulent bacteria or viruses may cause undesirable side effects. For example, vaccination with an avirulent non-capsulating *B.anthracis* strain in Russia proved to be safe and preventive but was not highly recommended due to the large number of spores introduced per individual in order to have protection against anthrax (Shlyakov and Rubinstein, 1994). Vaccination of infants with measles virus resulted in increased frequency of measles epidemics in vaccinated populations of developed countries. The protection rate of the vaccine in the infants is only 50-70 % due to the presence of maternal antibodies, which provide protection against measles in early life but interfere with the immune response following vaccination up to twelve month after birth (Obeid and Steward, 1994). It was suggested that the presence of maternal antibodies at the time of vaccination may reduce the immune response due to neutralization and elimination of the virus or bacteria.

Here, we have investigated the immune responses to a particulate (*B.subtilis* spores) and a soluble antigen (tetanus toxoid) alone or together in giant vesicles or in DRV. The ability of these liposomes to protect the particulate antigen from antibodies was also assessed by fluorescence microscopy.

4.4. METHODS

DRV and GV were prepared as described in Sections 2.2.4.3 and 2.2.8, respectively. The amounts of toxoid or spores mixed with the empty vesicles before freeze-drying are presented in Table 4.1. The unentrapped material was separated from the liposomal fraction using ultracentrifugation for the toxoid (Section 2.2.4.3.) or sucrose gradient fractionation for *B.subtilis* spores (Section 2.2.8.1). When both of these antigens were co-entrapped in the same vesicles, free molecules were first separated by ultracentrifugation followed by sucrose gradient fractionation. Percent entrapment calculations were based on ^{125}I labelled tracers of the toxoid and the spore. In the case of co-entrapment, two identical liposome formulations were prepared, one containing the radiolabelled toxoid together with unlabelled spores and the other containing labelled spores and unlabelled toxoid. Vesicles were then diluted accordingly in PBS so that mice (in groups of 5) received a dose of 1 μg in a total volume of 0.1 μL (Section 2.2.10.). Anti-toxoid antibody titers were assessed by ELISA as in Section 2.2.11. Anti-spore titers were assessed using the same technique with the following modifications (Kaspar and Hartman, 1987): The plates were coated with spores (1 x 10^8 spores/mL in PBS; 100 μL/well) by drying at 37°C overnight and washed three times. The remaining steps were identical to those for the toxoid except that the peroxidase conjugated mouse anti-IgG was diluted 1/1000 times.

To test whether liposomes (DRV or GV) could protect the spores against antibodies, a series of experiments were designed where a rabbit was immunized with spores (Section 2.2.9.1) and IgG was purified from the plasma of the same animal and an unimmunized rabbit as described in Sections 2.2.9.2 and 2.2.9.3. Purified control and immune IgGs were labelled with FITC and then incubated with spore-containing intact or Triton X-100-treated vesicles (Section 2.2.9.4). The existence of an antigen-antibody complex formation was then checked by fluorescence microscopy.

4.3. RESULTS

4.3.1. Toxoid and spore content of liposomes

Table 4.1. presents the amounts of spores or toxoid used in the preparation of various formulations injected to animals and their entrapment values. The entrapment of toxoid in GV alone was calculated to be 11.4 %, but it was doubled to 22.76 % when spores were co-entrapped together with the toxoid. This increase in the entrapment might be due to a retardation of release of the protein from the vesicles since the spores have a higher affinity towards the inner membrane surface due to their hydrophobic nature. Entrapment of toxoid in DRV was between 21.73-22.4 % with no significant change observed when spores were also co-entrapped. Spore entrapment for both GV and DRV liposomes was quite high with values of 52.8 (spores) and 33.1 % (co-entrapped) and 61.8 (spores) and 58.18 % (coentrapped), respectively.

Table 4.1. Preparation conditions and entrapment values of DRV and GV formulations used in immunization studies

Preparations	Amount of TT mixed with SUV or GV (μg)	Toxoid entrapment (% of used)	Number of spores mixed with SUV or GV (cfu/mL)	Spore entrapment (% of used)	Number of spores in the final preparation
spores+TT	-	-	-	-	$3.0x10^7$
TT	-	-	-	-	-
spores+GV(TT)	158	11.40	-	-	$3.0x10^7$
GV(TT)	158	11.40	-	-	-
SepEnt.GV	158	11.40	$1.0x10^8$	52.80	$2.9x10^7$
CoEnt.GV	150	22.76	$1.0x10^8$	33.11	$9.7x10^6$
DRV(TT)	50	24.29	-	-	-
spores+DRV(TT)	70	21.97	-	-	$2.5x10^7$
SepEnt.DRV	100	21.73	$1.0x10^8$	61.80	$1.0x10^7$
CoEnt.DRV	50	22.40	$1.0x10^8$	58.18	$5.2x10^7$

Empty SUV composed of equimolar PC and cholesterol or empty GV composed of PC:chol:PG:TO (4:4:2:1) were mixed with toxoid alone, spores alone or a mixture of these at various concentrations together with their radiolabelled tracers and were freeze-dried. Percent entrapment was based on ^{125}I radioactivity measurements. The DRV and GV were finally diluted with PBS so that each animal received a dose of 1 μg toxoid (in a total volume of 0.1 mL).

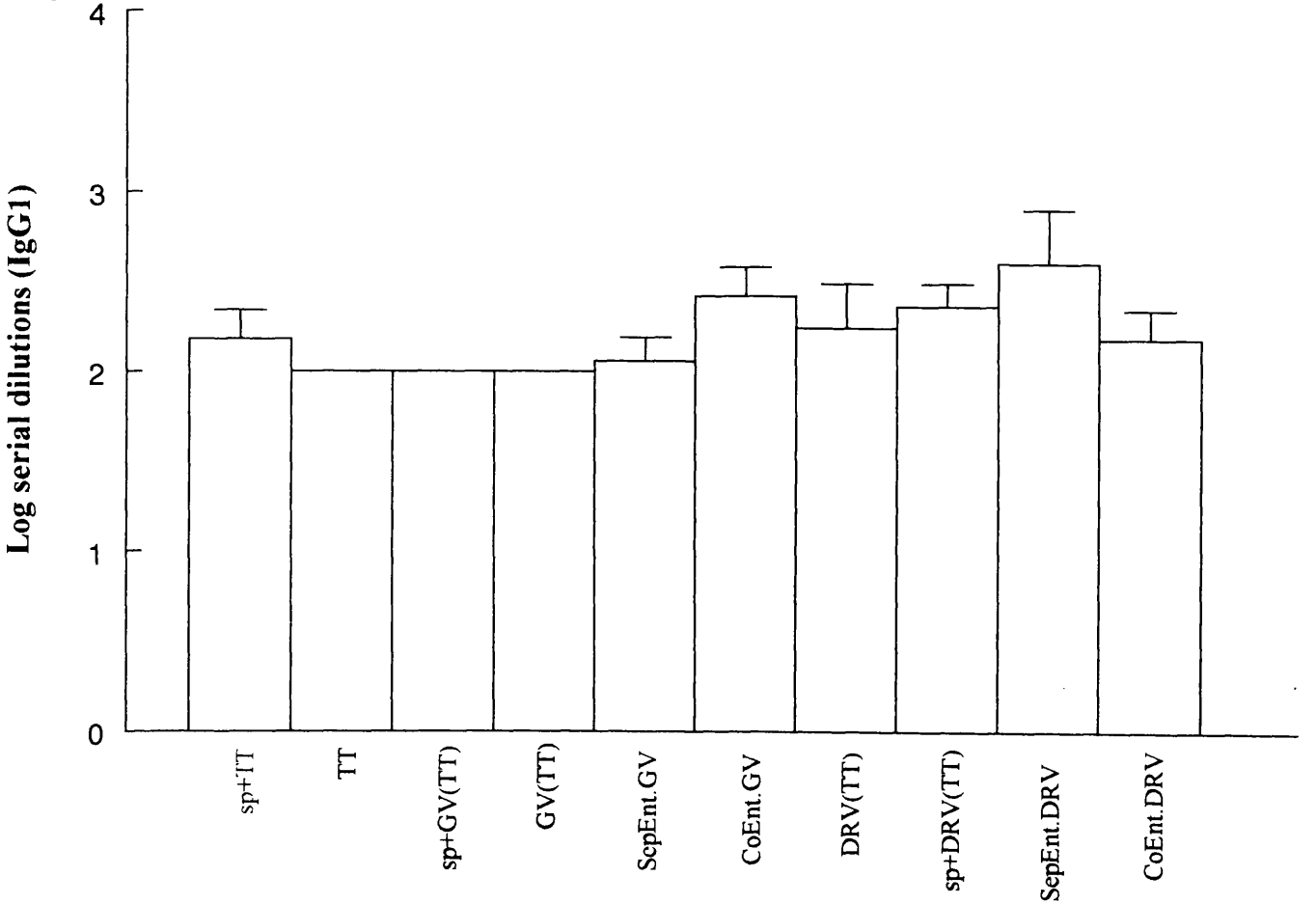
4.3.2. Immunization studies

4.3.2.1. Primary and secondary responses to tetanus toxoid entrapped in GV or DRV

Figures 4.1 to 4.6 present the IgG_1, IgG_{2a} and IgG_{2b} primary and secondary responses to tetanus toxoid entrapped in various GV or DRV formulations, in the presence or absence of *B.subtilis* spores. Spore-containing GV (co-entrapped with TT) and DRV (the separately entrapped and free spore-entrapped TT combination) boosted the IgG_1 primary responses significantly. GV (with co-entrapped spores and TT) and DRV entrapping toxoid alone were both effective in increasing the primary IgG_{2a} anti-toxoid titers when compared to free toxoid or toxoid entrapped in DRV separately from spores. The toxoid-spore mixture (sp+TT) and toxoid entrapped in GV alone elevated the primary IgG_{2b} levels when compared to free or separately entrapped (in GV) toxoid (for a detailed comparison of primary responses see Table 4.2).

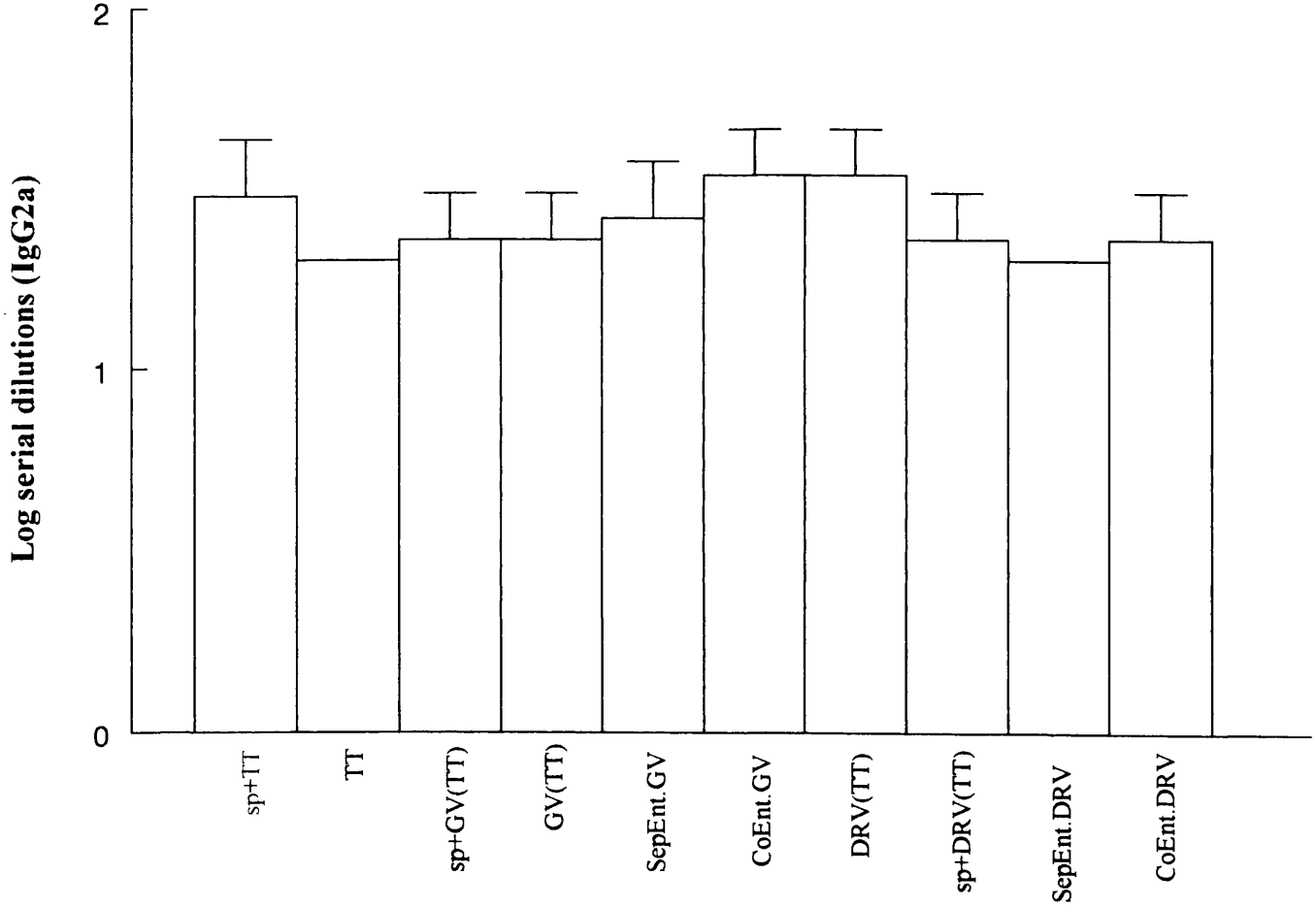
All DRV preparations were superior to free toxoid, toxoid entrapped in GV alone or co-injected together with free spores in terms of IgG_1 secondary responses. However the same preparations (except for the free spores+entrapped toxoid combination) were not significantly different than the free toxoid-spores mixture. This might be an indication that spores act as a weak adjuvant. The co-entrapped GV formulation was as effective as the DRV formulation for this subclass. The same preparation was the only one found to boost IgG_{2a} secondary titers significantly when compared to all other preparations except for the separately entrapped DRV formulation. Interestingly, all GV formulations were ineffective in increasing the secondary IgG_{2b} responses while all

Figure 4.1. Comparison of IgG_1 primary responses to tetanus toxoid entrapped in DRV or GV with or without *B.subtilis* spores



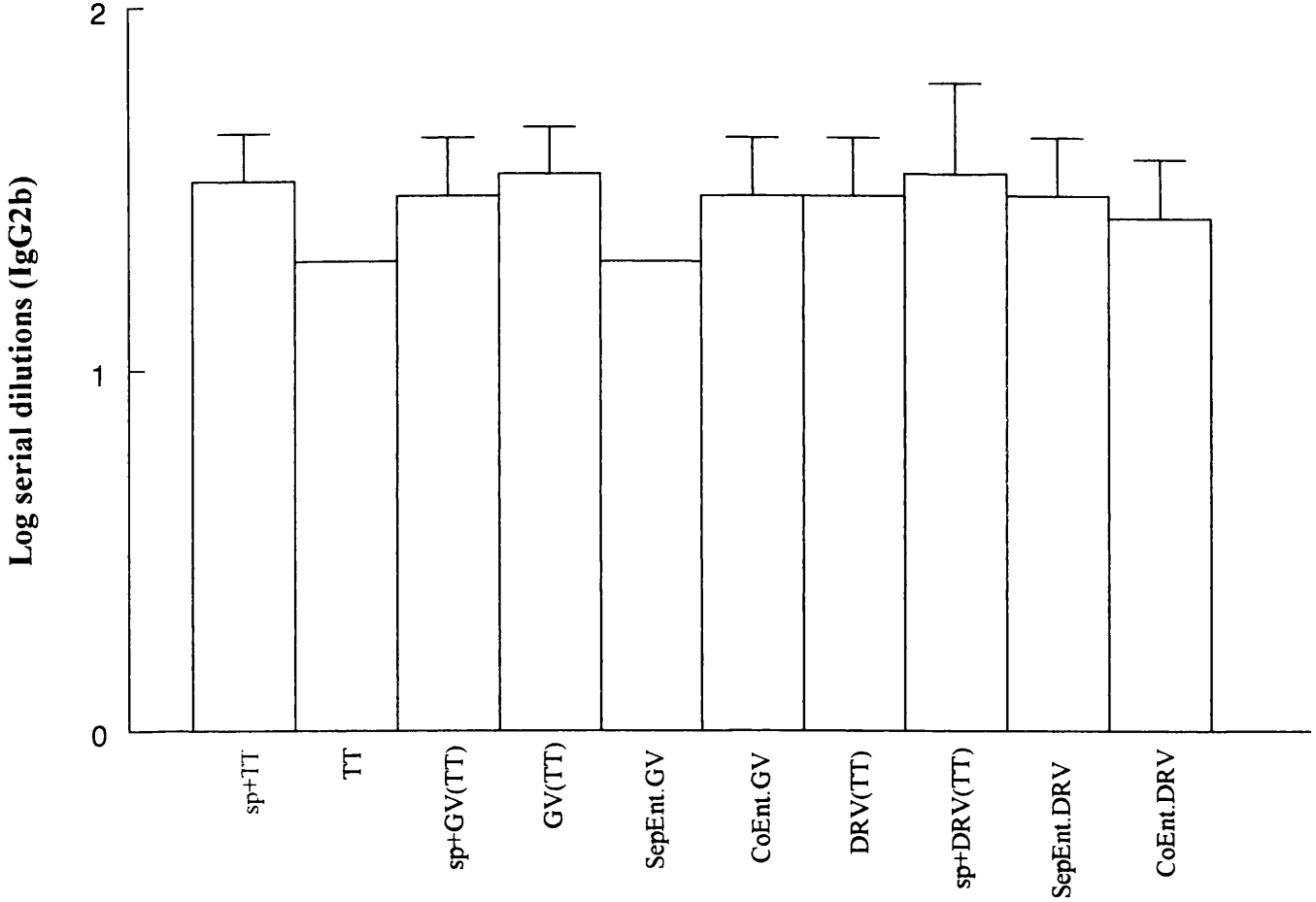
Teranus toxoid and/or spores were entrapped in giant vesicles (GV) composed of PC:cholesterol:PG:TO (4:4:2:1 molar ratios) or DRV composed of PC:cholesterol (1:1 molar ratio). Sp: spores; SepEnt.: TT and spores separately entrapped (in GV or DRV); CoEnt.: TT and spores co-entrapped in GV or DRV. Values are expressed as mean of logarithm serum dilutions ± S.D.

Figure 4.2. Comparison of IgG_{2a} primary responses to tetanus toxoid entrapped in DRV or GV with or without *B.subtilis* spores



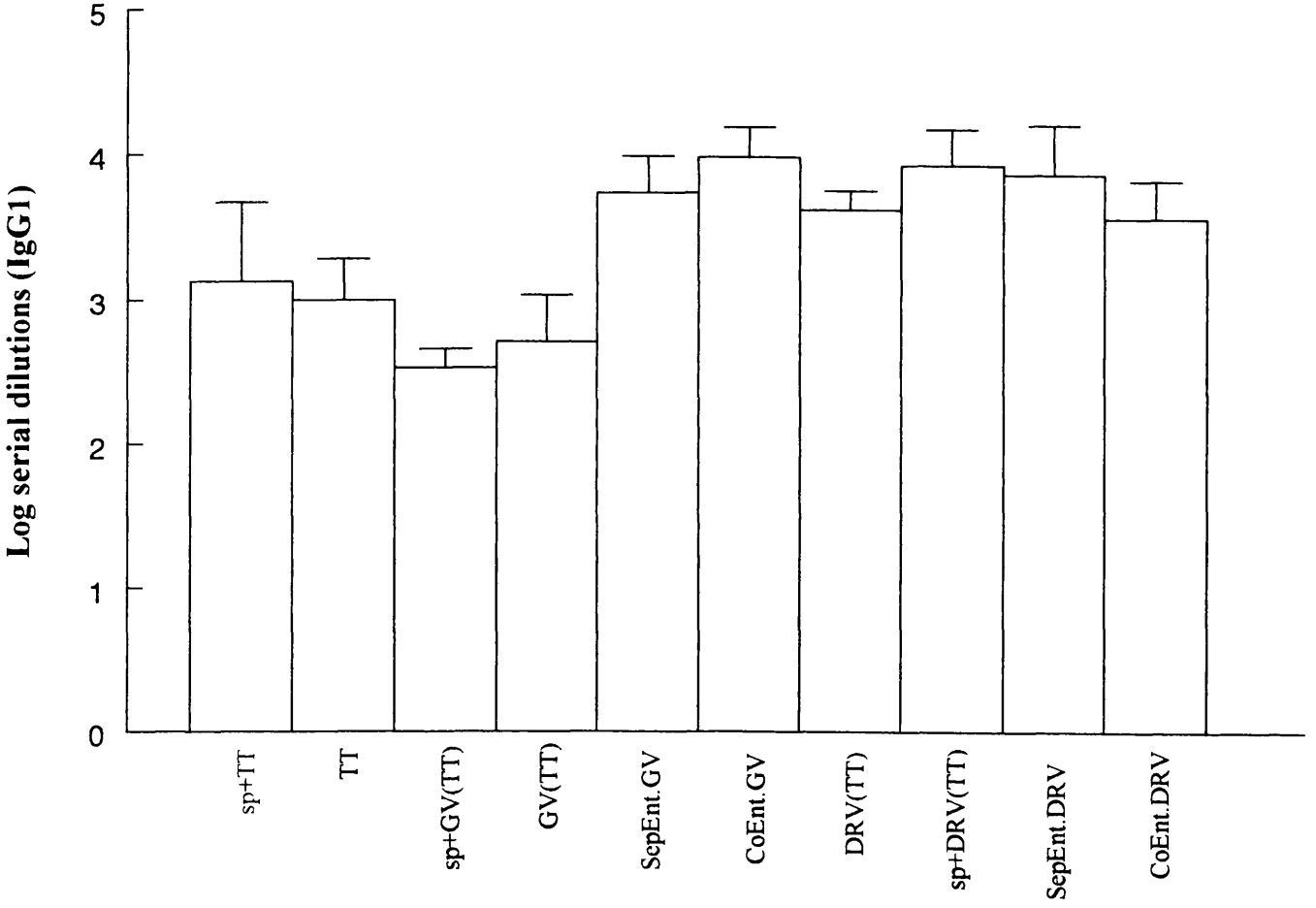
Teranus toxoid and/or spores were entrapped in giant vesicles (GV) composed of PC:cholesterol:PG:TO (4:4:2:1 molar ratios) or DRV composed of PC:cholesterol (1:1 molar ratio). Sp: spores; SepEnt.: TT and spores separately entrapped (in GV or DRV); CoEnt.: TT and spores co-entrapped in GV or DRV. Values are expressed as mean of logarithm serum dilutions ± S.D.

Figure 4.3. Comparison of IgG_{2b} primary responses to tetanus toxoid entrapped in DRV or GV with or without *B.subtilis* spores



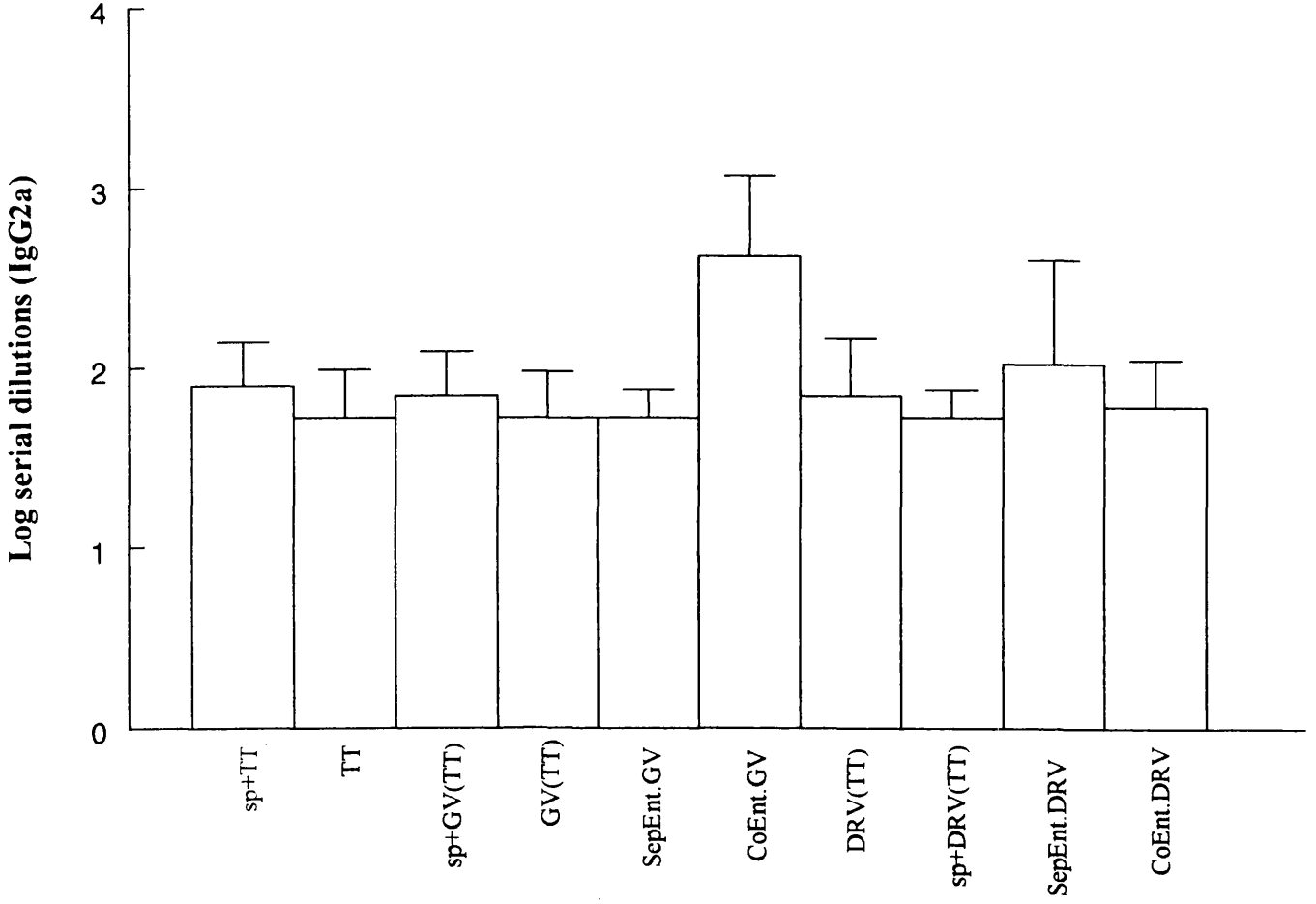
Teranus toxoid and/or spores were entrapped in giant vesicles (GV) composed of PC:cholesterol:PG:TO (4:4:2:1 molar ratios) or DRV composed of PC:cholesterol (1:1 molar ratio). Sp: spores; SepEnt.: TT and spores separately entrapped (in GV or DRV); CoEnt.: TT and spores co-entrapped in GV or DRV. Values are expressed as mean of logarithm serum dilutions ± S.D.

Figure 4.4. Comparison of IgG_1 secondary responses to tetanus toxoid entrapped in DRV or GV with or without *B.subtilis* spores



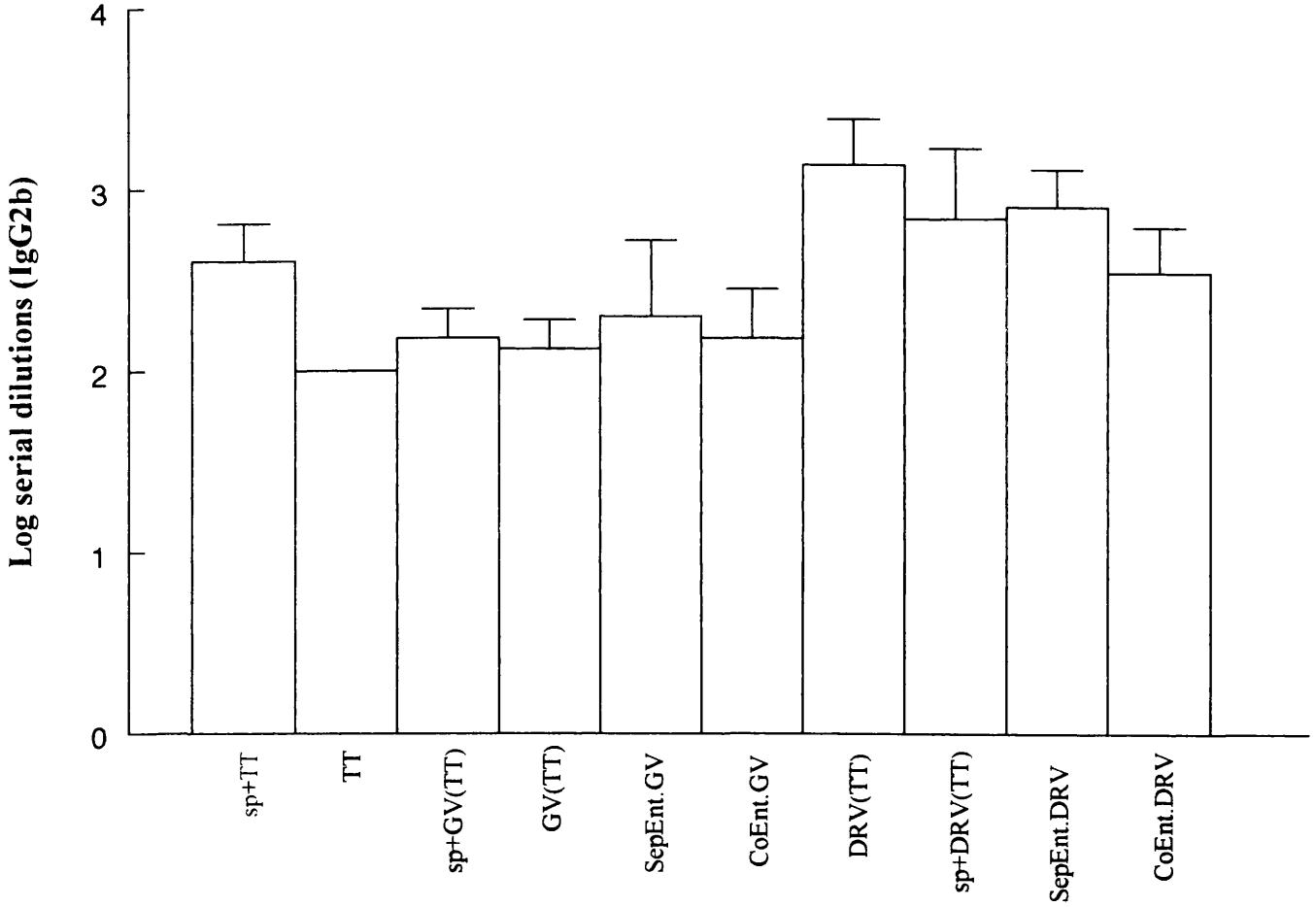
Teranus toxoid and/or spores were entrapped in giant vesicles (GV) composed of PC:cholesterol:PG:TO (4:4:2:1 molar ratios) or DRV composed of PC:cholesterol (1:1 molar ratio). Sp: spores; SepEnt.: TT and spores separately entrapped (in GV or DRV); CoEnt.: TT and spores co-entrapped in GV or DRV. Values are expressed as mean of logarithm serum dilutions ± S.D.

Figure 4.5. Comparison of IgG_{2a} secondary responses to tetanus toxoid entrapped in DRV or GV with or without *B.subtilis* spores



Teranus toxoid and/or spores were entrapped in giant vesicles (GV) composed of PC:cholesterol:PG:TO (4:4:2:1 molar ratios) or DRV composed of PC:cholesterol (1:1 molar ratio). Sp: spores; SepEnt.: TT and spores separately entrapped (in GV or DRV); CoEnt.: TT and spores co-entrapped in GV or DRV. Values are expressed as mean of logarithm serum dilutions ± S.D.

Figure 4.6. Comparison of IgG_{2b} secondary responses to tetanus toxoid entrapped in DRV or GV with or without *B.subtilis* spores



Teranus toxoid and/or spores were entrapped in giant vesicles (GV) composed of PC:cholesterol:PG:TO (4:4:2:1 molar ratios) or DRV composed of PC:cholesterol (1:1 molar ratio). Sp: spores; SepEnt.: TT and spores separately entrapped (in GV or DRV); CoEnt.: TT and spores co-entrapped in GV or DRV. Values are expressed as mean of logarithm serum dilutions ± S.D.

Table 4.2. Comparison of primary responses

Preparations	sp+TT	GV(TT)	CoEnt.GV		DRV(TT)	sp+DRV(TT)	SepEnt.DRV
	IgG_{2b}	IgG_{2b}	IgG_1	IgG_{2a}	IgG_{2a}	IgG_1	IgG_1
sp+TT							
TT	*	**	***	*	*	***	**
sp+GV(TT)			***			***	**
GV(TT)			***			***	**
SepEnt.GV	*	**	***			*	*
CoEnt.GV							
DRV(TT)							
sp+DRV(TT)							
SepEnt.DRV				*	*		
CoEnt.DRV							

Tetanus toxoid (TT) or *B.subtilis* spores (sp) were either entrapped alone or together in giant vesicles (GV) composed of PC:cholesterol:PG:TO (4:4:2:1 molar ratios) or DRV composed of PC:cholesterol (1:1 molar ratios). Only the formulations that improved the antibody titers significantly are presented in the Table. Stars represent the significance levels (*, $P<0.05$; **, $P<0.02$; ***, $P<0.01$). For explanation of codes, see legend to Figure 4.1.

Table 4.3. Comparison of secondary responses

	sp+TT	TT	ScpEnt.GV	CoEnt.GV		DRV(TT)		sp+DRV(TT)		SepEnt.DRV		CoEnt.DRV	
	IgG_{2b}	IgG_1	IgG_1	IgG_1	IgG_{2a}	IgG_1	IgG_{2b}	IgG_1	IgG_{2b}	IgG_1	IgG_{2b}	IgG_1	IgG_{2b}
sp+TT				*	*		*	*					
TT	***		**	***	**	**	****	***	***	**	****	*	***
sp+GV(TT)	*	*	****	****	*	****	***	****	*	***	***	***	
GV(TT)	**		***	***	**	***	***	***	**	***	***	**	*
SepEnt.GV					**		**				*		
CoEnt.GV							***		*		***		
DRV(TT)				*	*								
sp+DRV(TT)					**								
ScpEnt.DRV													
CoEnt.DRV					*		**						

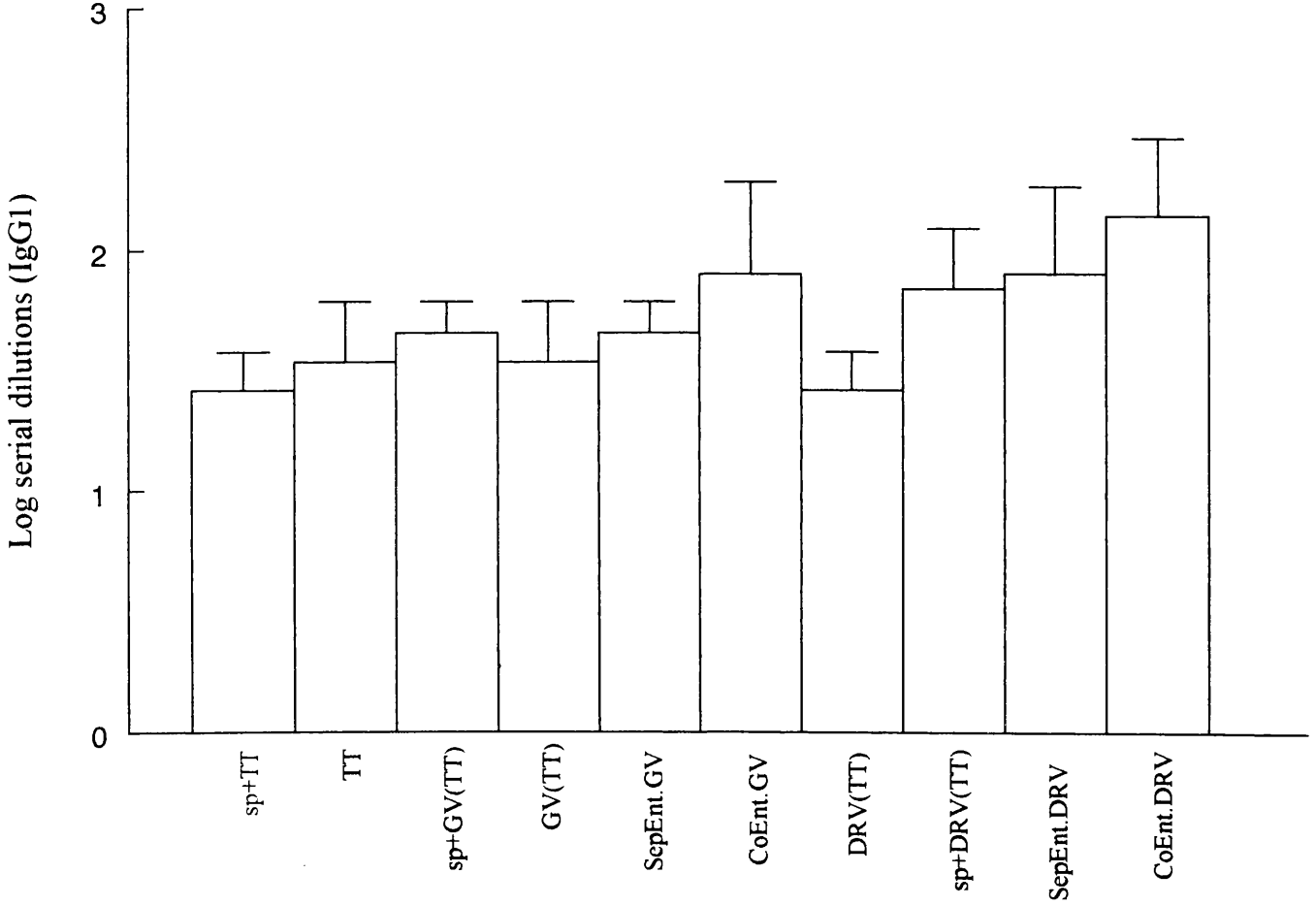
Tetanus toxoid (TT) or *B.subtilis* spores (sp) were either entrapped alone or together in giant vesicles (GV) composed of PC:cholesterol:PG:TO (4:4:2:1 molar ratios) or DRV composed of PC:cholesterol (1:1 molar ratios). Only the formulations that improved the antibody titers significantly are presented in the Table. Stars represent the significance levels (*, $P<0.05$; **, $P<0.02$; ***, $P<0.01$; ****, $P<0.001$). For explanation of codes, see legend to Figure 4.1.

DRV formulations improved responses significantly (see Table 4.3). The free spores-toxoid mixture was also better than the free toxoid alone. Thus, one may assume that the presence of spores is not the factor responsible for the low IgG_{2b} titers but the lipid composition might be of importance. The presence of a negatively charged lipid (PG) in the structure of giant vesicles in addition to the fatty acid triolein may influence the interaction of the carrier with the antigen presenting cells.

4.3.2.2. Secondary responses to spores entrapped in GV or DRV in the presence or absence of toxoid

The primary IgG titers (all subclasses) and secondary IgG_{2a} and IgG_{2b} titers for the spores were too low to detect with any of the formulations tested. Only the secondary IgG_1 anti-spore titers could be detected and these are presented in Figure 4.7. Of the groups tested, the free spore-entrapped TT (DRV) combination was significantly better than the free spores-toxoid mixture ($P<0.05$). However, the coentrapped spores and TT in DRV was the most effective group since it was found to be superior to free toxoid, and all GV formulations ($P<0.05$) except for the coentrapped GV. The same formulation was also superior to free spores-toxoid mixture ($P<0.02$). The presence of anti-spore antibodies in the groups immunized with free or liposomal toxoid alone was somewhat surprising. This may be the result of a non-specific interaction in the ELISA or the toxoid preparation may also contain some common epitopes shared with *B.subtilis spores*. In this respect, it is of interest that Amorena et al (1994) also observed an enhancement in immune responses against whole bacterial cells in the presence of bacterial toxoids. They suggested that either the toxoids attracted leucocytes to the site of vaccination or the toxoids contained some specific cell wall components.

Figure 4.7. Comparison of IgG_1 secondary responses to *B.subtilis* spores free or entrapped in DRV or GV with tetanus toxoid

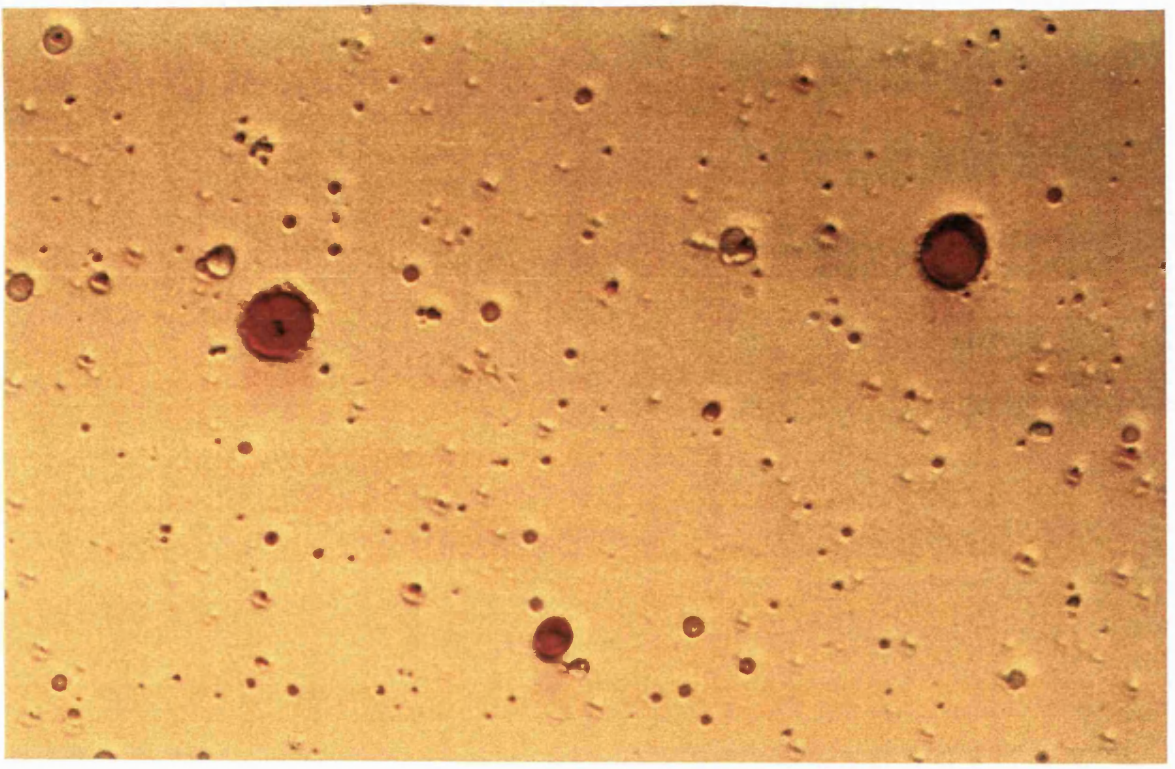


Teranus toxoid and/or spores were entrapped in giant vesicles (GV) composed of PC:cholesterol:PG:TO (4:4:2:1 molar ratios) or DRV composed of PC:cholesterol (1:1 molar ratio). Sp: spores; SepEnt.: TT and spores separately entrapped (in GV or DRV); CoEnt.: TT and spores co-entrapped in GV or DRV. Values are expressed as mean of logarithm serum dilutions ± S.D.

4.3.3. Spore-anti spore IgG binding studies

In order to ascertain whether entrapment of spores within liposomes could protect the former from binding to anti-spore antibodies, a series of experiments were conducted where the spores, as such or within liposomes, were incubated with FITC-labeled antibodies purified from the immunized or control rabbits. Figure 4.8 presents the appearance of oil-red-O stained spore-containing giant vesicles incubated with FITC labelled anti-spore IgG under the fluorescence microscope with the UV light off (a) and on (b), respectively. There is no detectable fluorescence to be seen. However, when the same vesicles were treated with the vesicle destabilizing Triton X-100 and then incubated with the anti-spore IgG, spores (released from GV) that could be clearly seen under normal light (Figure 4.9 a) began to fluoresce under the UV light (Figure 4.9 b). The same was observed when liposomes used were of the DRV type. Thus, Figure 4.10 (a and b) and 4.11 (a and b) show intact DRV and DRV mixed with Triton X-100 that were incubated with anti-spore IgG under normal and UV light, respectively. When the same experiment was repeated with FITC-labeled IgG from the unimmunized rabbit, fluorescence observed under UV light (Figure 4.12) was very weak perhaps indicating non-specific binding. One might therefore conclude that both vesicle types are able to accomodate particulate antigens while preventing them from binding to specific antibodies raised against the antigens.

A.



B.

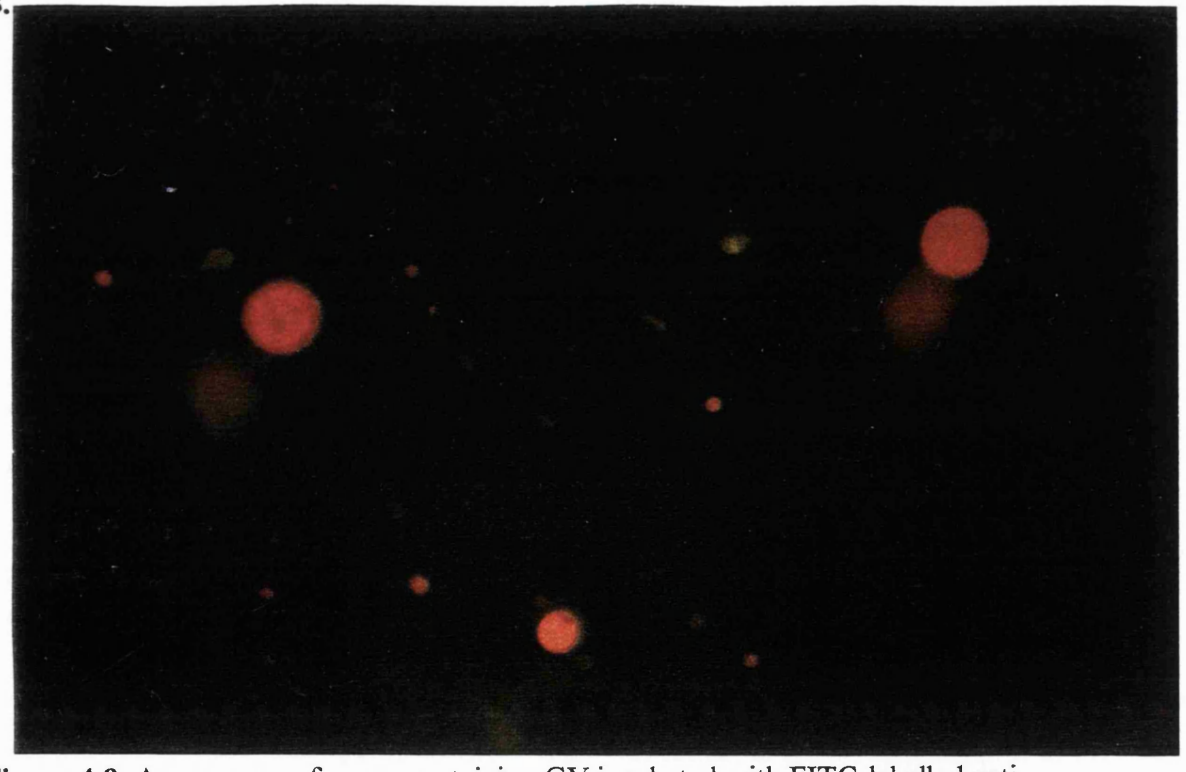
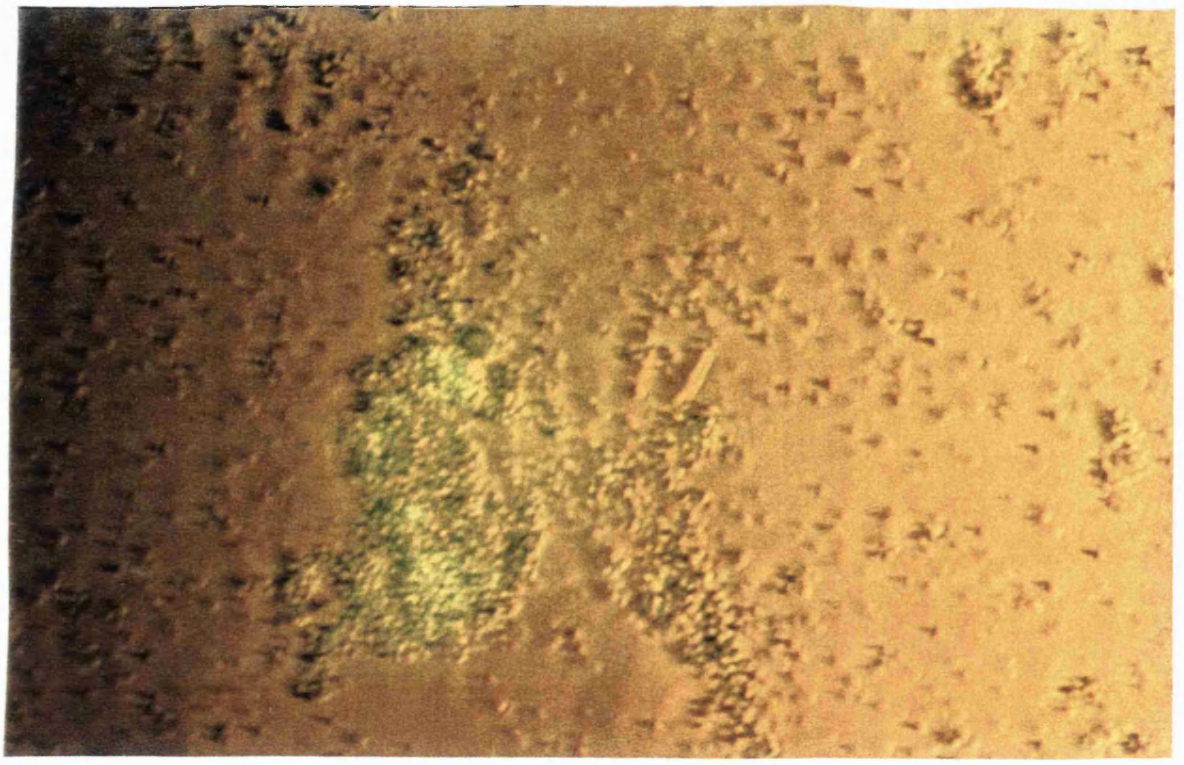


Figure 4.8. Appearance of spore-containing GV incubated with FITC-labelled anti-spore IgG under fluorescence microscope. UV off (a); UV on (b); magnification = 400 X

A.



B.

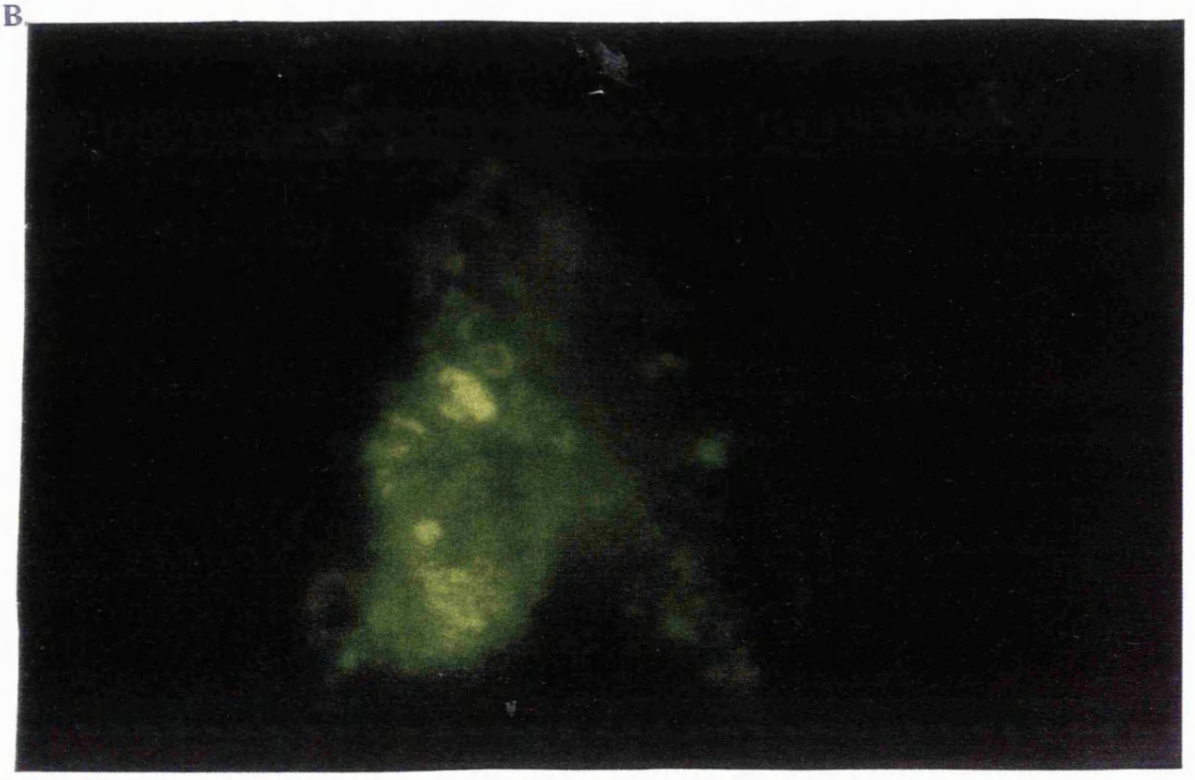
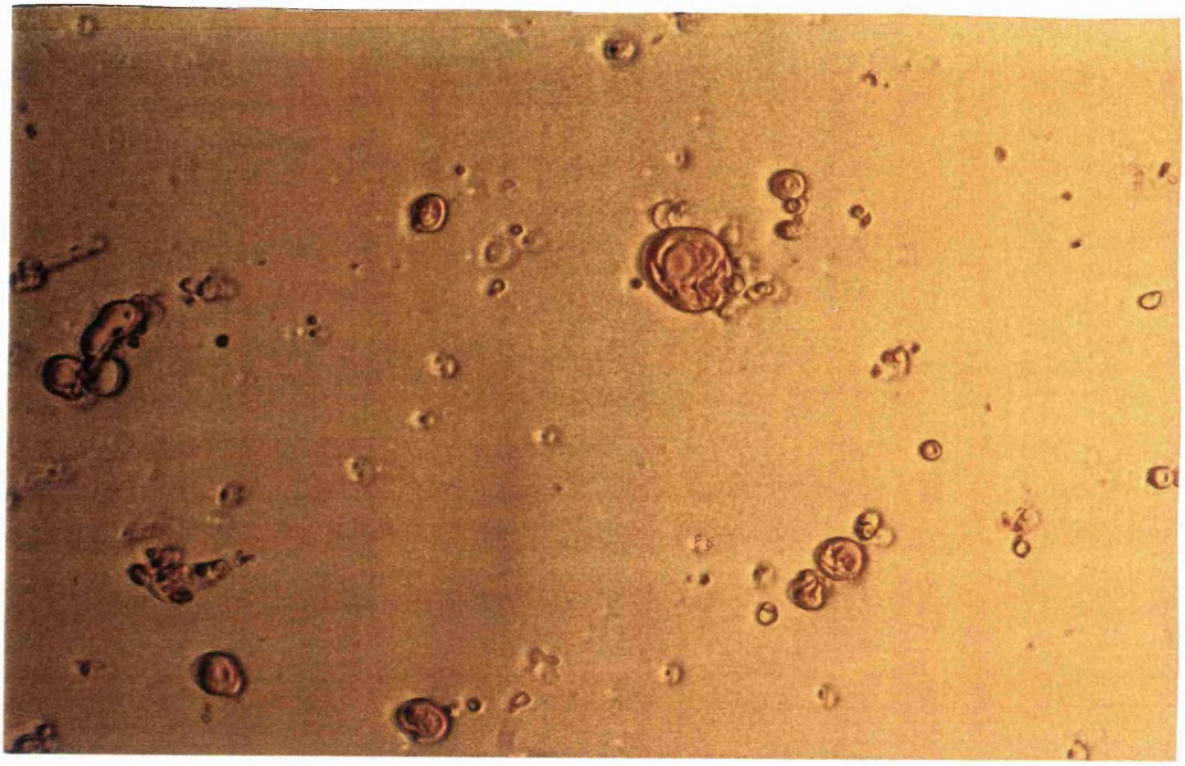


Figure 4.9. Appearance of Triton X-100 treated spore-containing GV incubated with FITC-labelled anti-spore IgG under fluorescence microscope.
UV off (a); UV on (b); magnification = 600 X

A.



B

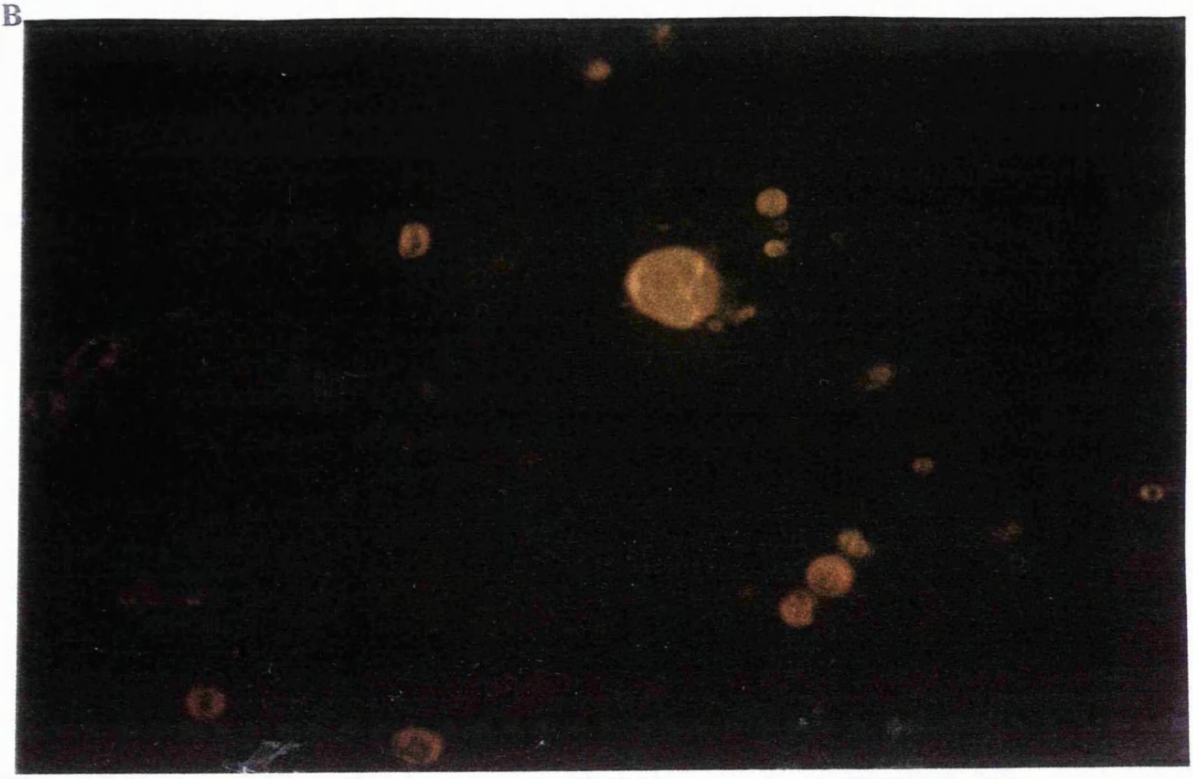
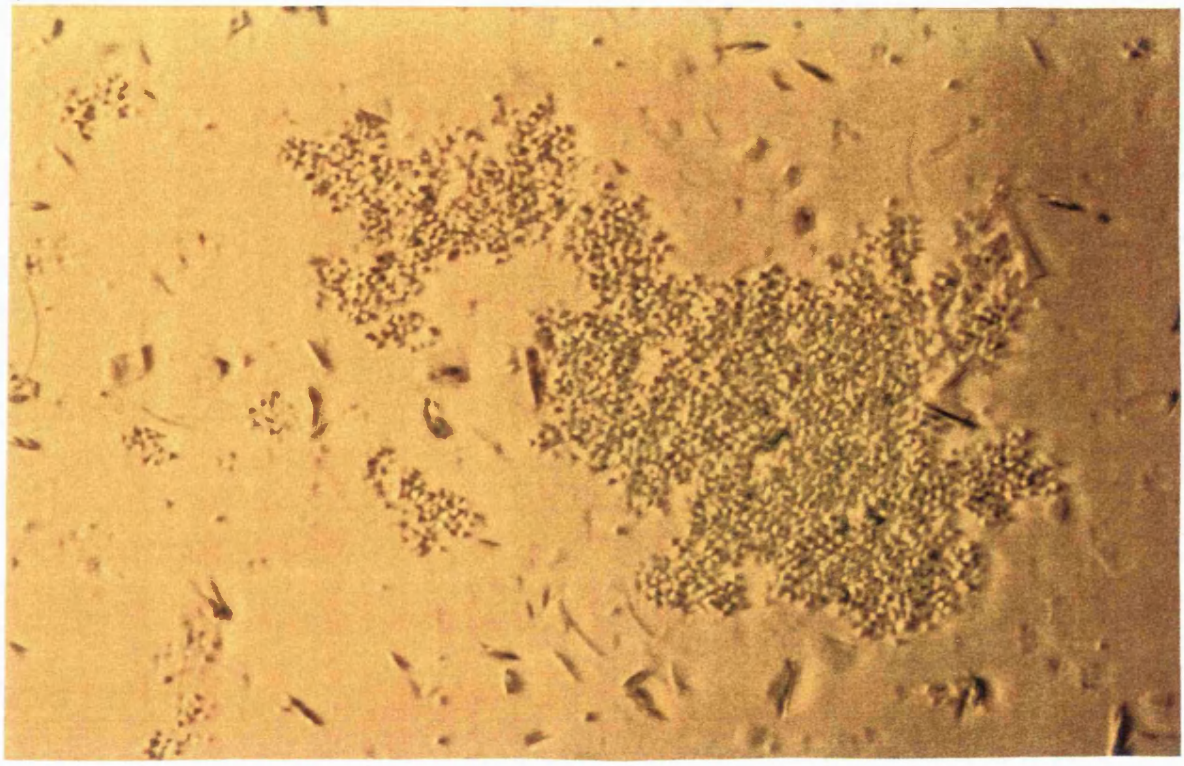


Figure 4.10. Appearance of spore-containing DRV incubated with FITC-labelled anti-spore IgG under fluorescence microscope. UV off (a); UV on (b); magnification = 400 X

A.



B

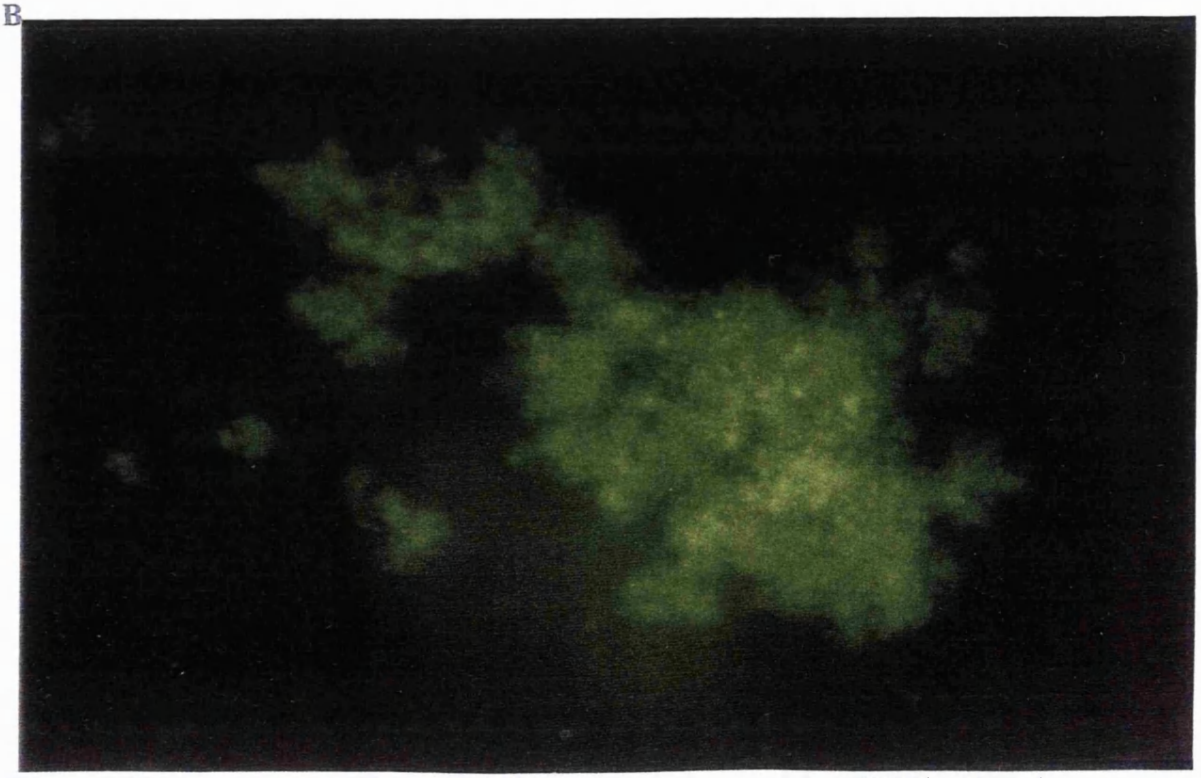


Figure 4.11. Appearance of Triton X-100 treated spore-containing DRV incubated with FITC-labelled anti-spore IgG under fluorescence microscope. UV off (a); UV on (b); magnification = 600 X

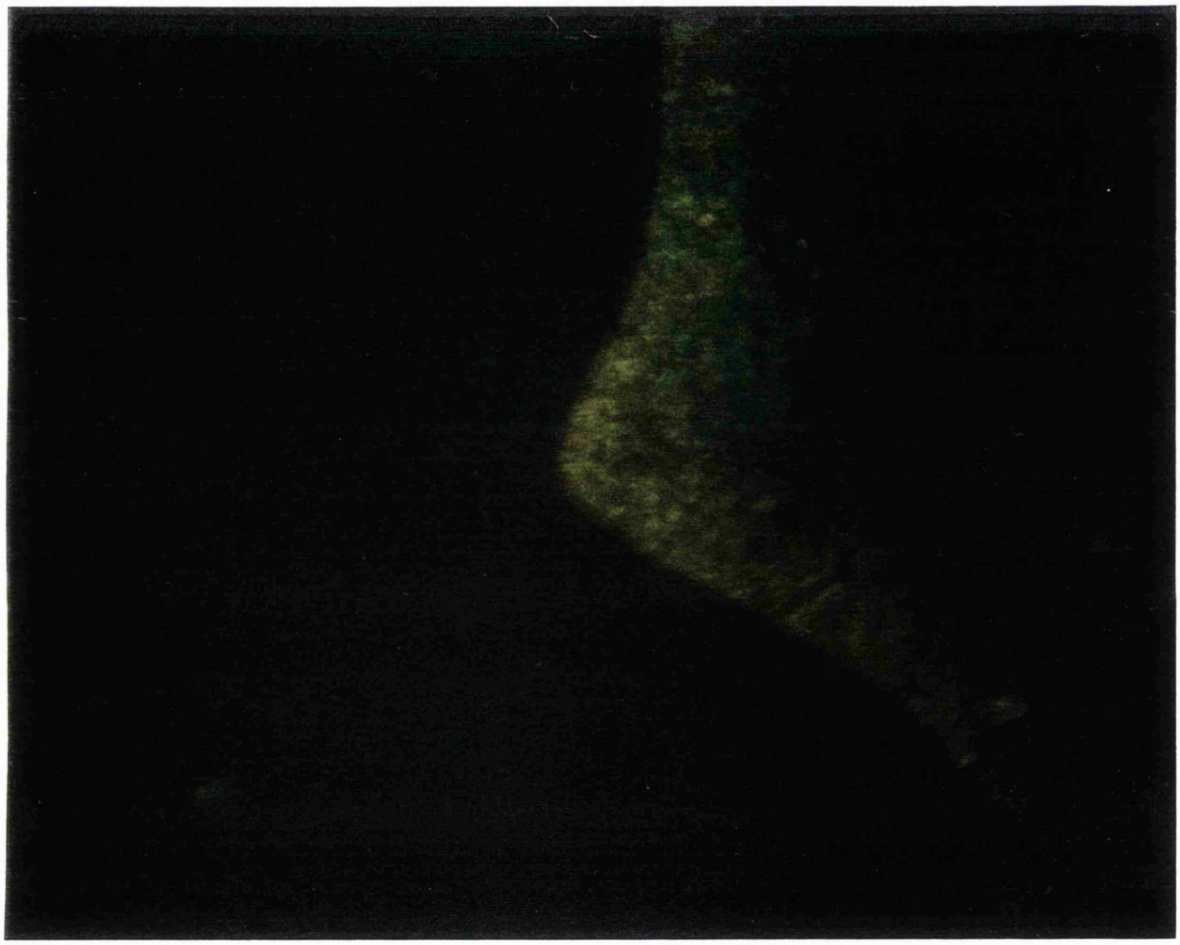


Figure 4.12. Appearance of Triton X-100 treated spore-containing DRV incubated with FITC-labelled control IgG under fluorescence microscope. UV on; magnification = 600 X

4.4. CONCLUSION

In this chapter, the potential of giant vesicles as multiple vaccine carriers was investigated and compared with the well established DRV system. Furthermore, both carriers were analyzed for their ability to protect their contents from binding to antibodies. Results show that giant vesicles, although able to potentiate immune responses to the soluble antigen they entrapped were not superior to DRV (especially when the IgG_{2b} secondary responses were considered). When we started to test this new system and planned the immunization studies, we did not expect to find that spores could be entrapped in DRV since these vesicles were reported to be around 0.5 μm in diameter (Gregoriadis et al, 1993) , a size too small to accomodate *B.subtilis* spores (0.8 μm in diameter; Antimisiaris et al, 1993). Surprisingly however, DRV were very effective in entrapping the particulate antigen as well as their more favorable entrapment values for the toxoid used in this study. It may be that on rehydration of the dry powder consisting of flattened fused bilayers (produced by the dehdration of SUV) in intimate contact with the spores, liposomes form around the spores to end up as giant vesicles rather than exclude the spores to form smaller vesicles. This would also explain the high yield incorporation of spores into such vesicles since most of the spores would be surrounded by lipid during DRV generation.

The differences observed with the anti-toxoid titers between the two vesicle types (GV and DRV) might be due to the different lipid compositions used. However, it should be noted that results with DRV would not necessarily apply to larger particulate antigens (eg. attenuated or killed bacteria) as entrapment within DRV would be unlikely,

leaving the GV as the only possible carrier suitable for multiple vaccine preparation. Also, the ability of both liposome types to protect their content from antibody attack is a further advantage especially with respect to the applications where neutralization of the antigen due to the presence of maternal antibodies is a problem.

CHAPTER 5

USE OF INTERLEUKIN-1β (163-171) AS A CO-ADJUVANT IN LIPOSOMES

5.1. INTRODUCTION

Interleukin-1 is one of the first cytokines found to enhance secondary antibody responses to an antigen (BSA) in vivo (Staruch and Wood, 1983).Since then, there have been a number of studies with this cytokine as an immunological adjuvant. For example, Andrews et al (1994) used recombinant ovine IL-1β as an adjuvant in sheep and mice. The cytokine, when administered in association with a slow release system, resulted in significant enhancement (4-8 fold increase) of antibody levels in both species as well as a stronger delayed type hypersensitivity, indicating the involvement of IL-1β in both humoral and cell mediated immunity. An important observation was that the adjuvant effect occured only when IL-1β was administered together with the antigen to a site drained by the same lymph node. However, systemic administration of IL-1β into animals or humans is known to result in fever, chills, myalgias, joint aches, headache, neutrophilia and at high doses, hypotension and a capillary leak syndrome are observed which require supportive therapy (Dinarello, 1994). Antoni et al (1986) identified the possible active sites on the molecule by analysing its structure and synthesized a nonapeptide (corresponding to sequence 163-171) which showed very similar biological activity as the whole cytokine itself but without inducing any side effects. The peptide was able to restore the immune responses of immunodepressed mice and enhanced the responses to a T-dependent and a T-independent antigen although the dose required for an adjuvant effect was one million-fold higher than that

required by the whole molecule (Nencioni et al, 1987; Frasca et al, 1988; Tagliabue and Boraschi, 1993).

In the light of these findings, we have attempted to use the IL-1β (163-171) peptide in liposomes as a co-adjuvant using our model antigen tetanus toxoid.

5.2. METHODS

The peptide was either coentrapped with the toxoid (Section 2.2.4.3) or covalently coupled to DRV-incorporated toxoid (Section 2.2.7.2). In all cases the pH of the peptide was adjusted to 7.4 using 0.1 N NaOH before use. The percent entrapment for the toxoid was based on ^{125}I radioactivity measurements, while the peptide in Triton X-100 lysed liposomal pellet was quantified using the bicinchoninic protein assay (Section 2.2.6). Since phospholipids and Triton X-100 interfered with color development, appropriate amounts of empty DRV and detergent were also incorporated in the standard tubes. The amounts of toxoid and peptide used in liposome preparations are shown in Table 5.1. For immunization studies, three different doses of free peptide, mixed with DRV-entrapped toxoid, a single co-entrapped preparation and two (peptide) surface-linked preparations with toxoid entrapped in the same vesicles were used. A DRV-entrapped toxoid preparation was also injected to one group as a control. All preparations were diluted accordingly with PBS so that mice (in groups of 4) received a dose of 0.1 μg each in a total volume of 0.1 mL (Section 2.2.10). Anti-toxoid titers were assessed by ELISA as previously described (Section 2.2.11).

5.3. RESULTS

5.3.1. Toxoid and IL-1β(163-171) content of liposomes

Table 5.1 presents the amounts of toxoid and peptide used for incorporation into DRV and values of entrapment and/or surface linkage obtained. It was not possible to obtain DRV entrapping the peptide when amount used exceeded 2.0 mg. Above this amount, the peptide formed a crystal like precipitate during the rehydration step of the freeze-dried material and no liposome pellet could be obtained by centrifugation. It is possible that the peptide destabilizes the liposome structure at high concentrations. However, surface-linkage at a concentration of 4 mg was achievable. As Table 5.1 shows, 24.38 % of the peptide used (2 mg) was entrapped in DRV while values of 32.9 and 22.0 % were observed for coupling with 1.352 and 4.0 mg peptide, respectively. Toxoid entrapments ranged between 23.06 to 30.0 % alone or together with the peptide with no significant differences observed.

5.3.2. Primary and secondary responses to tetanus toxoid entrapped in DRV with or without IL-1β(163-171)

In the immunization studies, a dose of 0.1 μg toxoid was chosen on the basis of dose-response experiments presented in chapter 3. The reason for using this dose (inducing a very low antibody response in the liposomal form) was to unable us to observe an adjuvant effect (if any) of the cytokine.

Analysis of primary and secondary anti-toxoid IgG_1, IgG_{2a} and IgG_{2b} responses did not show any differences among the groups. All the groups gave the base-line levels that

Table 5.1. Preparation conditions and incorporation values of IL-1β(163-171) and toxoid containing DRV used in immunization studies

Preparation	Amount of TT mixed with SUV (μg)	Toxoid entrapment (%)	IL-1β (163-171) mixed with SUV or DRV (mg)	IL-1β (163-171) entrapment or linkage (%)	Amount of IL-1β (163-171) in the final preparation (mg)	Injected IL-1β (163-171) (mg/kg body weight)
DRV(TT) & IL-1β	50.0	30.00	-	-	0.3981	1.991
DRV(TT) & IL-1β	50.0	30.00	-	-	0.9995	4.975
DRV(TT) & IL-1β	50.0	30.00	-	-	1.9980	9.990
Co.Ent. TT & IL-1β	4.0	24.19	2.000	24.38	0.4876	2.438
SL IL-1β & Ent. TT	4.0	23.06	1.352	32.90	0.3705	1.852
SL IL-1β & Ent. TT	4.0	30.00	4.000	22.00	0.8800	4.400

SUV composed of equimolar PC and cholesterol were mixed with toxoid alone or together with IL-1β(163-171) and freeze-dried to generate DRV. DRV composed of PC, cholesterol and APSA (1:1:0.05 molar ratios), entrapping tetanus toxoid were used to covalently couple IL-1β (163-171) to the vesicle surface. All preparations were finally diluted with PBS so that each animal received a final dose of 0.1 μg toxoid (in a total volume of 0.1 mL). For other details, see the text.

are obtained with liposomal toxoid alone. The free peptide dose required to observe an adjuvant effect in the work by Nencioni et al (1987) was 100 mg/kg body weight, although a 50 mg/kg dose also resulted in improved antibody responses. The highest dose of the peptide used here was 9.99 mg/kg in free form and 4.4 mg/kg in liposomal form, amounts clearly well below those shown to be effective by other workers.

5.4. CONCLUSION

None of the formulations used here were able to boost antibody responses against the toxoid. Since it was not possible to entrap higher amounts of the peptide in liposomes, the peptide dose injected per mouse could not be increased using liposomes as a carrier. Although the idea behind these experiments was attractive in the sense that one might be able to reduce the injected peptide doses by entrapping the peptide in a slow-release system and also contain the antigen and the cytokine in the same physical vesicles for presentation to the same antigen presenting cell, liposomes do not seem to be a suitable carrier in this case. However, use of other carrier systems (such as microcapsules) might prove more promising in this respect. The work by Andrews et al (1994) has clearly shown that IL-1β can boost antibody responses only if the cytokine is delivered with the antigen in a slow release formulation. Nencioni et al (1987) using the peptide as an adjuvant pointed out that the pharmacokinetics of the peptide might be quite different to the whole IL-1β molecule and thus there is a need to inject large amounts of the former. In short, an appropriate formulation that can accomodate higher amounts of the peptide together with the antigen might improve even further the adjuvant effects of the free peptide and also reduce significantly the dose of the peptide required.

CHAPTER 6

USE OF INTERLEUKIN-2 AS A CO-ADJUVANT IN LIPOSOMES

6.1. INTRODUCTION

IL-2 has been used as an immunological adjuvant and could be especially useful when non-responsiveness or low-responsiveness to antigens is a problem with certain populations of vaccinees. For instance, haemodialysis patients who do not respond to the hepatitis B vaccine are known (Meuer et al, 1989) to produce antibodies against the hepatitis B surface antigen when re-vaccinated in conjunction with interleukin-2 (IL-2). IL-2 occupies a central position in the cascade of cellular events involved in immune responses (Smith, 1988). It supports the growth and proliferation of antigen-activated T cells (Carding et al, 1991) and the generation of effector T cells, including helper (Lederer et al, 1994), suppressor (Taylor et al, 1991) and cytotoxic T cells (Reiter and Rappaport, 1993). Proliferating T cells produce other lymphokines besides IL-2 which affect cells of the B cell (Brooks and Vitetta, 1986) and macrophage (Malkovski et al, 1987) lineages. Indeed, experiments with Herpex simplex virus (HSV) (Weinberg and Merigan, 1988) and *Haemophilus pleuropneumoniae* (Anderson et al, 1987) vaccines suggest that IL-2 may function as a potent adjuvant increasing specific responses to vaccine immunogens. Unfortunately, use of IL-2 as such requires high dosages which can lead to toxicity (Anderson et al, 1992).

It has been shown (Tan and Gregoriadis, 1989) that administration of IL-2 together with the antigen co-entrapped in the same liposomes improves the cytokine's

coadjuvant effect. Results suggesting such a co-adjuvant effect have been substantiated in subsequent related work with a variety of liposomal vaccines, for instance influenza A virus (Mbawuike et al, 1990), HSV-recombinant glycoprotein D (Ho et al, 1992), bacterial polysaccharide (Abraham and Shah, 1992) and MCA-102 sarcoma (Sencer et al, 1991) vaccines. In these studies, the antigens were used as free in mixture with liposome-entrapped IL-2 except for the bacterial polysaccharide which was coentrapped in the liposomes together with IL-2. Here, we have undertaken a systematic investigation of the role of IL-2 and antigen (tetanus toxoid) mode of localization in liposomes (i.e. entrapped in the aqueous phase of the vesicles or bound to their surface) and mode of IL-2 administration (ie. entrapped together with the antigen in the same liposomes or in separate vesicle populations mixed before injection) in terms of co-adjuvant activity of the cytokine

6.2. METHODS

DRV entrapping tetanus toxoid and/or IL-2 were prepared as previously described (Section 2.2.4.3) in the presence of their ^{125}I-labelled tracers. For the co-entrapped materials only, the labelled toxoid was used and entrapment of IL-2 was assumed on the basis of its previous entrapment values. Covalent coupling of proteins to the surface of SUV has been described in Section 2.2.7.1 and these same protein-coupled vesicles were used to entrap the antigen (in the case of surface-linked cytokine) or IL-2 (in the case of surface-linked toxoid). The amount of protein exposed on the vesicle surface was determined by protease treatment (Section 2.2.7.3). The amounts of toxoid and IL-2 mixed with vesicles is given in Table 6.1. The liposome formulations were then injected into animals (groups of 4) after diluting the samples in PBS so that

each animal received a dose of 0.1 μg toxoid in a volume of 0.1 mL. Primary, secondary and long-term anti-toxoid responses were determined by ELISA.

6.3. RESULTS

6.3.1. Toxoid and IL-2 content of liposomes

Values of tetanus toxoid and IL-2 entrapment or coupling for each of the liposome formulations prepared, as well as the extent of coupled protein exposure on the liposomal surface are shown in Table 6.1. Initially, IL-2 entrapment in liposomes was planned to be calculated using the ELISA (Section 2.2.12) kit for the cytokine as a quantitative method. However, the kit which was suitable for the detection of human IL-2 in a concentration dependent manner proved unsuitable for the recombinant IL-2 used in this study. Although there was colour development, it was not linear over the wide range of concentrations tested (12-6000 pg/mL). On contacting the company it was confirmed that the 3 amino acid changes in the structure of the recombinant IL-2 and the absence of sugar groups (the cytokine was expressed in *E.coli*) might have been the factors affecting the binding of the second anti-IL-2 antibody and thus this approach was abandoned and instead. ^{125}I labelled IL-2 was used for the calculation of entrapment or linkage values. As expected from previous studies (Gregoriadis et al, 1987; Tan and Gregoriadis, 1989) with toxoid-containing DRV, entrapment of the antigen was considerable and reproducible (31.95-33.80 % of the amount used) even when the protein was co-entrapped with IL-2 (38.7±6 %). However, entrapment values for IL-2 were higher (52.7±1.3 %) and similar to those observed (Tan and

Table 6.1. Preparation conditions and toxoid and IL-2 entrapment or covalent coupling values in DRV used in immunization studies

Preparations	Amount of TT mixed with SUV	% Toxoid entrapment or surface linkage	% surface exposure	Units of IL-2 mixed with SUV	% IL-2 entrapment or surface linkage	% surface exposure	Units of IL-2 in the final preparation
0.1 μg Free TT	-	-		-			-
0.1 μg Ent. TT	50.0 μg	31.19		-			-
0.1μg SL TT	0.75 mg	15.00	35.85	-			-
Sep. Ent. TT&IL-2	50.0 μg	25.04		$2.5x10^4$	51.82		$1.453x10^3$
Sep. Ent. TT&IL-2	50.0 μg	25.04		$2.5x10^5$	53.74		$1.816x10^4$
CoEnt.TT&IL-2	20.0 μg	43.00		$2.5x10^4$	50 (assumed)		$1.453x10^3$
CoEnt.TT&IL-2	20.0 μg	34.43		$2.5x10^5$	50 (assumed)		$1.816x10^4$
SLIL-2&Ent.TT	20.0 μg	31.95		$2.5x10^5$	21.12	24.85	$8.272x10^3$
SLIL-2&Ent.TT	10.00 μg	33.80		$2.5x10^5$	21.12	24.85	$1.562x10^4$
SLTT&Ent.IL-2	0.75 mg	12.11	34.23	$2.5x10^5$	50 (assumed)		$1.375x10^3$
SLTT&Ent.IL-2	0.75 mg	12.11	34.23	$2.5x10^6$	50 (assumed)		$1.375x10^4$

SUV composed of equimolar PC and cholesterol and APSA in the case of surface linkage studies (1:1:0.05) were mixed with toxoid alone, IL-2 alone or a mixture of those to generate DRV. When the toxoid was surface-linked on SUV, IL-2 was entrapped in the same vesicles ansd when the cytokine was linked, antigen was entrapped in the same liposomes. Entrapment or linkage calculations are based on ^{125}I radioactivity measurements. All preparations were finally diluted with PBS accordingly so that each animal received a final dose of 0.1 μg toxoid in a total volume of 0.1 mL.

Gregoriadis, 1989) previously whether or not the cytokine was co-entrapped with the antigen. It has been suggested (Tan and Gregoriadis, 1989) that the increased entrapment values for IL-2 are the result of its hydrophobic nature which enables the incorporation of some of the protein into the lipid bilayers of DRV.

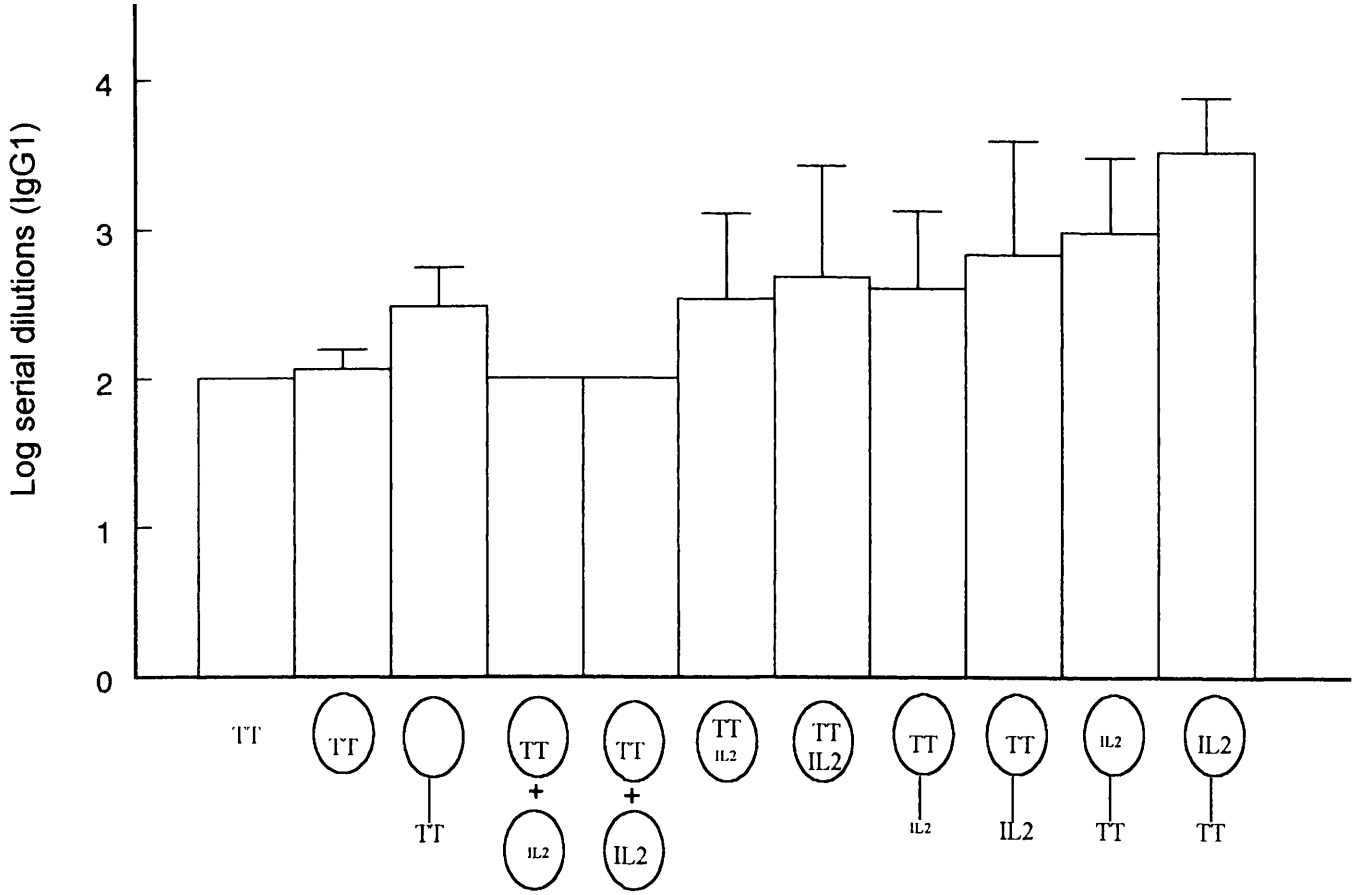
Covalent coupling of the toxoid or IL-2 onto the surface of SUV and subsequent generation of DRV resulted in 12.11-15.0 % (toxoid) and 21.2 % (IL-2) of the protein used being associated with the vesicles (Table 6.1). As noted before (Garcon et al, 1986, Gregoriadis et al, 1993) and also observed morphologically with freeze-fracture electron microscopy (Skalko, N. and Gregoriadis, G, unpublished results), a significant proportion of the coupled toxoid, i.e. a minimum (Gregoriadis et al, 1993) of 35 % was exposed on the DRV surface, available to the action of protease. As discussed elsewhere (Gregoriadis et al, 1993), the appearance of over a third of the amount of toxoid originally coupled to the surface of SUV, on the surface of the multilamellar DRV could only be explained by the preferential utilization of membrane fragments with coupled toxoid (formed on dehydration of SUV) towards the formation of the outer DRV bilayer during the process of rehydration. It now appears that a similar preferential localization on the liposomal surface is also true for coupled IL-2 (24.8 % of the total protein; Table 6.1).

6.3.2. Immunization studies

6.3.2.1. Primary responses to liposomal toxoid and IL-2

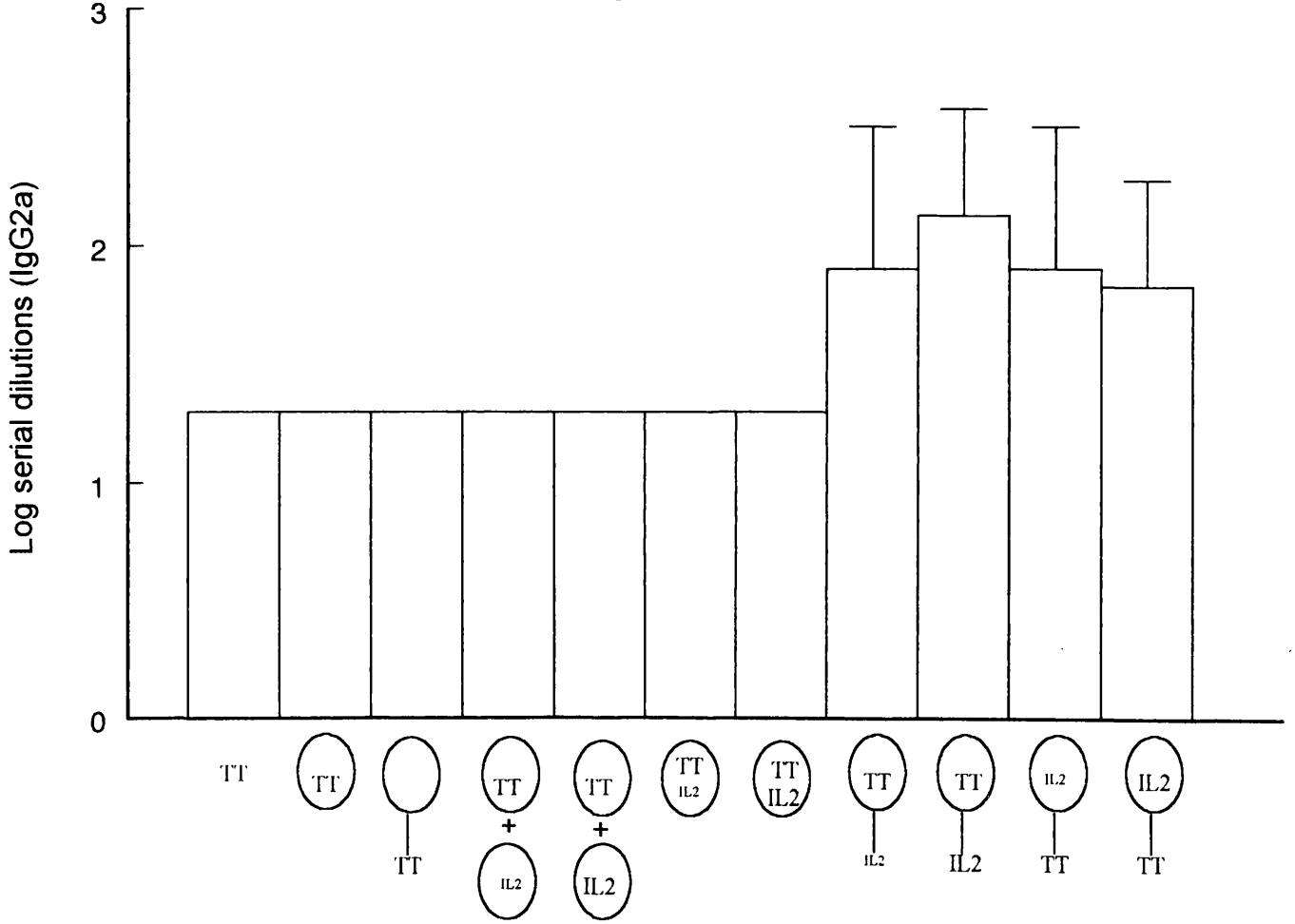
Figures 6.1-6.3 present the primary IgG_1, IgG_{2a} and IgG_{2b} anti-toxoid responses. The only formulation that boosted the anti-toxoid IgG_1 primary responses significantly was

Figure 6.1. Comparison of IgG_1 primary responses to free or liposomal tetanus toxoid with or without IL-2



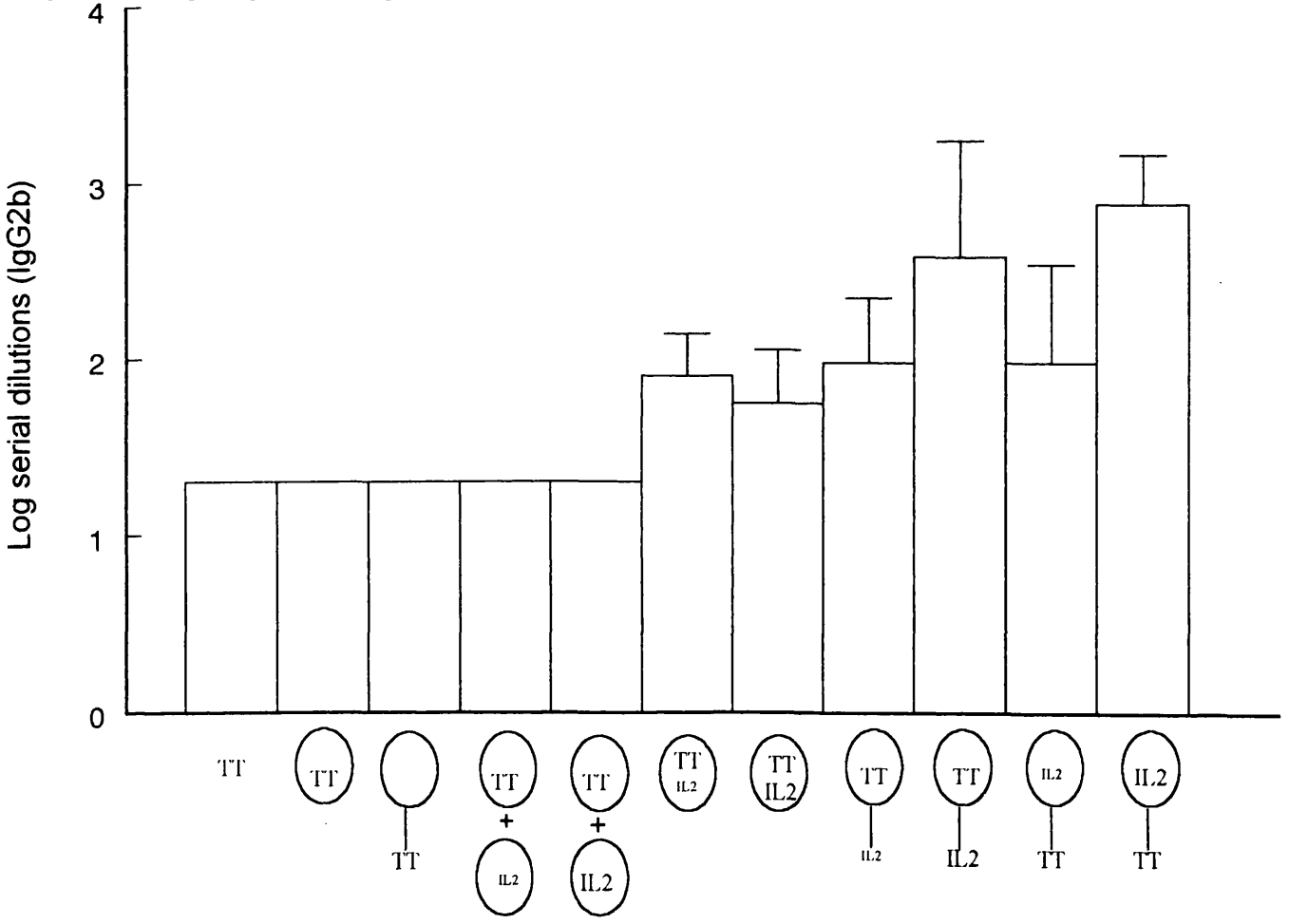
Tetanus toxoid and/or IL-2 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-2: low dose; IL-2: high dose.

Figure 6.2. Comparison of IgG_{2a} primary responses to free or liposomal tetanus toxoid with or without IL-2



Tetanus toxoid and/or IL-2 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-2: low dose; IL-2: high dose.

Figure 6.3. Comparison of IgG_{2b} primary responses to free or liposomal tetanus toxoid with or without IL-2



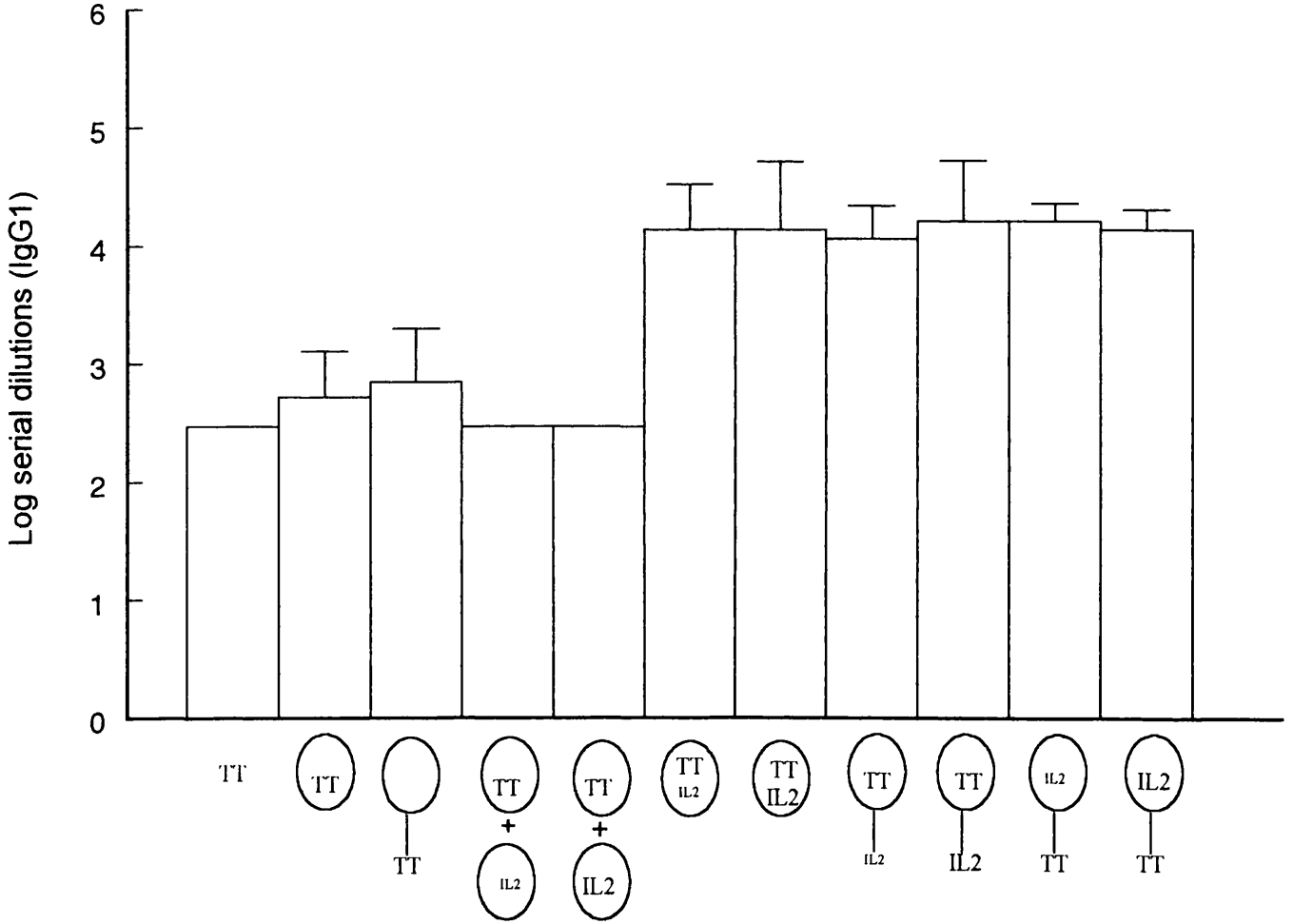
Tetanus toxoid and/or IL-2 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-2: low dose; IL-2: high dose.

the covalently coupled toxoid containing IL-2 in its aqueous space (both IL-2 doses) when compared to IL-2 lacking groups or the separately entrapped preparations. Surface linked IL-2-entrapped toxoid combination (higher dose) proved to be superior to all IL-2 lacking, separately entrapped or co-entrapped formulations in terms of IgG_{2a} primary responses. The co-entrapped (lower dose), surface-linked IL-2 entrapping toxoid inside (both doses) and surface linked toxoid containing IL-2 inside Higher dose) were all effective in increasing the IgG_{2b} titers with respect to free or liposomal toxoid alone or to separately entrapped preparations. Surface linked antigen was the most powerful of all in doing so being also superior to the co-entrapped (both doses) and surface linked IL-2 (lower dose) formulations. Table 6.2 presents a detailed statistical comparison of different formulations. A two way analysis of variance test was also done to see whether IL-2 dose affected the responses but no significant difference was observed for the IL-2 doses used here.

6.3.2.2. Secondary responses to liposomal toxoid and IL-2

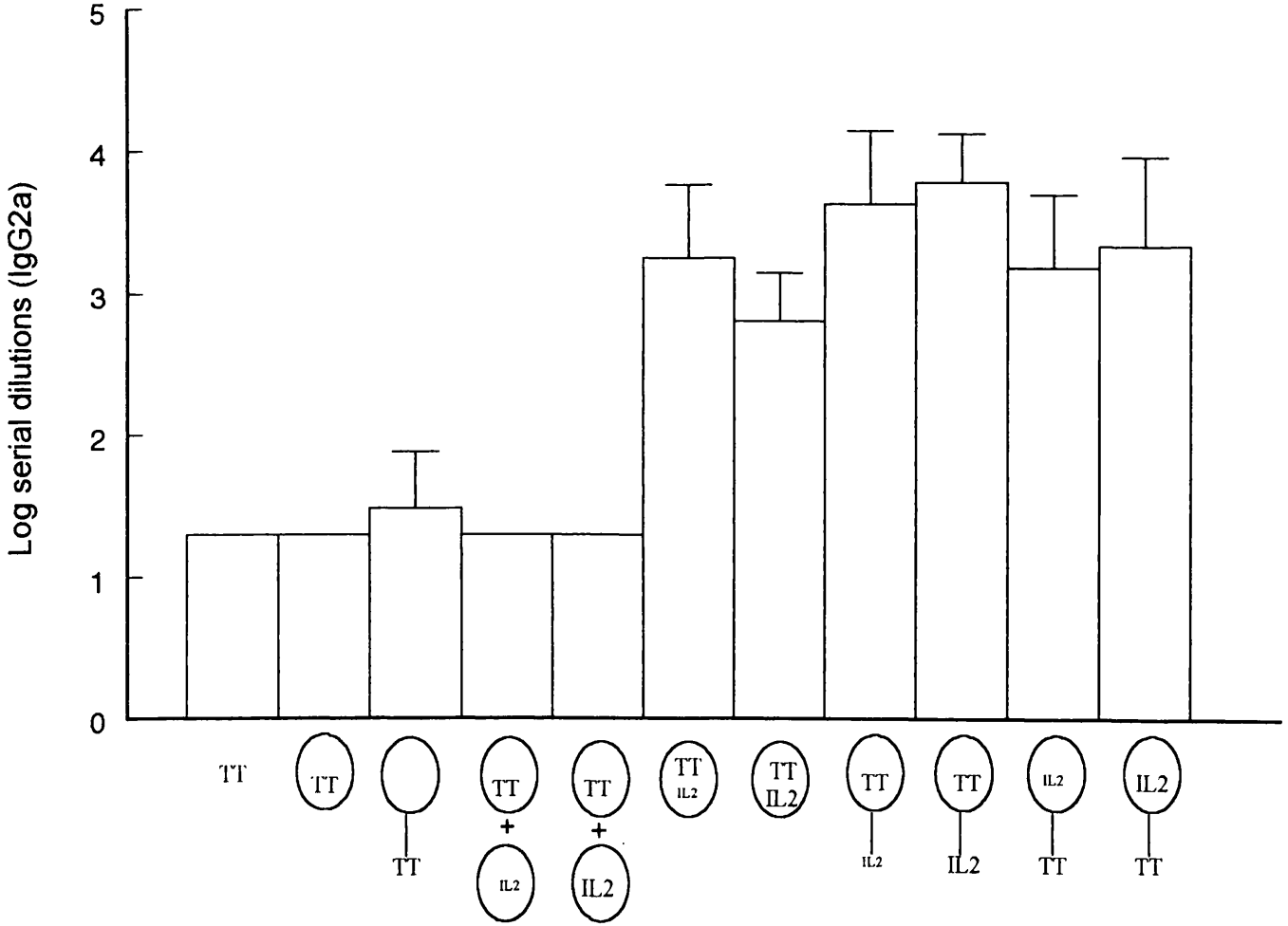
Figures 6.4-6.6 present the secondary IgG subclass responses obtained. The co-adjuvant effect of IL-2 was most striking when the anti-toxoid secondary responses were analyzed. All IL-2 containing preparations except for the separately entrapped formulation boosted the IgG titers (all subclasses tested) significantly when compared to free or liposomal toxoid alone or to separately entrapped preparations. When the significance levels were analyzed(Table 6.3) a preference of the surface-linked toxoid containing IL-2 inside in boosting especially the IgG_1 responses was observed while the most effective formulation in increasing the IgG_{2a} titers was the surface linked cytokine (higher dose) containing the toxoid inside which was also superior to the co-

Figure 6.4. Comparison of IgG_1 secondary responses to free or liposomal tetanus toxoid with or without IL-2



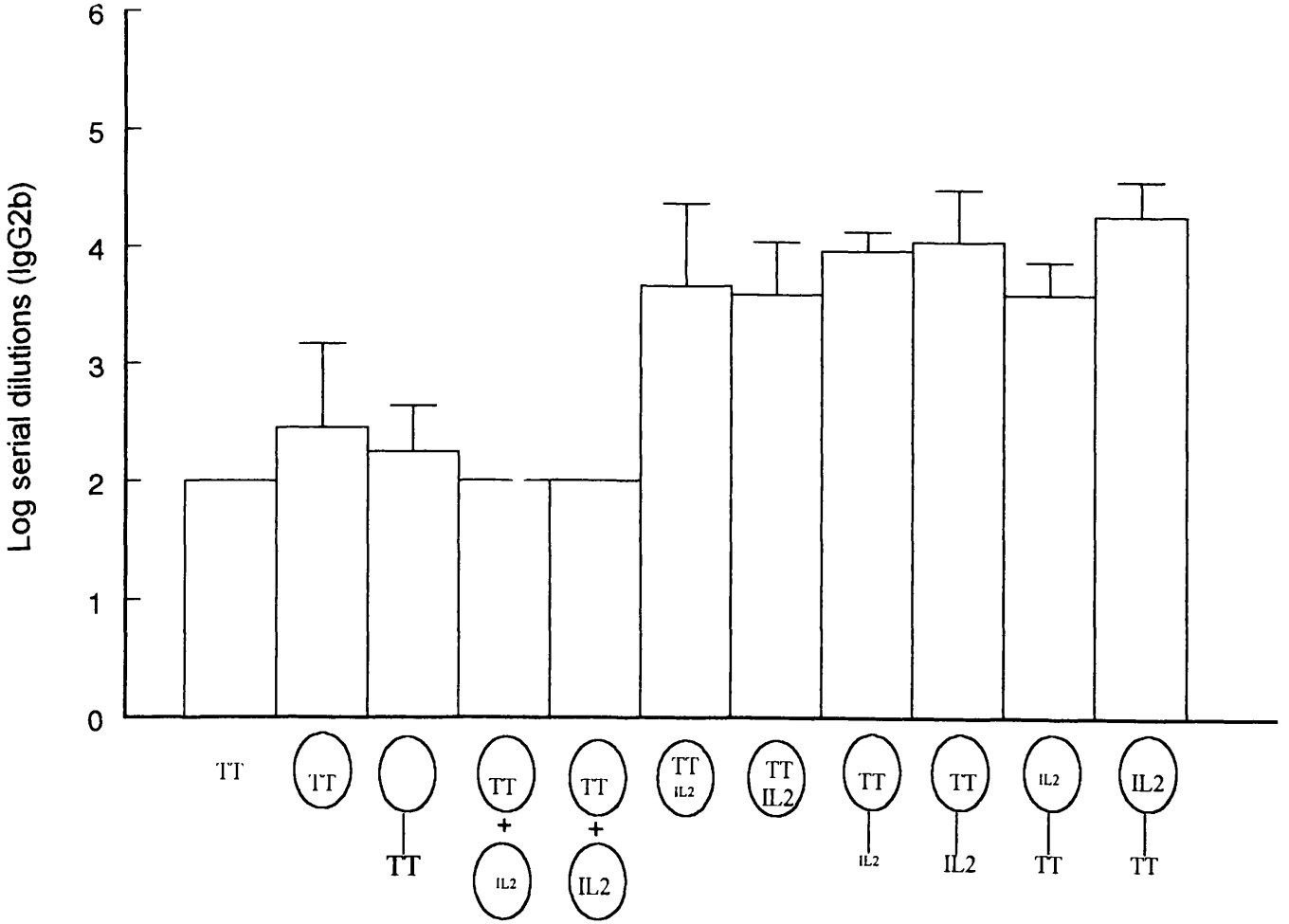
Tetanus toxoid and/or IL-2 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-2: low dose; IL-2: high dose.

Figure 6.5. Comparison of IgG_{2a} secondary responses to free or liposomal tetanus toxoid with or without IL-2



Tetanus toxoid and/or IL-2 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-2: low dose; IL-2: high dose.

Figure 6.6. Comparison of IgG_{2b} secondary responses to free or liposomal tetanus toxoid with or without IL-2



Tetanus toxoid and/or IL-2 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-2: low dose; IL-2: high dose.

Table 6.2. Comparison of primary responses

Preparations	Co-Ent. ($1.453x10^2$)	SL IL-2 ($8.272x10^2$)	SL IL-2 ($1.562x10^3$)		SL TT ($1.375x10^2$)	SL TT ($1.375x10^3$)	
	IgG_{2b}	IgG_{2b}	IgG_{2a}	IgG_{2b}	IgG_1	IgG_1	IgG_{2b}
TT	**	*	*	*	*	***	***
Ent. TT	**	*	*	*	*	***	***
SL TT	**	*	*	*		*	***
Sep. Ent. ($1.453x10^2$)	**	*	*	*	*	***	***
Sep. Ent. ($1.816x10^3$)	**	*	*	*	*	***	***
Co-Ent. ($1.453x10^2$)			*				**
Co-Ent. ($1.816x10^3$)			*				**
SL IL-2 ($8.272x10^2$)							*

Tetanus toxoid and/or IL-2 were either entrapped alone or together in DRV composed of PC:cholesterol (1:1 molar ratios) or were covalently coupled to the surface of SUV composed of PC:cholesterol:APSA (1:1:0.05 molar ratios). The SUV with either the toxoid or the cytokine coupled to their surface were then used to generate DRV in the presence of either protein destined for entrapment. Only the formulations that improved the antibody titers are given in the Table. Stars denote significance levels (*, $P<0.05$; **, $P<0.02$; ***, $P<0.01$). Numbers in parantheses denote dose of IL-2 received/mouse in each group. TT: tetanus toxois; SL: surface linked; Sep.Ent.: separately entrapped in different liposomes but co-injected; Co-Ent: co-entrapped in the same vesicles.

Table 6.3. Comparison of secondary responses

Preparations	Co-Ent. ($1.453x10^2$)			Co-Ent. ($1.816x10^3$)			SL IL-2 ($8.272x10^2$)			SL IL-2 ($1.562x10^3$)			SL TT ($1.375x10^2$)			SL TT ($1.375x10^3$)		
	IgG_1	IgG_{2a}	IgG_{2b}	IgG_1	IgG_{2a}	IgG_{2b}	IgG_1	IgG_{2a}	IgG_{2b}	IgG_1	IgG_{2a}	IgG_{2b}	IgG_1	IgG_{2a}	IgG_{2b}	IgG_1	IgG_{2a}	IgG_{2b}
TT	***	***	**	**	***	***	***	***	****	***	****	***	****	***	***	****	***	****
Ent. TT	**	***		*	***		**	***	*	*	****	*	***	***		**	***	**
SL TT	*	**	*	*	**	*	*	***	***	*	***	**	**	**	**	**	**	***
Sep. Ent. ($1.453x10^2$)	***	***	**	**	***	***	***	***	****	***	****	***	****	***	***	****	***	****
Sep. Ent. ($1.816x10^3$)	***	***	**	**	***	***	***	***	****	***	****	***	****	***	***	****	***	****
Co-Ent. ($1.453x10^2$)																		
Co-Ent. ($1.816x10^3$)											*							

Tetanus toxoid and/or IL-2 were either entrapped alone or together in DRV composed of PC:cholesterol (1:1 molar ratios) or were covalently coupled to the surface of SUV composed of PC:cholesterol:APSA (1:1:0.05 molar ratios). The SUV with either the toxoid or the cytokine coupled to their surface were then used to generate DRV in the presence of either protein destined for entrapment. Only the formulations that improved the antibody titers are given in the Table. Stars denote significance levels (*, $P<0.05$; **, $P<0.02$; ***, $P<0.01$; ****, $P<0.001$). Numbers in parantheses denote dose of IL-2 received/mouse in each group. TT: tetanus toxois; SL: surface linked; Sep.Ent.: separately entrapped in different liposomes but co-injected; Co-Ent: co-entrapped in the same vesicles.

entrapped preparation (higher dose). Although there was no significant differences between toxoid alone or separately entrapped formulations, the latter did not even induce the minimal response. In that sense, one can assume that IL-2 when entrapped in separate vesicles with the toxoid supressed the responses rather than enhancing. A two way ANOVA showed that IL-2 dose used here did not affect the responses significantly. Both doses were equally effective although for the covalently coupled cytokine the higher dose seems to favour the IgG_{2a} responses more.

Despite the good secondary responses obtained with IL-2 as a co-adjuvant, long term responses were disappointing. When the antibody responses (all subclasses) in mice were checked three months after booster injection, all titers dropped to levels obtained with liposomal toxoid alone. Whether this lack of memory response is due to the inability of the cytokine to do so or is due to the very low levels of antigen used here remains to be determined.

6.4. CONCLUSION

The immunization studies with tetanus toxoid reported here indicate that the spatial distribution of an antigen and a co-adjuvant within the same liposome may alter the extent of antibody production. Our results suggest that for IL-2 to act as an adjuvant, its presence together with the antigen in the same liposome is required. The inability of this cytokine to potentiate the immune response when it is separately entrapped from the antigen suggests a necessity for the cytokine and the toxoid to contact the same antigen-presenting cell as supported by the findings of Heath and Playfair (1990) where they reported an increased adjuvanticity of IFN-γ upon conjugating with the antigen

and assumed that the conjugate while ensuring that IFN-γ contacts with cells contacting with antigen, it might indeed target the optimal dose to these cells with no spill over to irrelevant cells. IL-2 was proven to be an ineffective adjuvant for influenza virus vaccine (Cao et al, 1992) but it is of importance to note that in this study the cytokine was administered in free form to the animals which alters its bioavailability drastically.

Adjuvants are thought to exert their effects in 3 general ways: by creating a depot for the antigen, via targeting to macropghages and by providing T-cell dependent help. Liposomes accomplish the first two criteria (Gregoriadis, 1992). They are also thought to enter the lymphatics and reach the regional lymph nodes (Hirano and Hunt, 1985) where they may interact with T and B lymphocytes. The incorporation of a T-cell stimulant such as IL-2 in liposomes is therefore of particular interest if T-cell help is required. The increased IgG_{2a} titers with IL-2 liposomes in this study indicates a preferential stimulation of Th1 clones that represent the cell-mediated arm of the immune response and favour IgG_{2a} secretion from B-cells via the action of cytokines they secrete (Snapper and Mond, 1993). The superiority of surface-linked IL-2 to other groups with the cytokine might be a facilitated and more efficient interaction of IL-2 with its receptor.

In the experiments presented in Chapter 3 where groups of mice were immunized with 1 μg toxoid in free or liposomal form, there was very little IgG_{2a} production whereas presence of IL-2 improved the titers of this subgroup even with 0.1 μg toxoid. It is known that TGF-β is a co-stimulus of B-cells for IgG2b class switching and also

induces IgA (Snapper and Mond, 1993). This cytokine suppresses several immune responses including T and B cell functions and deactivate monocyte/macrophages (Espinoza-Delgado et al, 1994). On the other hand, IL-2 leads to expression of IL-1β mRNA from monocytes among other effects, which affects Th1 cell function. It is a possibility that the inhibitory effect of TGF-β on T-cell functions is at least less prominent when IL-2 is supplied together with the antigen which may provide an improvement in IgG_{2a} antibody titers. Since of the IgG isotypes the 2a/2b class is more effective than other IgG subclasses in antibody driven cellular cytotoxicity reactions (Phillips and Emili, 1992) we feel that especially surface-linked IL-2 together with the antigen may prove to be effective especially for vaccines containing weak or non-immunogenic antigens.

CHAPTER 7

IMMUNE RESPONSES TO A PEPTIDE ANTIGEN IN LIPOSOMES WITH OR WITHOUT INTERLEUKIN-2 AS A CO-ADJUVANT

7.1. INTRODUCTION

Chlamydia trachomatis is an obligate intracellular organism which has a life cycle consisting of two morphological forms, one adapted to intracellular multiplication and the second to extracellular survival (Mackett and Williamson, 1995). Serovars A, B/Ba and C are the main causative agents of an ocular eye infection. In addition to eye infection by ocular strains, there are other serovars of *C.trachomatis* causing genital infections including nongonococcal urethritis in males and mucopurulent endocervicitis in females which may progress to more severe diseases such as lymphogranuloma venerum.

The infectious form of the organism , the elementary body, is very small (0.3 μm) with a double-layered outer membrane but no cell wall murein layer. Epitopes on the outer membrane protein (Omp) of *C.trachomatis* have been identified as important targets for the development of vaccines. Recently, Zhong et al (1993) demonstrated that a synthetic peptide composed of a sequence of the variable domain IV (VD IV) of serovar B and another sequence from the VD I of serovar C associated with a lipidic amino acid based polylysine carrier system (Toth, 1994) was able to elicit neutralizing antibodies to serovars A, B, and C although these were of low titers. The amino acid

sequence and octomeric structure of this 30-mer chimeric peptide is shown in Figure 7.1.

Sequence:

Ac-SAETIFDVTTLNPTIAG**SDVAGLQNDPTTN**-Amide

from B Omp VD IV from C Omp VD I

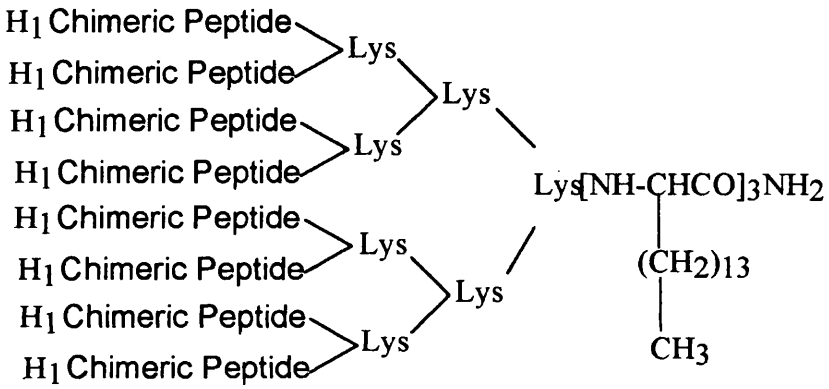


Figure 7.1. Amino acid sequence and octameric structure of the chimeric peptide

In the work by Zhong et al (1993), only the total IgG was measured against the peptide. Here, we have tested whether incorporation of this peptide (kindly supplied to us by Dr. Istvan Toth) in liposomes alone or together with IL-2 might improve IgG (and IgG subclasses) responses.

7.2. METHODS

Liposomes containing the peptide and/or IL-2 were prepared as described in Section 2.2.4.3. Peptide entrapment was assayed by the fluorescamine protein determination

method in the Triton X-100 treated liposomal pellet (Section 2.2.5). Since the presence of phospholipids and Triton X-100 interfered with fluorescence readings, similar amounts of detergent-treated empty DRV were also included in the standard tubes. IL-2 entrapment calculations were based on ^{125}I radioactivity measurements. The amounts of peptide and IL-2 used in the immunization studies are presented in Table 7.1. Since the recommended dose in the work by Zhong et al (1993) was 50 μg/mouse in the presence of the adjuvant NAGO, animals (in groups of four) were initially immunized with 30 and 50 μg of free, entrapped or co-entrapped (with either a low or a high dose of IL-2) peptide. In a different set of experiments, animals (in groups of 5) were immunized with 30 μg of free, or 10, 20 and 30 μg of entrapped or co-entrapped (together with a single dose of IL-2) peptide. Primary and secondary anti-peptide titers were determined by ELISA (Section 2.2.11) for both experiments while long term responses were tested only for the high antigen dose experiment.

7.3. RESULTS

7.3.1. Peptide (LCPH1) and IL-2 content of liposomes

Table 7.1. presents the percent entrapment results for the peptide and IL-2 in DRV. The entrapment of peptide in liposomes ranged between 23.0 to 44.44 % of the amount used. In the case of coentrapment with IL-2, these values varied drastically from 19.14 to 61.5 %. With a 1.0 mg initial peptide input, the highest entrapment was obtained whilst the highest input (4.875 mg), resulted in lowest entrapment results. Thus, it seems that for optimal encapsulation to be achieved, the initial amount of peptide mixed with SUV must not exceed 3.0 mg. IL-2 entrapment also tended to

Table 7.1. Preparation conditions and entrapment values of LCPH1 peptide and IL-2 in DRV used in immunization studies

Preparation	Amount of LCPH1 mixed with SUV(mg)	LCPH1 entrapment (% of used)	IL-2 mixed with SUV(units)	% IL-2 Entrapment (% of used)	Peptide in the final preparation (μg/mL)	IL-2 per mL in the final preparation (units)
Free LCPH1	-	-	-		300	-
Free LCPH1	-	-	-		500	-
Ent. LCPH1	4.500	44.44	-		100	-
Ent. LCPH1	4.500	44.44	-		200	-
Ent. LCPH1	4.500	44.44	-		300	-
Ent. LCPH1	4.875	23.00	-		300	-
Ent. LCPH1	4.875	23.00			500	-
CoEnt LCPH1&IL-2	1.000	61.5	$5x10^4$	51.80	100	$4.316x10^3$
CoEnt LCPH1&IL-2	3.000	36.92	$5x10^4$	43.42	200	$3.919x10^3$
CoEnt LCPH1&IL-2	4.000	36.53	$5x10^4$	42.18	300	$4.329x10^3$
CoEnt LCPH1&IL-2	4.875	19.14	$2.5x10^4$	31.28	300	$2.398x10^3$
CoEnt LCPH1&IL-2	4.875	19.14	$2.5x10^5$	33.44	300	$2.563x10^4$
CoEnt LCPH1&IL-2	4.875	19.14	$2.5x10^4$	34.50	500	$4.413x10^3$
CoEnt LCPH1&IL-2	4.875	19.14	$2.5x10^5$	30.88	500	$3.950x10^4$

SUV composed of equimolar PC and cholesterol were mixed with peptide alone or together with IL-2 and freeze-dried to generate DRV. Percent entrapment of peptide was based on protein content as determined by the fluorescamine method. IL-2 entrapment was calculated from ^{125}I radioactivity measurements. All preparations were finally diluted accordingly so that each animal received a final dose (0.1 mL) as shown in the last two columns.

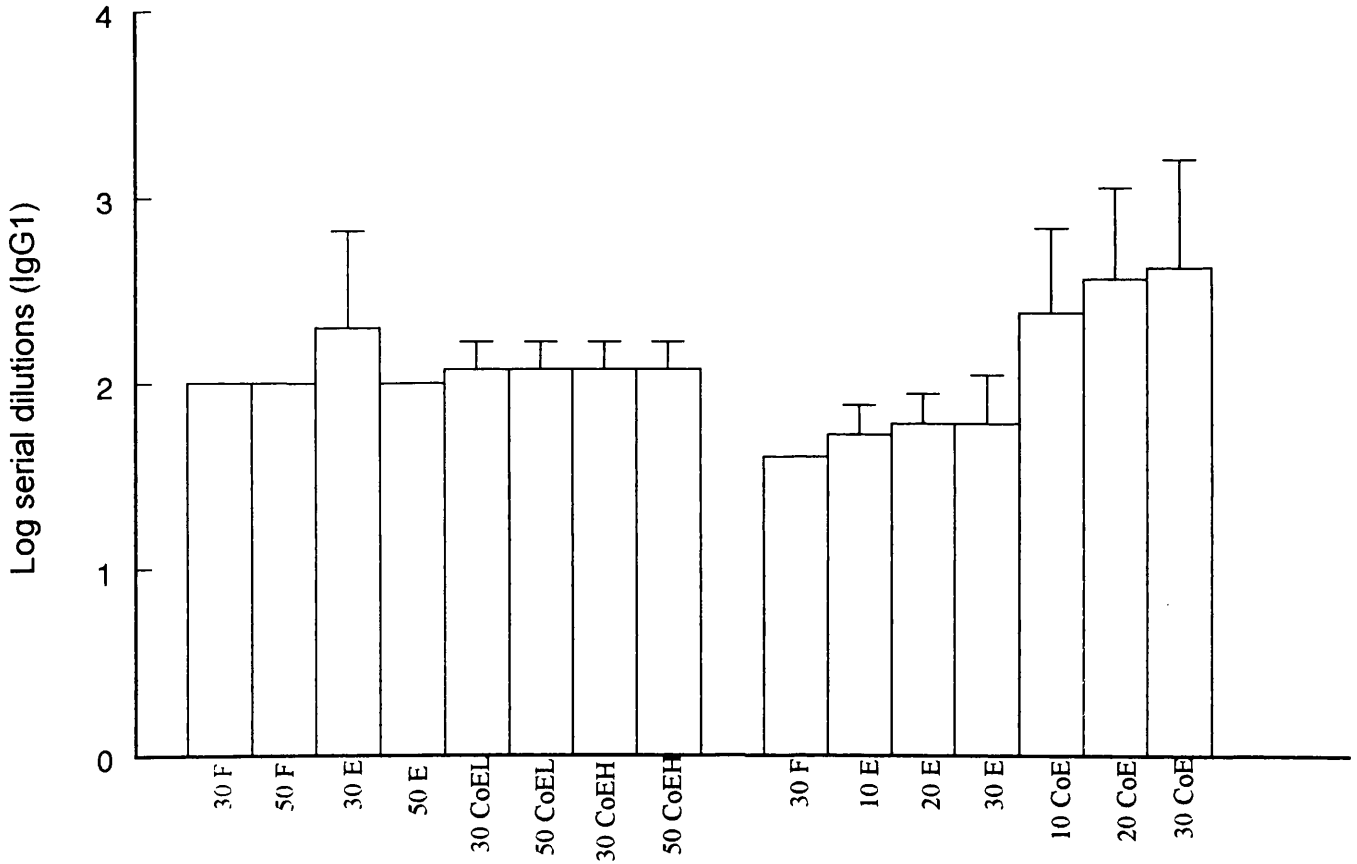
decrease with the higher amount of peptide used. For example, with a 1 mg peptide input, 51.8 % of the cytokine could be incorporated in DRV while this value decreased to c.a 30 % when the initial peptide input was 4.875 mg. Since it was assumed (Tan and Gregoriadis, 1989) that high IL-2 entrapment is probably due to its hydrophobic nature allowing interaction with the lipid bilayers, one can make a similar assumption for the peptide, owing to its membrane-like character (Toth, 1994).

7.3.2. Immune responses to LCPH1 entrapped in DRV with or without IL-2 (30 and 50 microgram doses)

Figures 7.2 to 7.7 present the anti-LCPH1 IgG_1, IgG_{2a}, and IgG_{2b} primary and secondary responses, respectively. With the 30 and 50 μg doses used, none of the groups differed significantly in terms of primary (all subclasses) and IgG_{2a} secondary responses. However, liposomal peptide alone or in combination with IL-2 (both the low and high doses) were superior to the free peptide in boosting the IgG_1 titers with an immunization dose of 30 μg ($P<0.06$ and $P<0.01$, respectively). Fifty μg free LCPH1 was equally effective as the liposomal preparations even when they contained IL-2 as the co-adjuvant. Only the 30 μg coentrapped formulation (higher IL-2 dose) increased the IgG_{2b} secondary responses significantly ($P<0.05$) when compared to the free peptide. No stastistically significant differences were observed between the entrapped and coentrapped preparations.

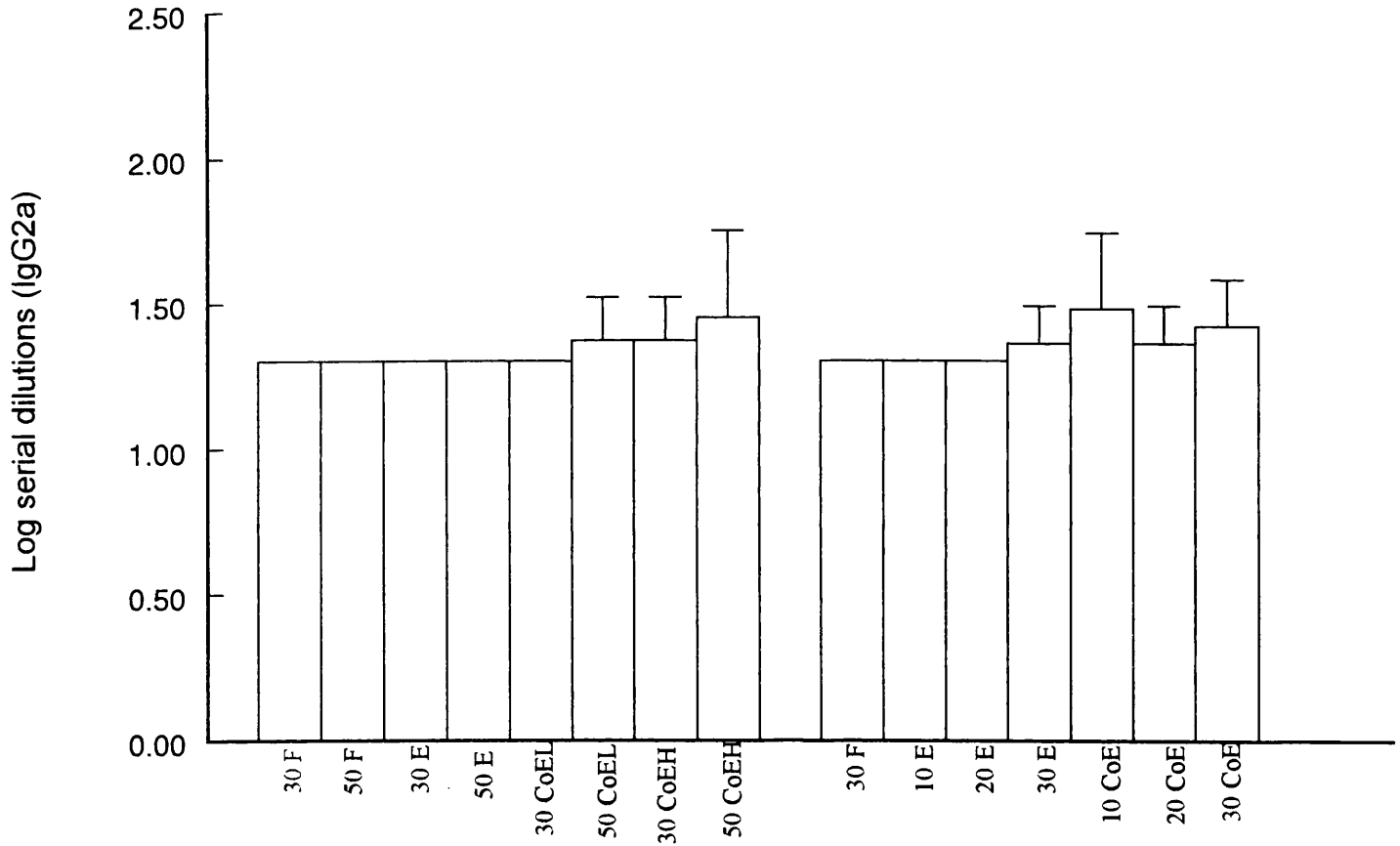
Antibody titers seen after the booster injection declined only slightly three months later, indicating that the peptide could evoke memory responses as well. The long term subclass responses are presented in Figures 7.8 to 7.10. Statistical analysis of IgG_1

Figure 7.2. Comparison of IgG_1 primary responses to free or liposomal LCPH1 with or without IL-2



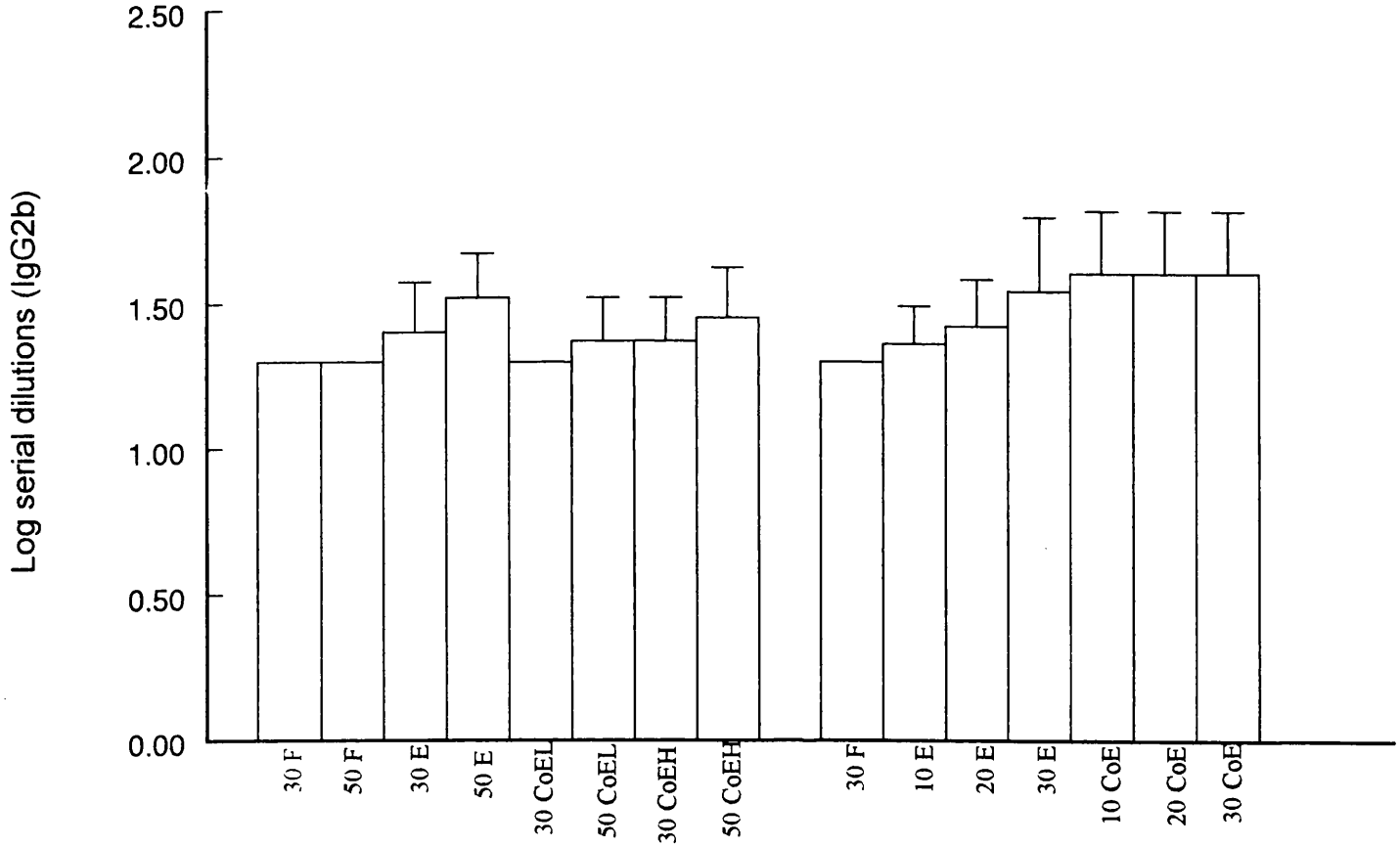
LCPH1 and/or IL-2 were entrapped in DRV composed of PC:cholesterol (1:1 molar ratio). All preparations were diluted with PBS so that each animal received a dose of 10, 20, 30 or 50 μg peptide. F: free peptide; E: entrapped; CoE: co-entrapped with IL-2; L: low IL-2 dose; H: high IL-2 dose.

Figure 7.3. Comparison of IgG_{2a} primary responses to free or liposomal LCPH1 with or without IL-2



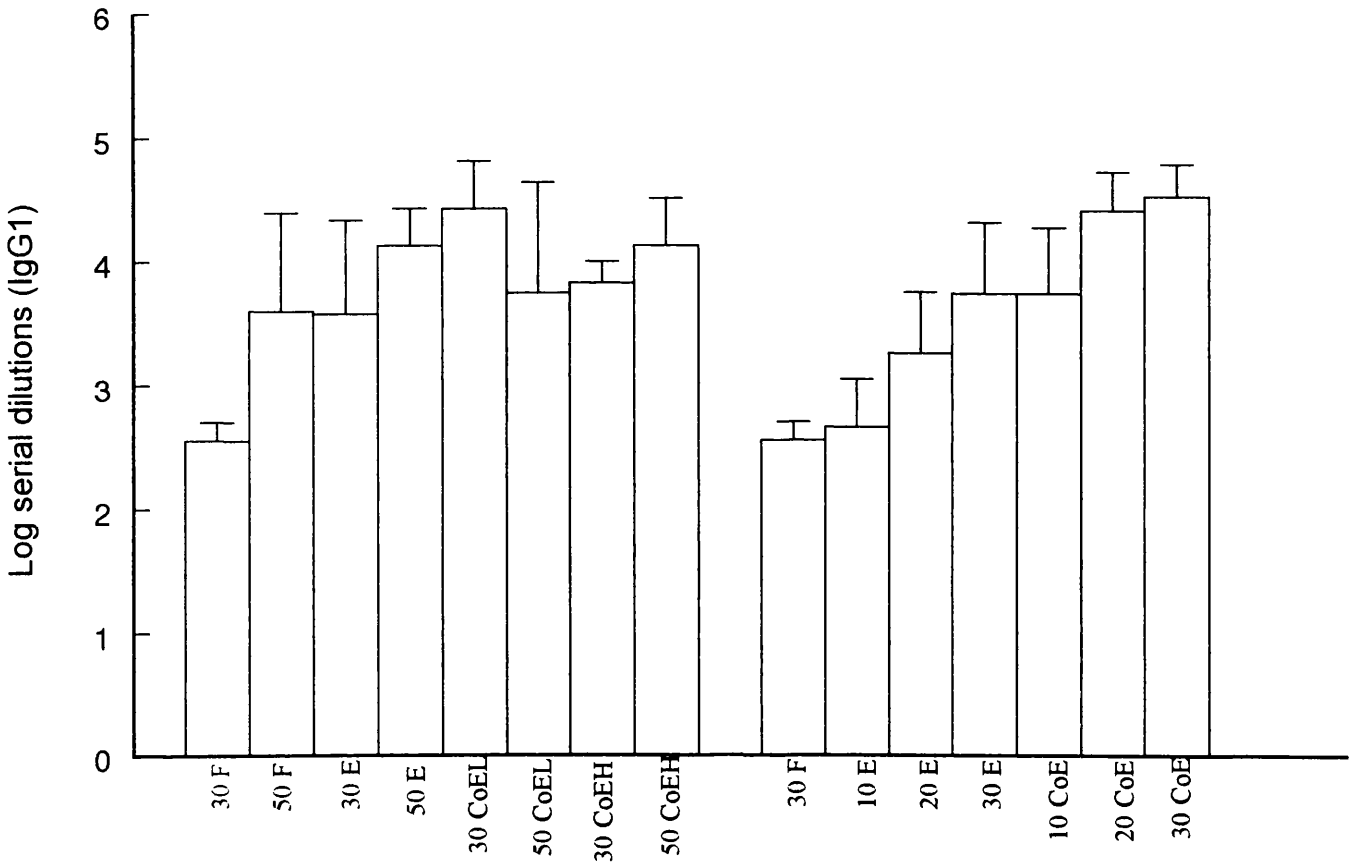
LCPH1 and/or IL-2 were entrapped in DRV composed of PC:cholesterol (1:1 molar ratio). All preparations were diluted with PBS so that each animal received a dose of 10, 20, 30 or 50 μg peptide. F: free peptide; E: entrapped; CoE: co-entrapped with IL-2; L: low IL-2 dose; H: high IL-2 dose.

Figure 7.4. Comparison of IgG_{2b} primary responses to free or liposomal LCPH1 with or without IL-2



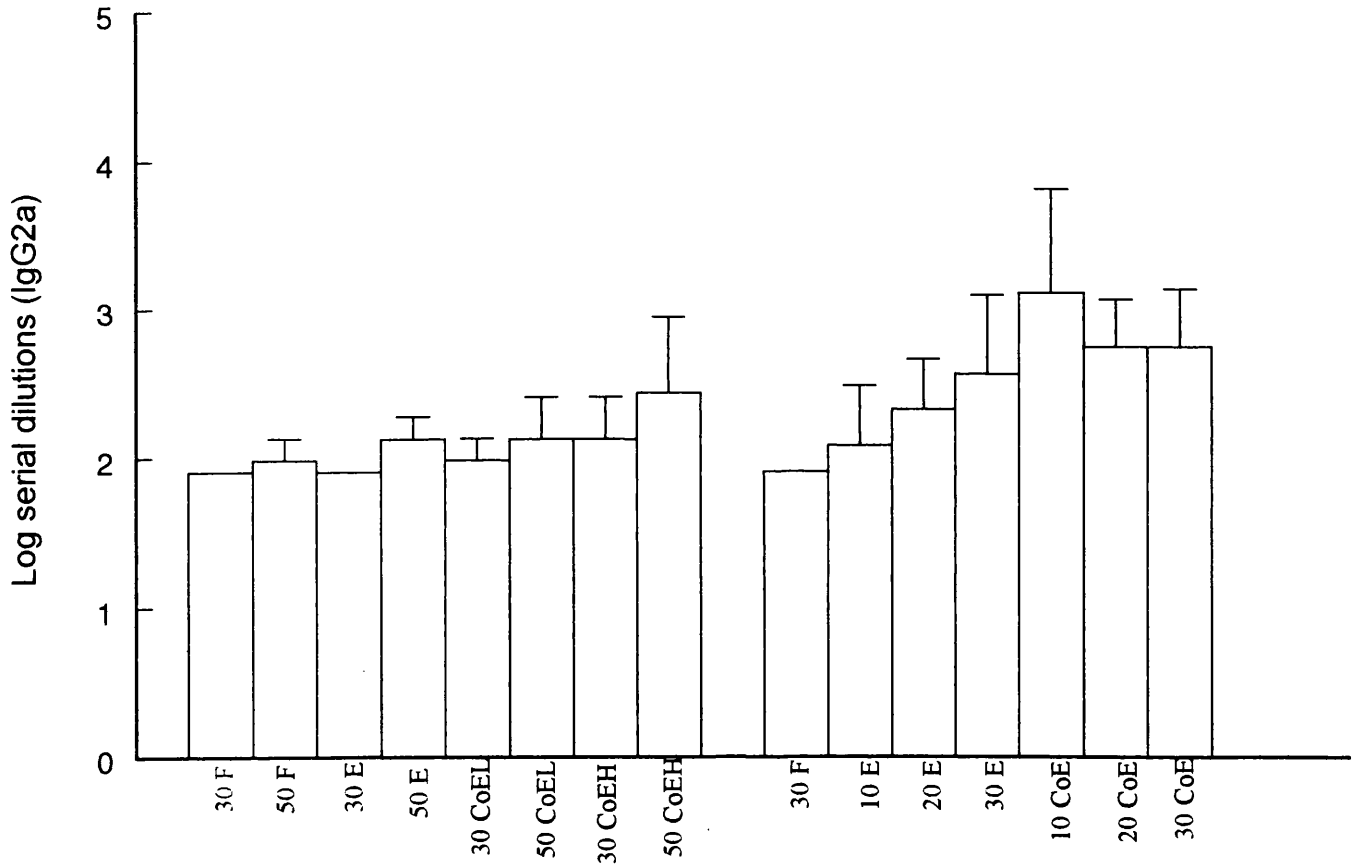
LCPH1 and/or IL-2 were entrapped in DRV composed of PC:cholesterol (1:1 molar ratio). All preparations were diluted with PBS so that each animal received a dose of 10, 20, 30 or 50 μg peptide. F: free peptide; E: entrapped; CoE: co-entrapped with IL-2; L: low IL-2 dose; H: high IL-2 dose.

Figure 7.5. Comparison of IgG_1 secondary responses to free or liposomal LCPH1 with or without IL-2



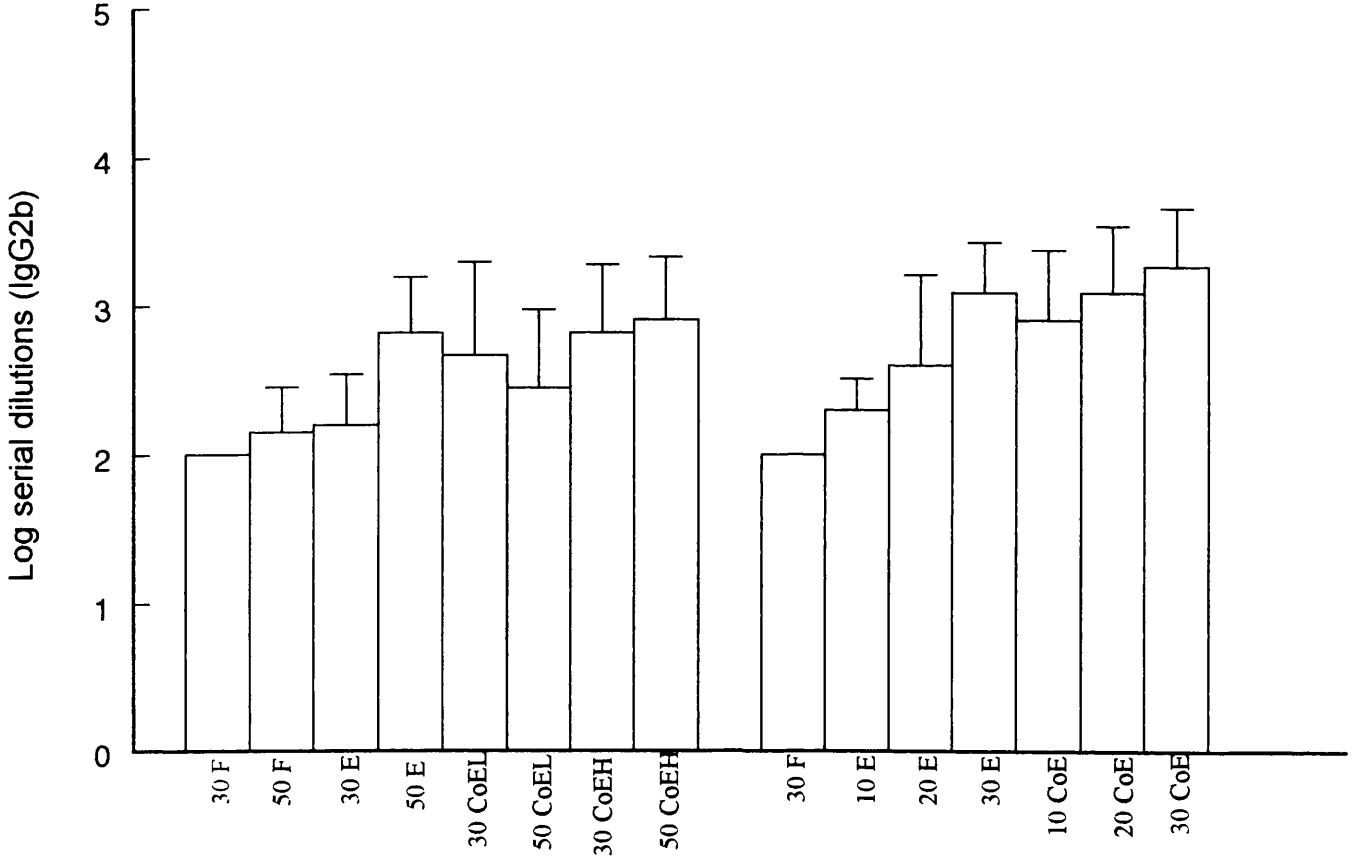
LCPH1 and/or IL-2 were entrapped in DRV composed of PC:cholesterol (1:1 molar ratio). All preparations were diluted with PBS so that each animal received a dose of 10, 20, 30 or 50 μg peptide. F: free peptide; E: entrapped; CoE: co-entrapped with IL-2; L: low IL-2 dose; H: high IL-2 dose.

Figure 7.6. Comparison of IgG_{2a} secondary responses to free or liposomal LCPH1 with or without IL-2



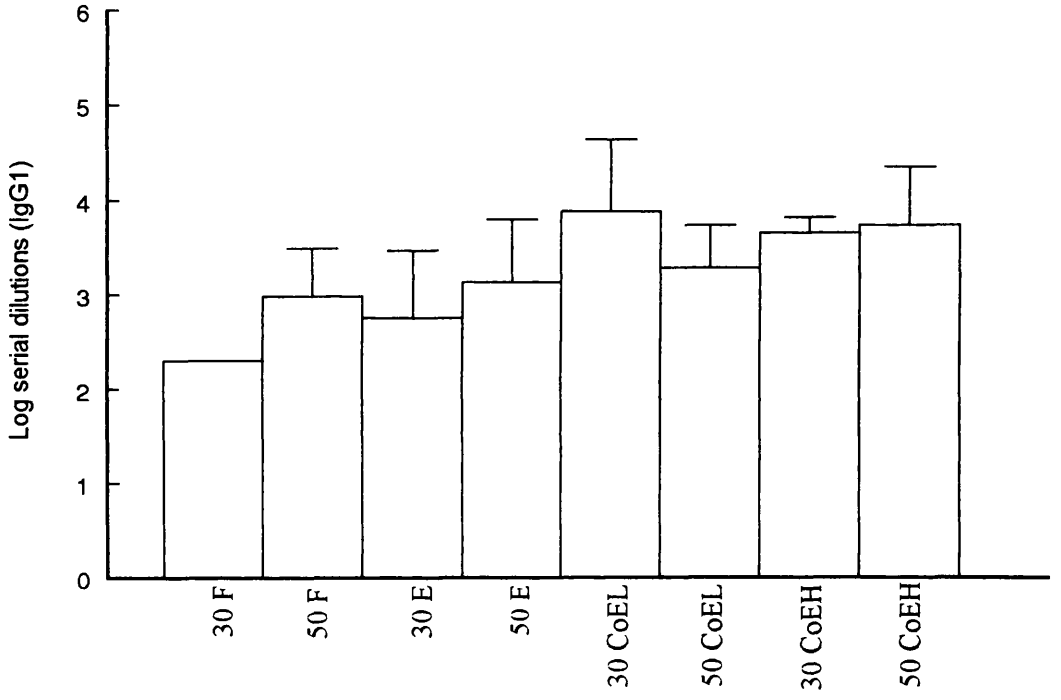
LCPH1 and/or IL-2 were entrapped in DRV composed of PC:cholesterol (1:1 molar ratio). All preparations were diluted with PBS so that each animal received a dose of 10, 20, 30 or 50 μg peptide. F: free peptide; E: entrapped; CoE: co-entrapped with IL-2; L: low IL-2 dose; H: high IL-2 dose.

Figure 7.7. Comparison of IgG_{2b} secondary responses to free or liposomal LCPH1 with or without IL-2



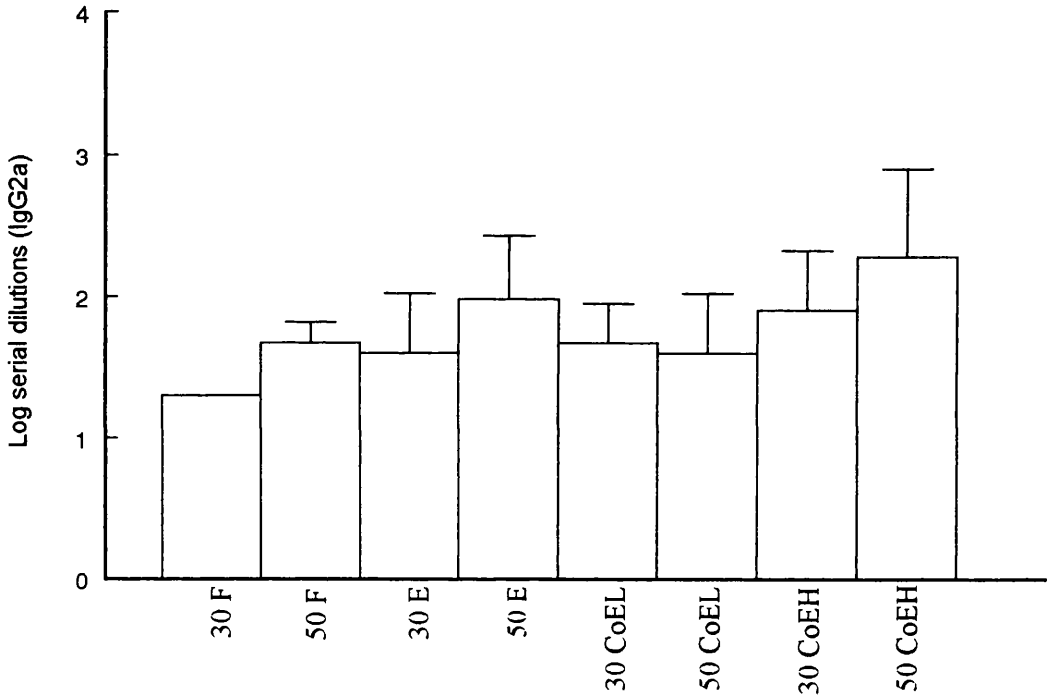
LCPH1 and/or IL-2 were entrapped in DRV composed of PC:cholesterol (1:1 molar ratio). All preparations were diluted with PBS so that each animal received a dose of 10, 20, 30 or 50 μg peptide. F: free peptide; E: entrapped; CoE: co-entrapped with IL-2; L: low IL-2 dose; H: high IL-2 dose.

Figure 7.8. Comparison of IgG_1 long-term responses to free or liposomal LCPH1 with or without IL-2



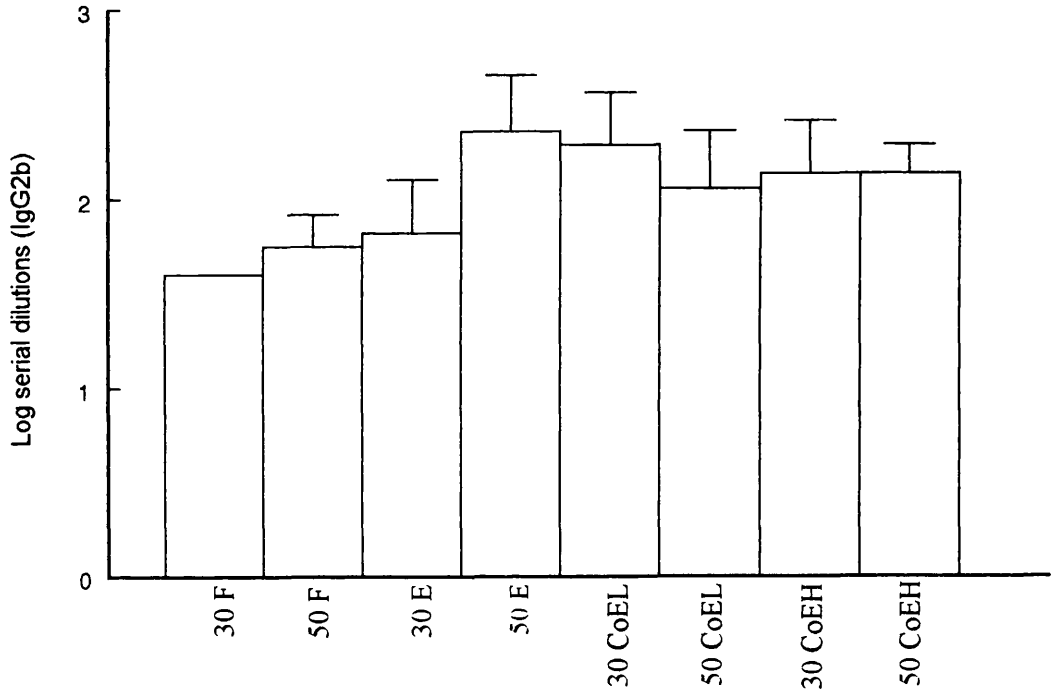
LCPH1 alone or together with IL-2 were entrapped in DRV composed of PC:cholesterol (1:1 molar ratio). All preparations were diluted with PBS so that each animal received a dose of 30 or 50 μg peptide. F: free peptide; E: entrapped; CoE: co-entrapped; L: low IL-2 dose; H: high IL-2 dose.

Figure 7.9. Comparison of IgG_{2a} long-term responses to free or liposomal LCPH1 with or without IL-2



LCPH1 alone or together with IL-2 were entrapped in DRV composed of PC:cholesterol (1:1 molar ratio). All preparations were diluted with PBS so that each animal received a dose of 30 or 50 μg peptide. F: free peptide; E: entrapped; CoE: co-entrapped; L: low IL-2 dose; H: high IL-2 dose.

Figure 7.10. Comparison of IgG_{2b} long-term responses to free or liposomal LCPH1 with or without IL-2



LCPH1 alone or together with IL-2 were entrapped in DRV composed of PC:cholesterol (1:1 molar ratio). All preparations were diluted with PBS so that each animal received a dose of 30 or 50 μg peptide. F: free peptide; E: entrapped; CoE: co-entrapped; L: low IL-2 dose; H: high IL-2 dose.

titers showed that only the 30 μg coentrapped formulations (both IL-2 doses) were still superior to the free peptide ($P<0.02$ for the low IL-2 and $P<0.001$ for the high IL-2 dose). The same was also true for the IgG_{2b} long term responses with the 30 μg coentrapped formulations being superior to the free peptide ($P<0.02$ for the low IL-2 dose and $P<0.05$ for the high IL-2 dose). There were no differences between groups in terms of IgG_{2a} long term responses.

7.3.3. Immune responses to LCPH1 entrapped in DRV with or without IL-2 (10, 20 and 30 microgram doses)

The primary and secondary IgG_1, IgG_{2a} and IgG_{2b} responses to 10, 20 and 30 μg entrapped and coentrapped peptide are presented in Figures 7.2-7.7. As was the case with higher peptide doses, there were no differences between free or entrapped and coentrapped preparations in primary responses for all subclasses tested. The 30 μg entrapped and coentrapped formulations elevated the IgG_1 secondary titers significantly when compared to the free sample ($P<0.02$ and $P<0.001$, respectively). Whilst responses obtained with 30 μg peptide of entrapped and coentrapped preparations did not differ from each other significantly, responses with the lower doses of 10 and 20 μg coentrapped formulations were superior to their (peptide only) entrapped counterparts ($P<0.05$ and $P<0.02$, respectively) for the same IgG subclass. These same preparations were even superior to the 30 μg free peptide. All the coentrapped peptide formulations augmented the IgG_{2a} responses significantly ($P<0.02$ with 10 μg and $P<0.01$ with 20 and 30 μg doses) when compared to the 30 μg free peptide. The entrapped peptide was, however, inefficient. The 20 μg coentrapped formulation was also superior to its (peptide only) entrapped counterpart ($P<0.05$).

Except for the 20 μg (peptide only) entrapped formulation, all others improved the IgG_{2b} titers significantly ($P<0.05$ for 10 μg entrapped, $P<0.02$ for 10 μg coentrapped and $P<0.01$ for 30 μg entrapped and 20 and 30 μg coentrapped preparations). Coentrapment did not prove to be superior over the entrapped peptide alone when the relevant doses were compared with each other.

7.4. CONCLUSION

Over the range of different peptide doses used in this study, it was found that an immunoadjuvant effect of liposomes could be seen when an intermediate peptide dose (30 μg) was administered to the animals. Above this amount, free peptide was equally effective and below the 30 μg dose, liposomes could not act as an adjuvant alone. However, incorporation of IL-2 together with the peptide antigen in the same liposomes maintained the antibody titers and even boosted the secondary responses (IgG_{2a}) when a low peptide dose was used (up to 30 μg). This might have a practical advantage in the development of a vaccine since the peptide dose used in the coentrapped formulation can be one fifth of the amount used in the free form.

In the work by Zhong et al (1993), although LCPH1 administration to animals resulted in high antibody titers, the neutralization titers for the whole organism were low suggesting that the conformation of the antigenic epitopes on *C.trachomatis* is of importance and the peptide construct does not fulfill this requirement completely. Entrapment or covalent coupling of an antigen in liposomes might alter the conformation of the molecule (Friede et al, 1994), and it thus remains to be seen

whether the neutralization ability of LCPH1 entapped in DRV (especially in the presence of IL-2 as a coadjuvant) is superior to the free peptide.

Although in the case of bacterial toxins antibodies of any subclass with sufficiently high binding affinities can protect the host, for protection against many microorganisms, antibodies of isotypes that can activate complement and bind to receptors on antibody-dependent effector cells are required. In mouse, IgG_{2a} is the isotype that induces better protection against infectious agents (Allison and Byars, 1986). The presence of IgG_{2a} specific for the infectious agent confers greater specificity for the cytolytic activity of natural killer cells by mediating antibody dependent cellular cytotoxicity (Yuan et al, 1994). Since *C.trachomatis* is an organism adapted to both intracellular and extracellular survival, antibodies of protective nature such as IgG_{2a} become of utmost importance in the provision of protection against the pathogen. This fact is well demonstrated with influenza virus vaccines which, due to very low levels of IgG_{2a} antibodies induced by the vaccine (Ahmeida et al, 1993) cannot induce an immunity in mice comparable to that seen following live virus infection. The results obtained here, indicate that IL-2 in liposomes might improve the titers of this subclass. Nevertheless, experiments related to the neutralization ability of these antibodies must be conducted and compared with those obtained by the free peptide alone.

CHAPTER 8

USE OF INTERLEUKIN-12 AS A CO-ADJUVANT IN LIPOSOMES

8.1. INTRODUCTION

Interleukin-12 (IL-12) is a cytokine produced by macrophages and B-cells (Kobayashi et al, 1989; D'Andrea et al 1992; Stern et al, 1990) and to a lesser extent from keratinocytes (Aragane et al, 1994) induces IFN-γ production (Tripp et al, 1993; Alzona et al, 1995; Seder et al, 1993) from T-cells, enhances proliferation and cytotoxic function of NK cells (Bonnema et al, 1994), promotes Th1-clone differentiation (Yamagida et al, 1994; McKnight et al, 1994; Trinchieri, 1993; Schmitt et al, 1994) and recently was also shown to be a potent co-stimulus of B-cell differentiation and proliferation (Jelinek and Braaten, 1995). The use of this cytokine in vivo was proven to be effective for the treatment of experimental visceral Leishmaniasis (Murray and Hariprashad, 1995), acted as an effective adjuvant in a vaccine against Leishmania major (Afonso et al, 1994), reversed some of the immune abnormalities observed in Sezary syndrome (Rook et al, 1995). Despite its attractive immunomodulating effects, IL-12, in doses in excess of 100 ng/day was shown to be hepatoxic and resulted in profound immunotoxicity in mice infected with lymphocytic choriomeningitis virus (Orange et al, 1994 and 1995). Use of IL-12 in liposomes might be particularly advantageous in reducing such unwanted side-effects and also would eliminate the requirement for multiple injections by reducing the clearance rate for the cytokine. Based on the information from the work by Tan and Gregoriadis (1989) and the results presented in Chapter 6, one can say that the formulation type in which the

antigen and the co-adjuvant is contained can alter the immune responses especially with respect to the IgG subclasses produced. Therefore, here we have investigated the role of IL-12 as a co-adjuvant as such or in different liposomal preparations using tetanus toxoid as a model antigen. The cytokine was either co-injected with the antigen in free form or it was administered in separately entrapped, coentrapped or co-surface linked formulations. An entrapped and surface linked toxoid alone was also used as controls. Three different IL-12 doses were used in all groups (a low, intermediate and a high dose) except for the surface linked preparations where only the low and intermediate doses were used.

8.2. METHODS

DRV entrapping tetanus toxoid and/or IL-12 were prepared as previously described in Section 2.2.4.3. Covalent coupling of proteins on the surface of the same vesicle was carried out as give in Section 2.2.7.2. The amounts of tetanus toxoid and IL-12 mixed with the vesicles are presented in Table 8.1. Entrapment and linkage values of toxoid were calculated using the ^{125}I tracer of the protein. The amount of IL-12 entrapped in or covalently linked on liposomes was assessed from the PHA-stimulated human T lymphocyte growth promoting ability of the cytokine (Section 2.2.13.5). For this, in preliminary experiments liposomes were disrupted by Triton X-100 filter sterilized, and the liberated cytokine was tried to be quantitated but since the detergent also probably lysed the cells no reproducible results could be obtained. Thus IL-12 was quantitated using the filter sterilized wash solutions devoid of any liposomes or detergent. This approach also avoids any interference that can be encountered due to presence of certain phospholipids in liposomal form which were previously reported to synergize

with growth promoting effects of some cytokines (Joeffret et al, 1990; Cano et al, 1992). The growth of cells against an IL-12 standard at various concentrations as assessed by the ability of the cells to metabolize the dye MTT, is presented in Figure 8.1. The liposome formulation were then injected to animals (groups of four) after diluting the samples in PBS accordingly so that each animal received a dose of 0.1 μg in a total volume of 0.1 mL. Primary and secondary responses were determined by ELISA.

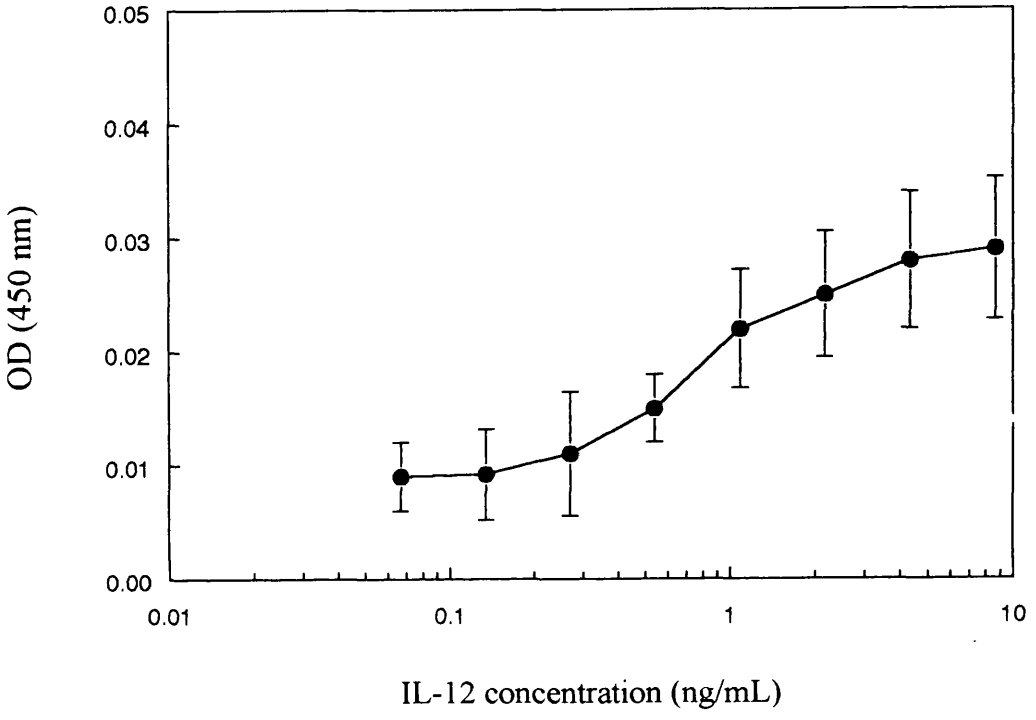
8.3. RESULTS

8.3.1. Toxoid and IL-12 content of liposomes

Values of tetanus toxoid and IL-12 entrapment or coupling for each of the liposome formulation prepared are shown in Table 8.1. Entrapment of the toxoid alone or together with IL-12 was quite substantial and reproducible (25.14-32.5 % of the amount used) and is in agreement with the previous studies. Entrapment values for IL-12 in liposomes was found to be 27.22 % which was lowered when the cytokine was co-entrapped with the toxoid (20.02-21.81 %). As presented in Chapters 6 and 9, entrapment of IL-2 and IL-15 in liposomes were much higher (ca. 50 %). This was attributed to a possible interaction of IL-2 and IL-15 with the lipid bilayers. The same does not seem to apply for IL-12.

Covalent coupling of the toxoid or IL-12 on the surface of DRV resulted in 22.09-24.00 % (toxoid) and 13.67-14.21 % (IL-12) of the protein used being associated with the vesicles (Table 8.1). The values obtained for IL-12 is based on PHA-stimulated

Figure 8.1. PHA-activated T-cell proliferation assay with IL-12



The biological activity of IL-12 was assessed using the ability of this cytokine to induce growth of PHA-activated human peripheral blood T lymphocytes. Growth of cells were deduced from the colour of metabolized mitochondrial stain MTT. The amount of IL-12 present in liposomes was quantitated from the unentrapped wash solutions by extrapolating the OD values for each sample to the linear portion of the graph.

Table 8.1. Preparation conditions and entrapment or covalent coupling values of DRV formulations used in immunizations studies

Preparations	Amount of TT mixed with SUV or DRV	% Toxoid entrapment or linkage	μg of IL-12 mixed with SUVor DRV	% IL-12 entrapment or linkage	μg of IL-12 in the final preparation
Free TT & IL-12	-	-	-	-	0.1000
Free TT & IL-12	-	-	-	-	1.0000
Free TT & IL-12	-	-	-	-	10.0000
Sep.Ent. TT & IL-12	50 μg	32.50	40.00	27.22	0.0926
Sep.Ent. TT & IL-12	50 μg	32.50	40.00	27.22	0.9260
Sep.Ent. TT & IL-12	50 μg	32.50	40.00	27.22	9.2600
Coentrapped TT & IL-12	4 μg	30.02	0.35	21.81	0.0635
Coentrapped TT & IL-12	4 μg	31.68	3.50	21.25	0.5868
Coentrapped TT & IL-12	4 μg	25.14	35.00	20.02	7.0007
Co-SL TT & IL-12	8 μg	22.09	0.50	14.21	0.0403
Co-SL TT & IL-12	8 μg	24.00	5.00	13.67	0.3561

SUV composed of equimolar PC and cholesterol and APSA in the case of surface linkage studies (1:1:0.05) were mixed with toxoid alone, IL-12 alone or a mixture of these to generate DRV. Toxoid and IL-12 were covalently coupled on the surface of the same vesicle. Entrapment or linkage calculations for the toxoid are based on ^{125}I radioactivity measurements. The same values for IL-12 was assayed by the ability of the cytokine to promote growth of PHA-stimulated human lymphoblasts. All preparations were diluted with PBS accordingly so that each animal received a dose of 0.1 μg toxoid in a total volume of 0.1 mL.

PMBC proliferation assay which measures the biologically active cytokine, thus the real values of linkage might in fact be higher for IL-12 but reflected as lower amounts due to a possible inactivation during the covalent coupling process.

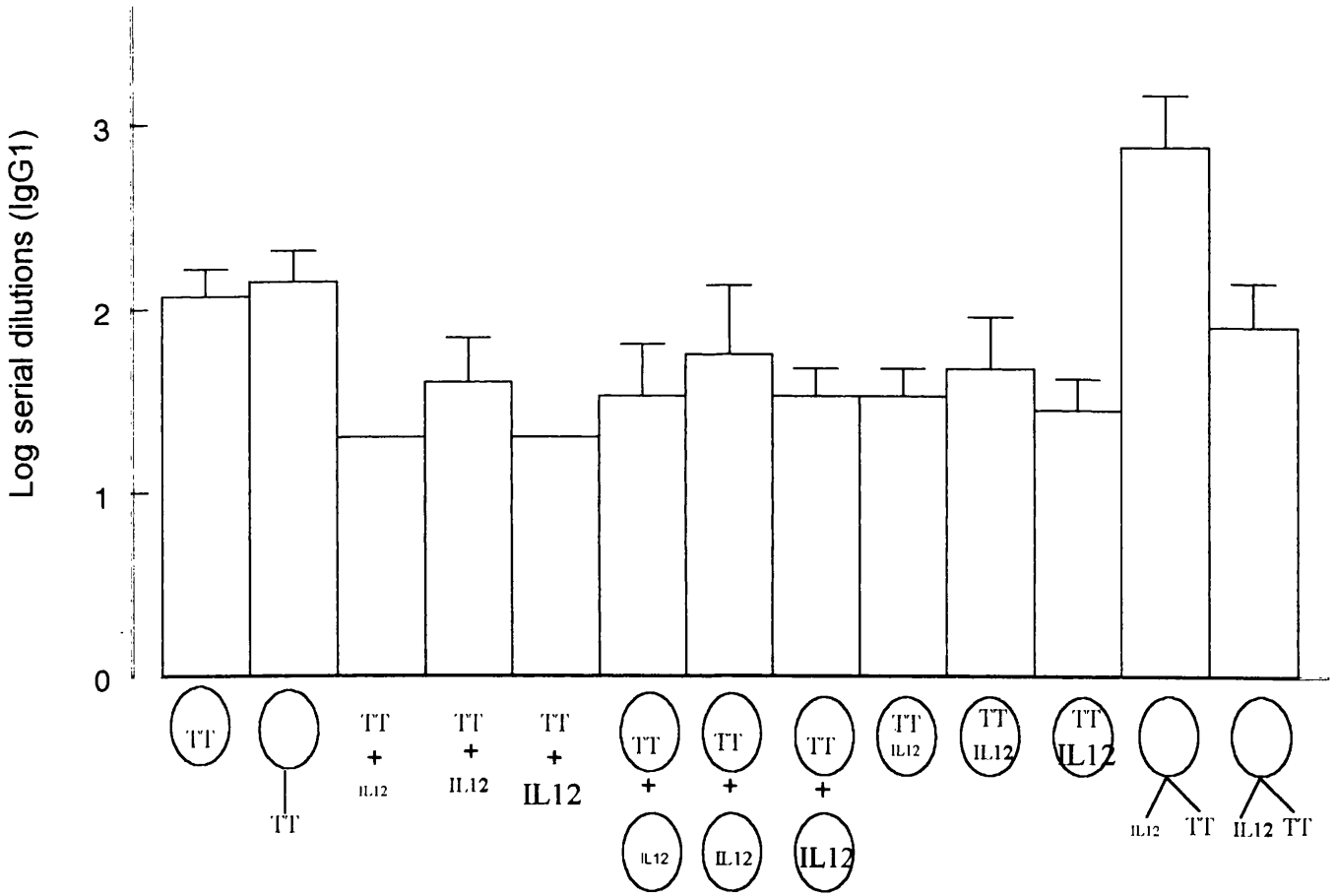
8.3.2. Immunization studies

8.3.2.1. Primary responses to liposomal toxoid and IL-12

The adjuvant activity of IL-12 in liposomes was investigated in experiments where groups of mice were immunized with 0.1 μg toxoid alone or in the presence of three doses of IL-12 (except for one preparation where only 2 doses were used) in various formulations. This dose of the toxoid was shown to be only very slightly immunogenic in liposomal form (Chapter 3). The responses obtained with the cytokine containing groups were compared among themselves and to those of entrapped or covalently coupled toxoid alone.

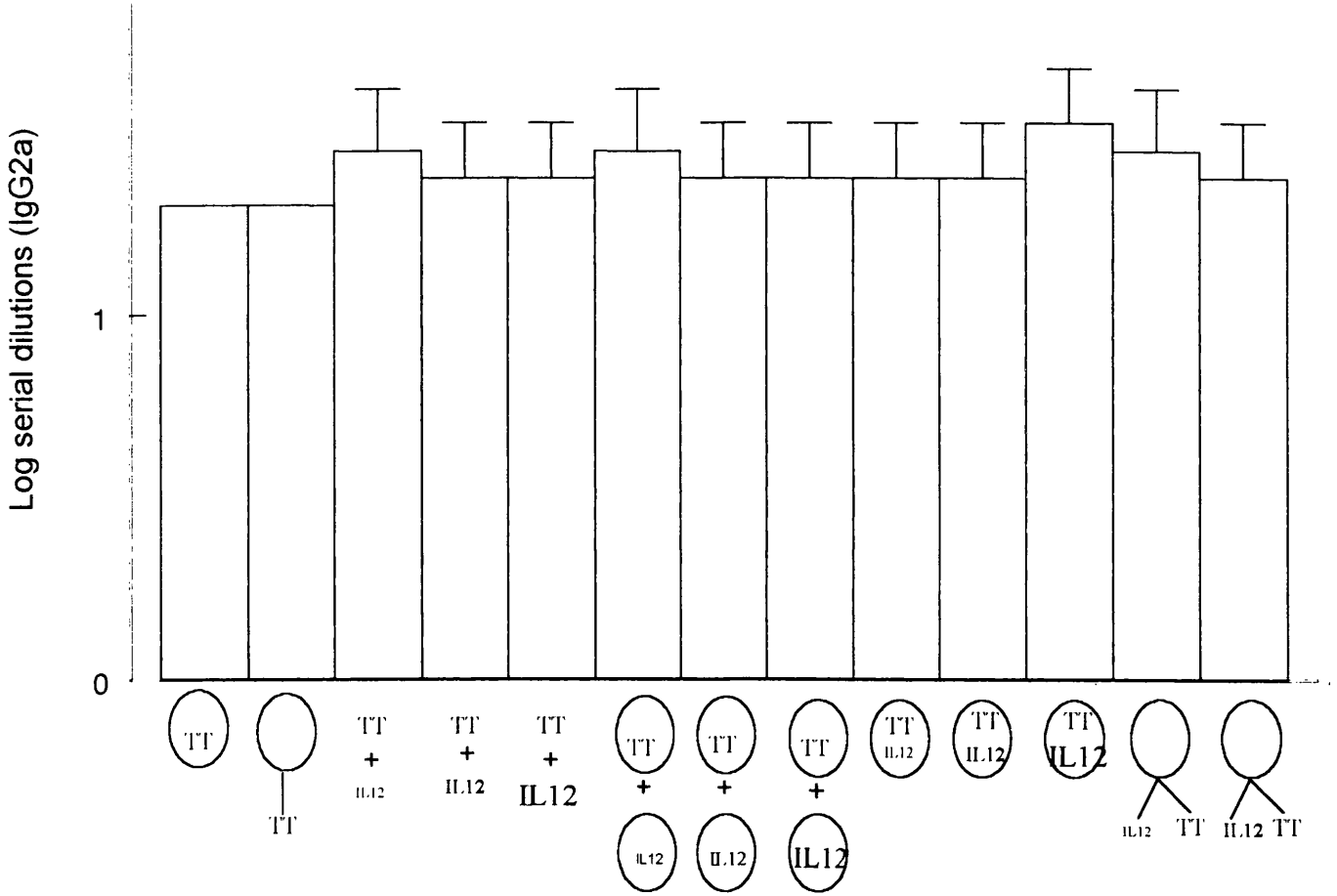
The primary IgG_1, IgG_{2a} and IgG_{2b} responses to tetanus toxoid in different formulations are shown in Figures 8.2 to 8.4, respectively. With all of the IL-12 containing formulations (except the co-surface linked preparation with the lowest dose of the cytokine), there was a suppression in IgG_1 primary responses. Liposomal toxoids alone were superior to the free IL-12-toxoid combination (low and high doses, the surface linked toxoid also being better than the intermediate dose), separately entrapped (high dose for both entrapped and surface linked toxoid and low dose in the case of covalent coupling) and coentrapped (low and high doses) formulations for this antibody subclass. Interestingly, IL-12 when covalently coupled on the surface of

Figure 8.2. Comparison of IgG_1 primary responses to free or liposomal tetanus toxoid with or without IL-12



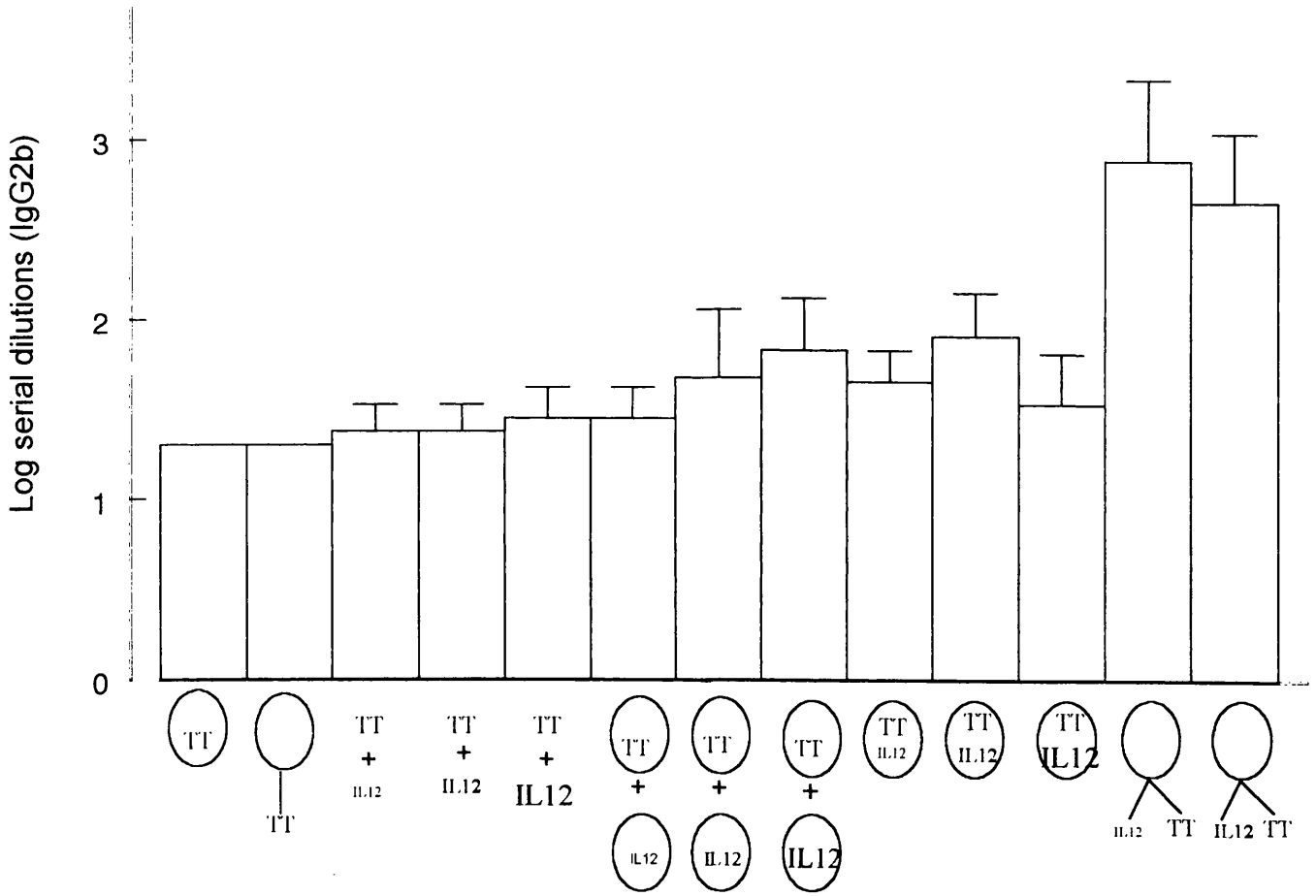
Tetanus toxoid and/or IL-12 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-12: low dose; IL-12:intermediate dose; IL-12: high dose.

Figure 8.3. Comparison of IgG_{2a} primary responses to free or liposomal tetanus toxoid with or without IL-12



Tetanus toxoid and/or IL-12 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-12: low dose; IL-12:intermediate dose; IL-12: high dose.

Figure 8.4. Comparison of IgG_{2b} primary responses to free or liposomal tetanus toxoid with or without IL-12



Tetanus toxoid and/or IL-12 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-12: low dose; IL-12:intermediate dose; IL-12: high dose.

Table 8.2. Comparison of primary responses

Preparations	Ent. TT	SL TT	Sep.Ent.(high)	CoEnt.(low))	CoEnt.(int)	Co-SL (low)		Co-SL(high)	
	IgG_1	IgG_1	IgG_{2b}	IgG_{2b}	IgG_{2b}	IgG_1	IgG_{2b}	IgG_1	IgG_{2b}
Ent. TT			*	*	**	**	***		***
SL TT			*	*	**	**	***		***
IL-12+TT (low)	***	***			*	***	***	**	***
IL-12+TT (int)		*			*	***	***		***
IL-12+TT (high)	***	***				***	***	**	**
Sep.Ent. (low)		*				***	***		**
Sep.Ent. (int)						**	*		*
Sep.Ent. (high)	*	**				***	*		*
CoEnt. (low)	*	**				***	**		**
CoEnt. (int)						***	*		*
CoEnt. (high)	**	**				***	**		**
Co-SL (low)									
Co-SL (int)						**			

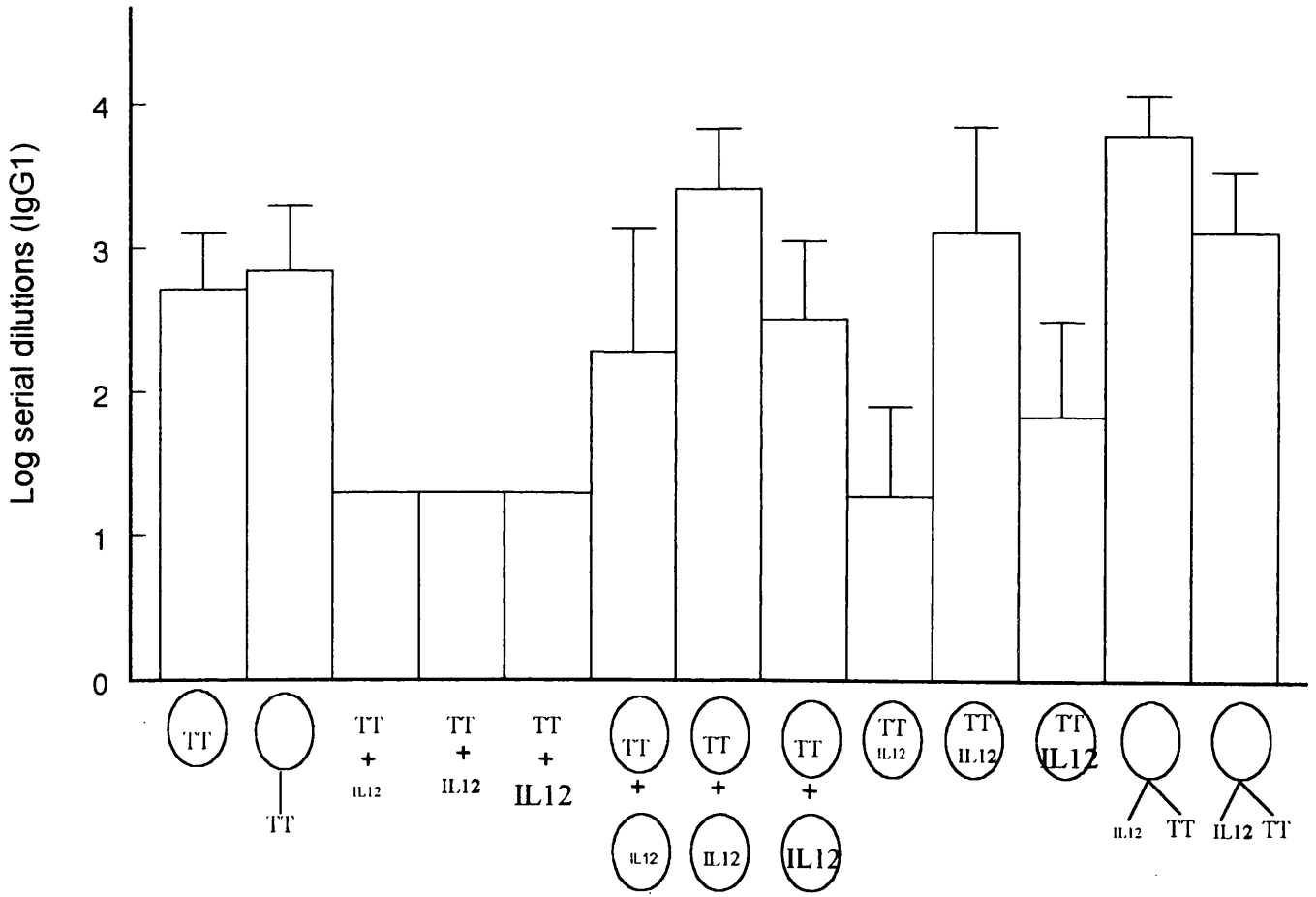
Tetanus toxoid and/or IL-12 were either entrapped alone or together in DRV composed of PC:cholesterol (1:1) or were covalently coupled on the surface of DRV composed of PC:cholesterol:APSA (1:1:0.05). Only the formulations that improved the antibody titers are given in the table. Stars represent the significance levels (*, $P<0.05$; **, $P<0.02$; ***, $P<0.01$). TT: tetanus toxoid; Ent.: entrapped; CoEnt.: coentrapped; Sep.Ent.: separately entrapped; Co-SL: co-surface linked; low: low IL-12 dose; int: intermediate IL-12 dose; high: high IL-12 dose.

liposomes (low dose) together with the antigen increased the IgG_1 primary titers significantly when compared to all other groups. However, the co-surface linked formulation with the intermediate IL-12 dose was only superior to the free cytokine-antigen combination (low and high doses). For a detailed statistical comparison among groups, see Table 8.2. None of the groups differed significantly in terms of IgG_{2a} primary responses. The co-surface linked formulations (both doses) enhanced the IgG_{2b} primary responses significantly when compared to all other formulations. The separately entrapped (highest dose) and the coentrapped (lowest and intermediate doses) preparations both improved the titers of this subclass when compared to liposomal toxoid alone. The coentrapped (intermediate dose) formulation was also superior to the free toxoid-IL-12 combination (lowest and intermediate doses).

8.3.2.2. Secondary responses to liposomal toxoid and IL-12

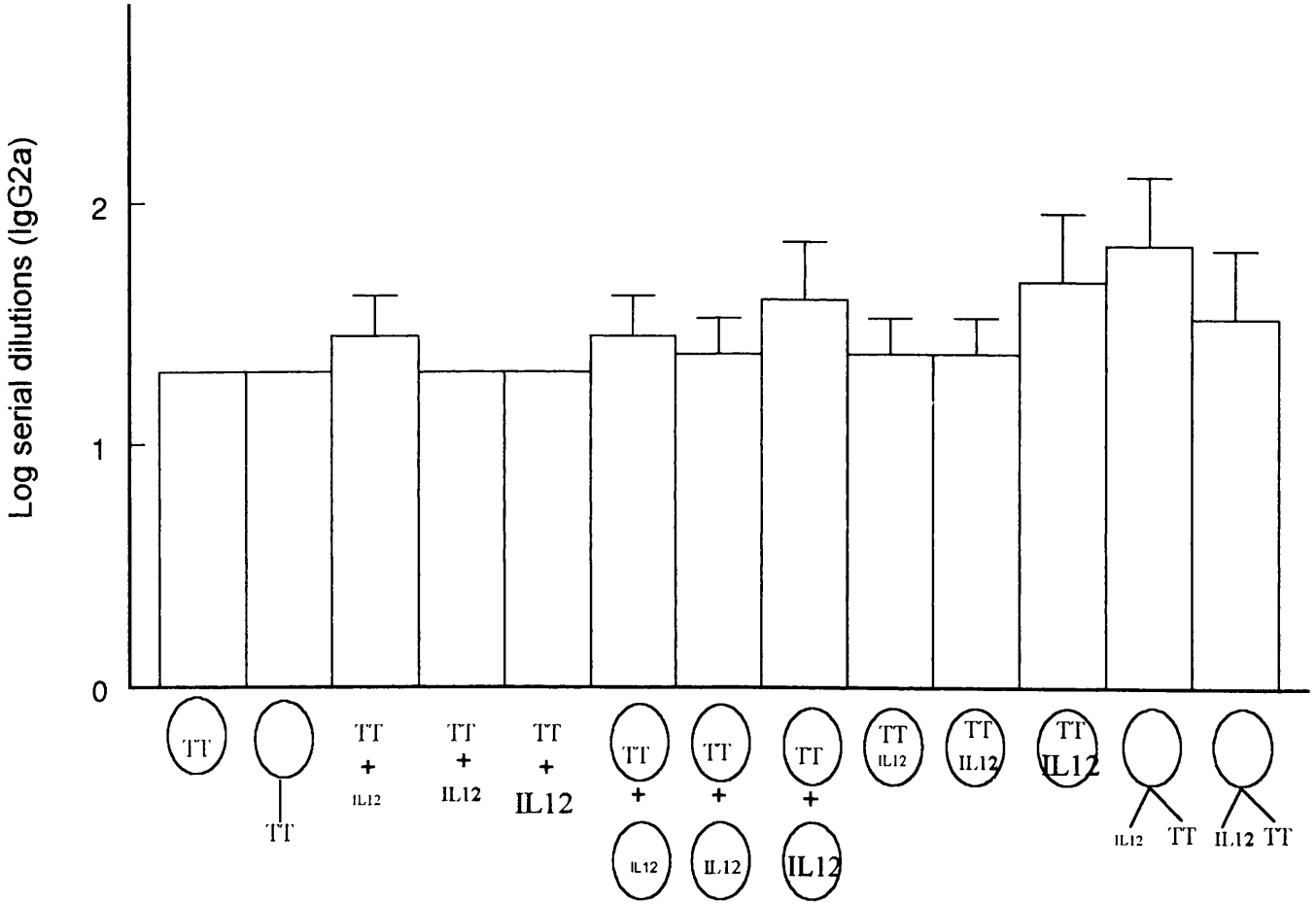
Figures 8.5 to 8.7 present the secondary anti toxoid IgG_1, IgG_{2a} and IgG_{2b} responses, respectively. When the IgG_1 secondary responses were analyzed, there was a severe supression seen in groups immunized with free antigen-free IL-12 combination (all three doses) when compared to liposomal toxoid alone ($P<0.01$ for both cases). As was the case with the primary IgG_1 primary response, the co-surface linked formulation (lowest dose) enhanced the titers significantly and was superior to all groups except for the separately entrapped (intermediate dose), co-entrapped (intermediate dose) and co-surface linked (intermediate dose) preparations. None of the separately entrapped or co-entrapped preparations differed significantly from the liposomal toxoid alone. Another observation was that, for the separately entrapped and the coentrapped formulations, the three IL-12 doses employed yielded a bell-shaped

Figure 8.5. Comparison of IgG_1 secondary responses to free or liposomal tetanus toxoid with or without IL-12



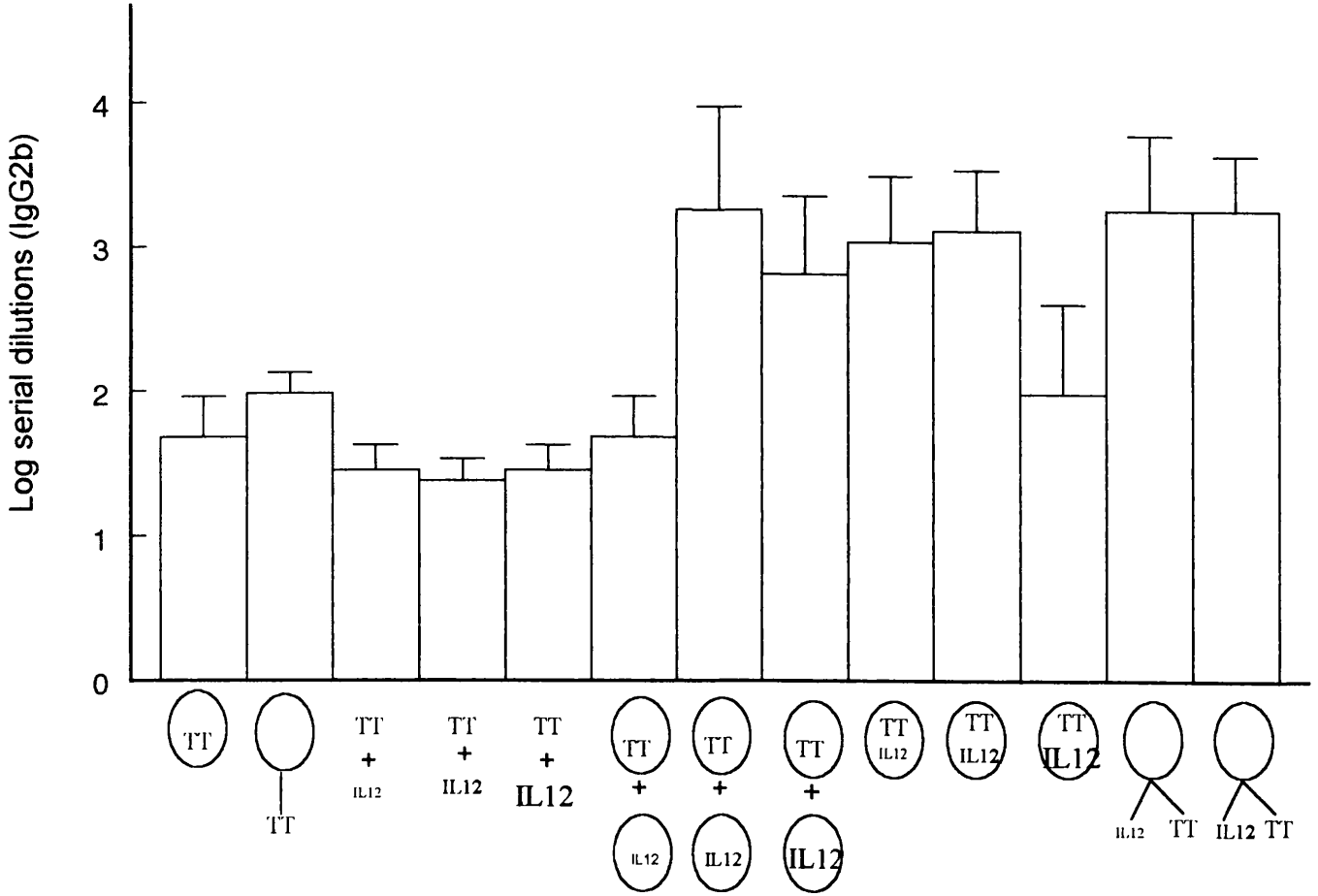
Tetanus toxoid and/or IL-12 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-12: low dose; IL-12:intermediate dose; IL-12: high dose.

Figure 8.6. Comparison of IgG_{2a} secondary responses to free or liposomal tetanus toxoid with or without IL-12



Tetanus toxoid and/or IL-12 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-12: low dose; IL-12:intermediate dose; IL-12: high dose.

Figure 8.7. Comparison of IgG_{2b} secondary responses to free or liposomal tetanus toxoid with or without IL-12



Tetanus toxoid and/or IL-12 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-12: low dose; IL-12:intermediate dose; IL-12: high dose.

Table 8.3. Comparison of secondary responses

Preparations	Ent. TT	SL TT		Sep.Ent.(int)		Sep.Ent.(high)		CoEnt.(low)	CoEnt.(int)		Co-SL(low)		Co-SL(int)		
	IgG_1	IgG_1	IgG_{2b}	IgG_1	IgG_{2b}	IgG_1	IgG_{2b}	IgG_{2b}	IgG_1	IgG_{2b}	IgG_1	IgG_{2b}	IgG_1	IgG_{2a}	IgG_{2b}
Ent. TT					*		*	**		**	*	**		*	***
SL TT					*			*		**	*	**		*	***
IL-12+TT (low)	***	***	**	***	**	*	**	***	**	***	****	***	***		***
IL-12+TT(int)	***	***	**	***	**	*	**	***	**	***	****	***	***	*	***
IL-12+TT(high)	***	***	**	***	**	*	**	***	**	***	****	***	***	*	***
Sep.Ent.(low)					*		*	**		**	*	**			***
Sep.Ent. (int)															
Sep.Ent. (hugh)											*				
CoEnt. (low)				*							**		*		
CoEnt.(int)															
CoEnt(high)				*							**		*		*
Co-SL(low)															
Co-SL(int)															

Tetanus toxoid and/or IL-12 were either entrapped alone or together in DRV composed of PC:cholesterol (1:1) or were covalently coupled on the surface of DRV composed of PC:cholesterol:APSA (1:1:0.05). Only the formulations that improved the antibody titers are given in the table. Stars represent the significance levels (*, $P<0.05$; **, $P<0.02$; ***, $P<0.01$; ****, $P<0.001$). TT: tetanus toxoid; Ent.: entrapped; CoEnt.: coentrapped; Sep.Ent.: separately entrapped; Co-SL: co-surface linked; low: low IL-12 dose; int: intermediate IL-12 dose; high: high IL-12 dose.

response with the lowest and highest doses effectively suppresing the IgG_1 titers while the intermediate doses evoked titers comparable to those obtained with liposomal toxoid alone. The free toxoid-IL-12 combination resulted in lowered IgG_{2b} titers (all doses) when compared to surface-linked toxoid alone ($P<0.02$). On the other hand, Separately entrapped (intermediate and high doses), co-entrapped (low and intermediate doses) and co-surface linked (both doses) formulations boosted the secondary IgG_{2b} responses significantly (see Table 8.3 for a detailed comparison among groups). Although IL-12 is known to be a potent Th1 stimulant, its adjuvant effect on IgG_{2a} secondary responses was very modest. Only the co-surface linked formulation (lowest dose) boosted the titers significantly with respect to liposomal toxoid alone and free toxoid-IL-12 combinations (intermediate and higher doses) ($P<0.05$). The increase in titers obtained with the co-surface linked formulation was quite similar to the values obtained when IL-15 or IL-2 were used as co-adjuvants (Chapter 6 and 9, respectively).

8.4. CONCLUSION

The results presented in this chapter imply that the type of formulation in which an antigen and a cytokine is contained can result in evoking different immune responses. Contrary to our expectations based on the fact that IL-12 is a Th1-cell inducer inhibiting IgG_1 production (McKnight et al, 1994, Hsieh et al, 1993, Yanagida et al, 1994, Morris et al, 1994) the co-surface linked formulation with the lowest dose of IL-12 indeed boosted the IgG_1 titers significantly when compared to all other groups (for a statistical comparison among groups, see Table 8.2). Recently, IL-12 was shown to enhance the proliferation and differentiation of human B-cells (Jelinek and Braaten,

1995). It has also been suggested earlier that antigens linked on the surface of liposomes might favour a more direct interaction with specific membrane receptors on B-cells, thus using also these as antigen presenting cells besides the normal macrophage processing route (Therien et al, 1990, Shahum and Therien, 1994). It is also beleived that presentation by B-cells favours Th2-cell line activation (Therien et al, 1991), that results in IgG_1 subclass production via the action of IL-4 secreted by these cells (Seder and Paul, 1994). Thus, the co-surface linked formulation employed in this study with the lowest dose of IL-12 might be using B-cells for antigen presentation besides macrophages, and this low dose might as well be stimulatory for the alternative pathway. At a higher dose, Th1 stimulating activity of IL-12 might be dominant and thus would result in decreased IgG_1 titers. The lack of an enhancement with the separately entrapped and co-entrapped formulations supports this idea since these preparations would not be available for B-cell processing and presentation due to the masking of antigens by the liposomal bilayers, rendering them available only for macrophage processing. Liposomal IL-12 seems to result in a very different response than its free counterpart on the basis of these findings, and this can be also partly attributed to its altered biodistribution in vivo in liposomal form.

CHAPTER 9

USE OF INTERLEUKIN-15 AS A CO-ADJUVANT IN LIPOSOMES

9.1. INTRODUCTION

With certain groups of antigens where low or non-responsiveness is the major problem, use of cytokines such as interleukin-2 (IL-2) in liposomes was shown to improve the outcome (Tan and Gregoriadis, 1989; Ho et al, 1992; Abraham and Shah, 1992; Sencer et al, 1991, Mbawuike et al, 1990). This approach reduces toxicities encountered by high cytokine doses and eliminates the neccessity for multiple injections by increasing the half-life of the agent in blood (Anderson et al, 1992). Another advantage of cytokine-liposome preparations is their extended storage life (Anderson et al, 1994).

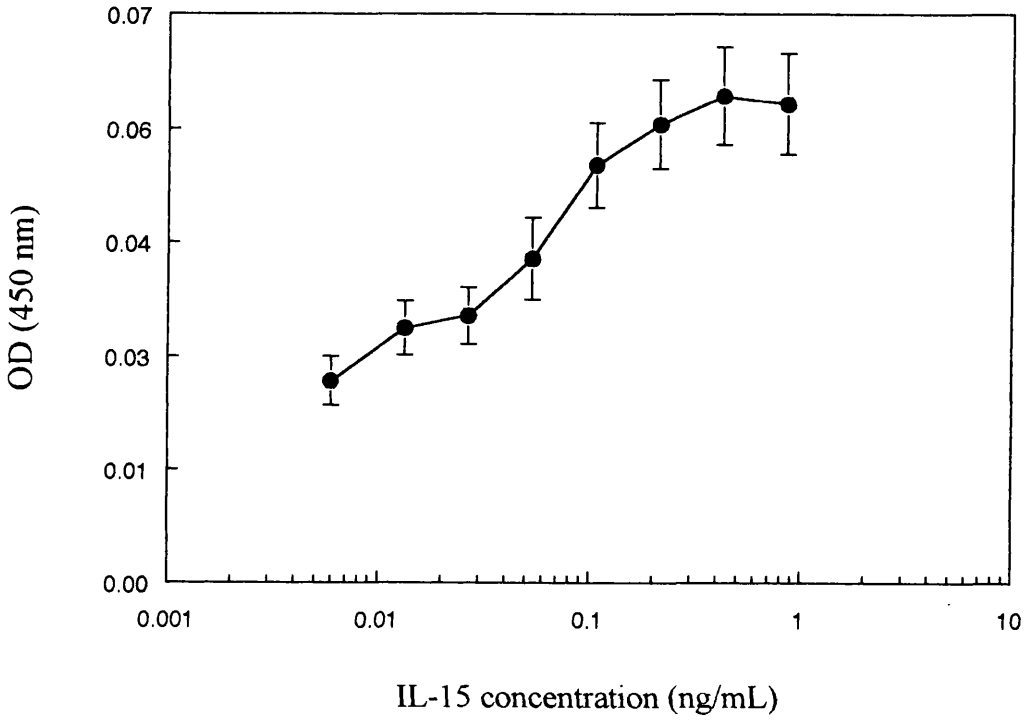
Interleukin-15 (IL-15) is a newly discovered cytokine (Grabstein et al, 1994) which has a very similar activity to that of IL-2 and interacts with the components of IL-2 receptor (Giri et al, 1994; Matthews et al, 1995; Carson et al, 1994). Another independent group has also isolated and characterized possibly the same cytokine and provisionally named it as IL-T (Burton et al, 1994; Bamford et al, 1994). Besides IL-15's T-cell growth promoting activity demonstrated in the studies mentioned above, the cytokine was also shown to be a chemoattractant for human blood T-cells (Wilkinson and Liew, 1995) and induced B-cell proliferation and differentiation in the presence of other stimuli such as phorbol ester (Armitage et al, 1995). Up to date there have been no in vivo results published with IL-15. Therefore,here we have analyzed

the co-adjuvant action of this new cytokine in various formulations together with our model antigen tetanus toxoid. Six different formulation containing IL-15 were administered to animals, each containing either a low or a high dose of the cytokine. IL-15 was either injected in free form together with the antigen (free or entrapped in liposomes) in the presence or absence of empty DRV, or in separately entrapped, co-entrapped and co-surface linked forms.

9.2. METHODS

DRV entrapping tetanus toxoid and/or IL-15 were prepared as previously described in Section 2.2.4.3. Covalent coupling of proteins on the surface of same liposomes was carried out as given in Section 2.2.7.2. The amounts of tetanus toxoid and IL-15 mixed with the vesicles are summarized in Table 9.1. Entrapment and covalent coupling values for the toxoid were obtained from ^{125}I radioactivity measurements. The amount of IL-15 entrapped in or covalently linked on DRV was assessed from the ability of the cytokine to promote the growth of murine CTLL-2 T lymphocytes (Section 2.2.13.3). Growth of cells were deduced from the colour of metabolized mitochondrial stain MTT (Section 2.2.13.6). IL-15 amounts in each sample was quantitated using the wash solutions (corresponding to the unentrapped or unlinked cytokine) to avoid any interferences coming from the presence of liposomal phospholipids and detergent by extrapolating the OD values for each sample to the linear portion of the graph. The growth of cells against different concentrations of an IL-15 standard is presented in Figure 9.1. The liposomal formulations were then injected to mice (in groups of 4) after diluting the samples in PBS accordingly so that each animal received a dose of μg in a total volume of 0.1 mL. Primary and secondary responses were determined by

Figure 9.1. T-cell proliferation assat with IL-15



The biological activity of IL-15 was assessed using the ability of this cytokine to induce the growth of murine CTLL-2 T lymphocytes. Growth of cells was deduced from the colour of metabolized mitochondrial stain MTT. The amount of IL-15 present in liposomes was quantitated from the unentrapped wash solutions by extrapolating the OD values for each sample to the linear portion of the graph.

ELISA. The amount of IL-15 adsorbed on DRV was assessed by incubating the cytokine (17.5 μg) with empty DRV at 4°C for a week (since this was the minimum time period taken for a liposomal IL-15 sample to be injected) and quantitating the protein in the liposomal pellet and the wash solutions using the bicinchoninic acid protein determination method (Section 2.2.6) against a BSA standard. Due to an enhancement in colour formation in the presence of detergent treated liposome, these in appropriate amounts were also included in standard tubes when assaying for the cytokine in the liposomal pellet.

9.3. RESULTS

9.3.1. Tetanus toxoid and IL-15 content of liposomes

Values of tetanus toxoid and IL-15 entrapment or coupling for each of the liposome formulation prepared are shown in Table 9.1. In agreement with previous studies (Gregoriadis et al, 1987; Tan and Gregoriadis, 1989,) entrapment of the toxoid was considerable and reproducible (29.7- 32.5 % of the amount used) even when the protein was co-entrapped with IL-15 (28.65-31.71 %). Interestingly, entrapment values for IL-15 were much higher (44.67 %) and very similar to values obtained when IL-2 was used (Tan and Gregoriadis,results presented in Chapter 6). It has been suggested (Tan and Gregoriadis, 1989) for IL-2 that the increased entrapment values might be the result of its hydrophobic nature which enables the incorporation of some of the protein into the lipid bilayers of DRV. Although IL-15 and IL-2 do not have sequence homologies, this might also be true for the former. In fact, the data which

Table 9.1. Preparation conditions and entrapment or covalent coupling values of DRV formulations used in immunizations studies

Preparations	Amount of TT mixed with SUV or DRV	% Toxoid entrapment or linkage	Units of IL-15 mixed with SUVor DRV	% IL-15 entrapment or linkage	Units of IL-15 in the final preparation
Free TT&IL-15	-	-	-	-	$1.5x10^3$
Free TT&IL-15	-	-	-	-	$1.50x10^4$
Free TT&IL-15+DRV	-	-	-	-	$1.50x10^3$
Free TT&IL-15+DRV	-	-	-	-	$1.50x10^4$
DRV(TT)+IL-15	50 μg	32.50	-		$1.50x10^3$
DRV(TT)+IL-15	50 μg	32.50	-		$1.50x10^4$
Sep.Ent. TT&IL-15	50 μg	32.50	$2.50x10^5$	44.67	$2.461x10^3$
Sep.Ent. TT&IL-15	50 μg	32.50	$2.50x10^5$	44.67	$2.461x10^4$
Coentrapped TT&IL-15	20 μg	28.65	$2.50x10^4$	31.79	$1.390x10^3$
Coentrapped TT&IL-15	20 μg	31.71	$2.50x10^5$	32.34	$1.269x10^4$
Co-SL TT&IL-15	8 μg	23.59	$6.25x10^3$	20.68	$7.341x10^2$
Co-SL TT&IL-15	8 μg	21.85	$6.25x10^4$	21.65	$7.049x10^3$

SUV composed of equimolar PC and cholesterol and APSA in the case of surface linkage studies (1:1:0.05) were mixed with toxoid alone, IL-15 alone or a mixture of these to generate DRV. Toxoid and IL-15 were covalently coupled on the surface of the same vesicle. Entrapment or linkage calculations for the toxoid are based on ^{125}I radioactivity measurements. The same values for IL-15 were assessed by the ability of the cytokine to promote growth of CTLL-2 cells. All preparations were diluted with PBS accordingly so that each animal received a dose of 0.1 μg toxoid in a total volume of 0.1 mL.

will be presented later in this chapter showing an interaction of IL-15 (ca. 50% of the amount used) with empty DRV supports this suggestion.

Covalent coupling of the toxoid or IL-15 on the surface of DRV resulted in 23.59-21.85 % (toxoid) and 20.68-21.65 % (IL-15) of the protein used being associated with the vesicles (Table 9.1). The values obtained for IL-15 is based on CTLL-2 proliferation assay which measures the biologically active cytokine, thus the real value of linkage might in fact be higher for IL-15 but reflected as lower amounts due to a possible inactivation during the covalent coupling process.

9.3.2. Immunization studies

9.3.2.1. Primary responses to tetanus toxoid with or without IL-15

The adjuvant activity of IL-15 in liposomes was investigated in experiments where groups of mice were immunized with 0.1 μg toxoid alone or in the presence of two different doses of IL-15 in various liposomal formulations. This dose of toxoid was shown to be only very slightly immunogenic in liposomal form (Chapter 3). The responses obtained with cytokine containing groups were compared to those of entrapped or covalently coupled antigen alone. There were six different formulations where the cytokine was administered together with the toxoid in free forms, free in the presence of empty DRV, entrapped antigen co-injected with free IL-15, separately entrapped antigen and cytokine mixed before injection, co-entrapped and co-linked on the surface of the same liposomes. The units of IL-15 in the final preparations is given

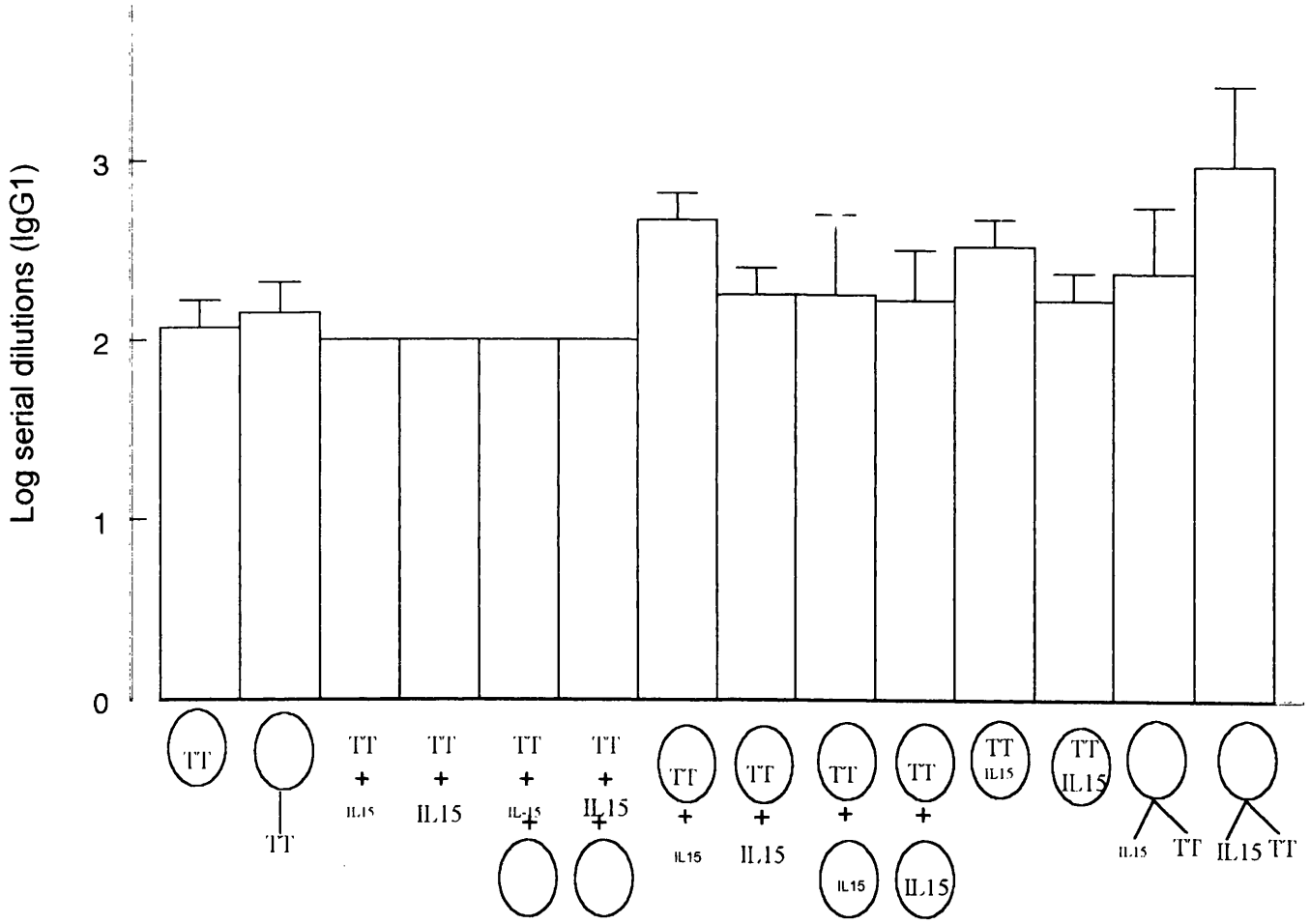
in Table 1, but the amounts received/mouse is one tenth of these values based on the volume injected (0.1 mL).

Figures 9.2-9.4 presents the primary IgG_1, IgG_{2a} and IgG_{2b} responses, respectively. Analysis of primary IgG_1 responses revealed that only the three formulations, entrapped toxoid-free IL-15 combination (lower dose), co-entrapped (lower dose) and co-surface linked (higher dose) preparations significantly boosted the production of this subclass ($P<0.02$ and $P<0.05$ for the first and the last two formulations, respectively). The entrapped toxoid-free cytokine combination proved to be the most effective type for this subclass on the basis of statistical analysis (for a detailed statistical comparison of groups, see Table 9.2). Analysis of IgG_{2a} and IgG_{2b} primary responses proved only the co-surface linked formulation (higher dose) to be effective in increasing the anti-toxoid titers when compared to the entrapped or surface linked toxoid alone ($P<0.05$ for both cases).

9.3.2.2. Secondary responses to tetanus toxoid with or without IL-15

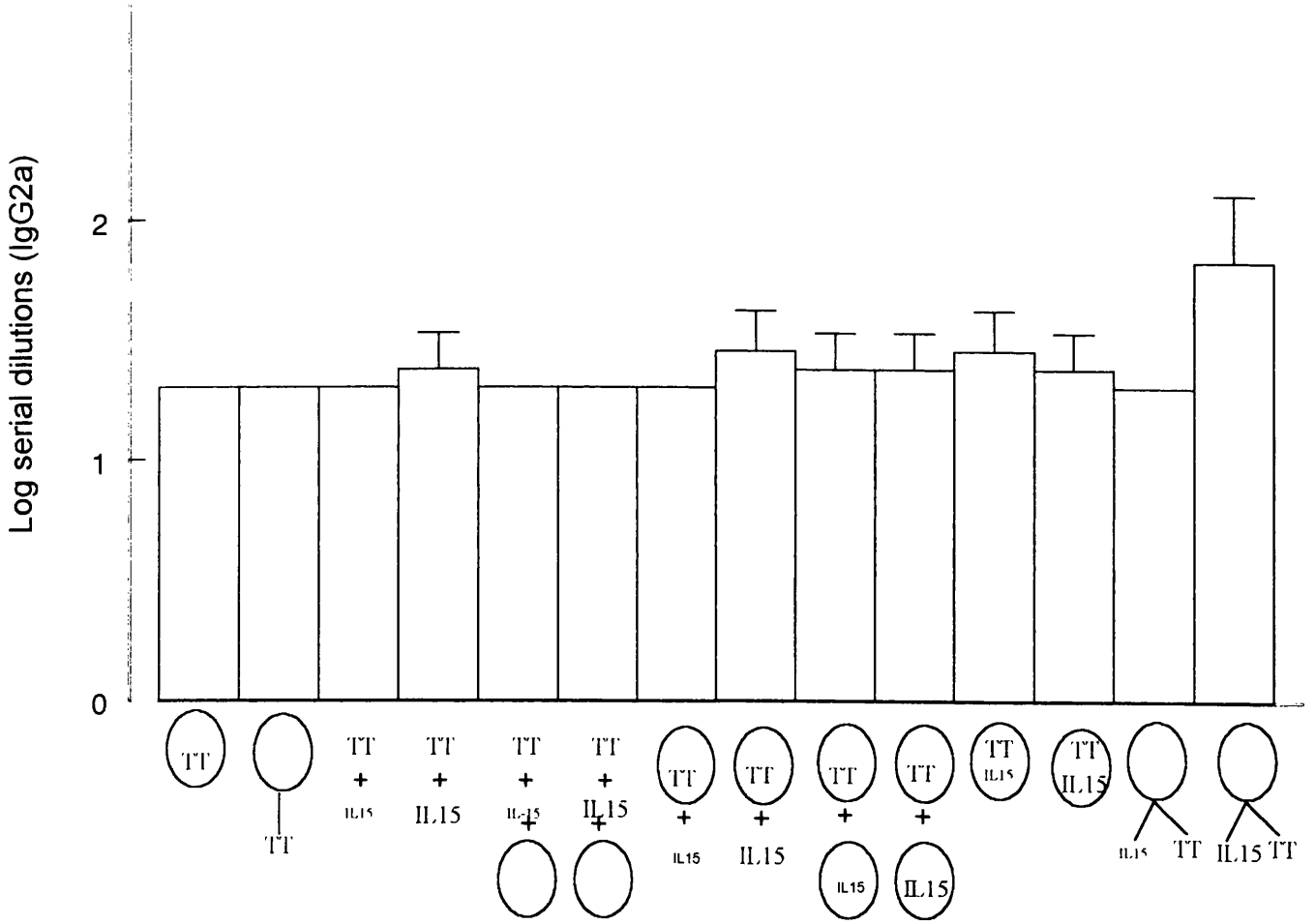
Figures 9.5-9.7 presents the secondary IgG_1, $IgG_{2a}$and IgG_{2b} responses, respectively. The adjuvant effect of IL-15 was much more pronounced for secondary responses. Entrapped toxoid-free IL-15 combination (lower dose), and the co-entrapped and co-surface linked formulations (both doses) all increased the IgG_1 titers when compared to IL-15 lacking groups or to those where the antigen or the cytokine were not physically associated with liposomes (for the levels of significance see Table 9.3). As was seen for the primary response, entrapped toxoid-free IL-15 combination was the most effective formulation for this subclass which was also significantly superior to all other

Figure 9.2. Comparison of IgG_1 primary responses to free or liposomal tetanus toxoid with or without IL-15



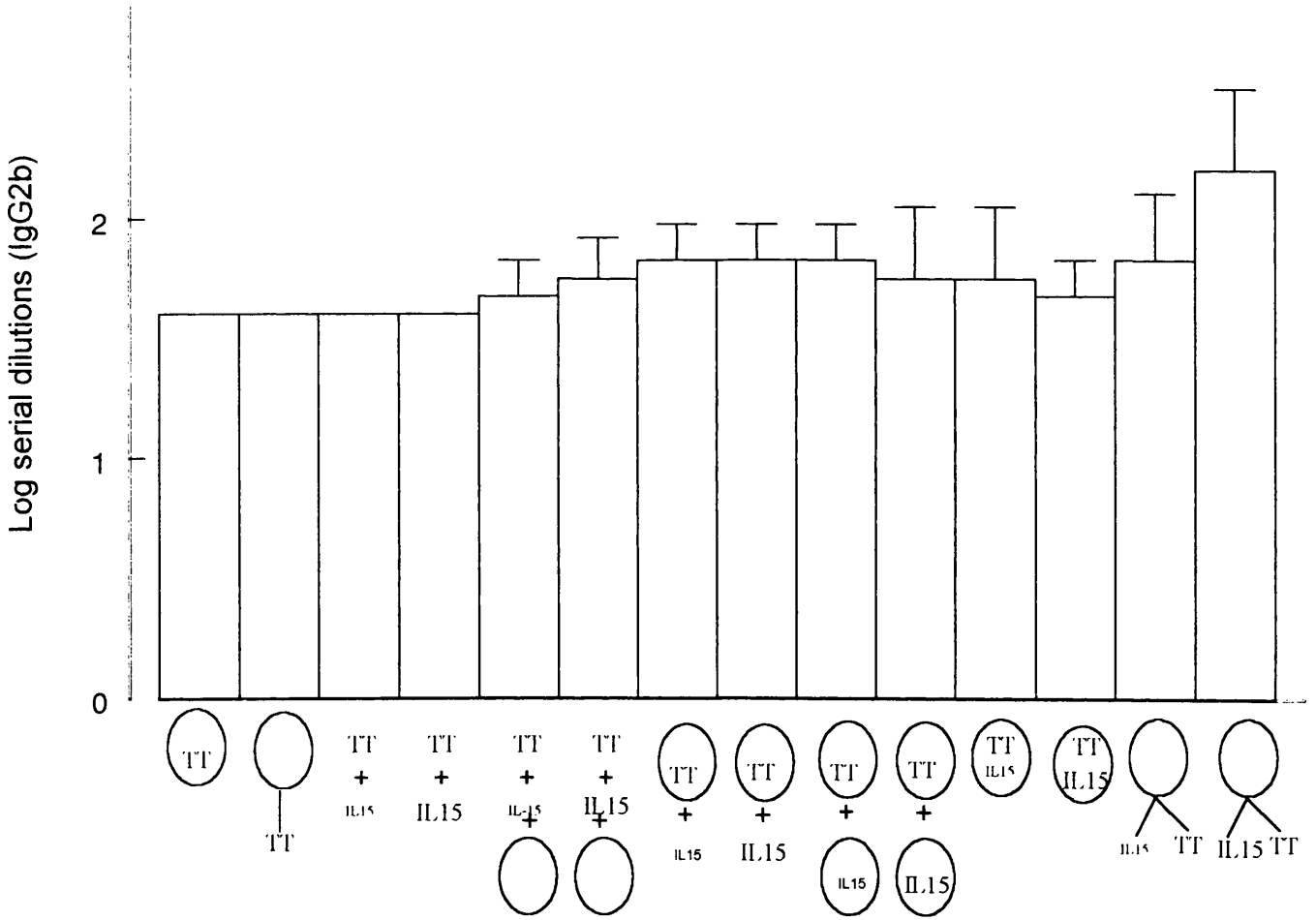
Tetanus toxoid and/or IL-15 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-15: low dose; IL-15: high dose.

Figure 9.3. Comparison of IgG_{2a} primary responses to free or liposomal tetanus toxoid with or without IL-15



Tetanus toxoid and/or IL-15 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-15: low dose; IL-15: high dose.

Figure 9.4. Comparison of IgG_{2b} primary responses to free or liposomal tetanus toxoid with or without IL-15



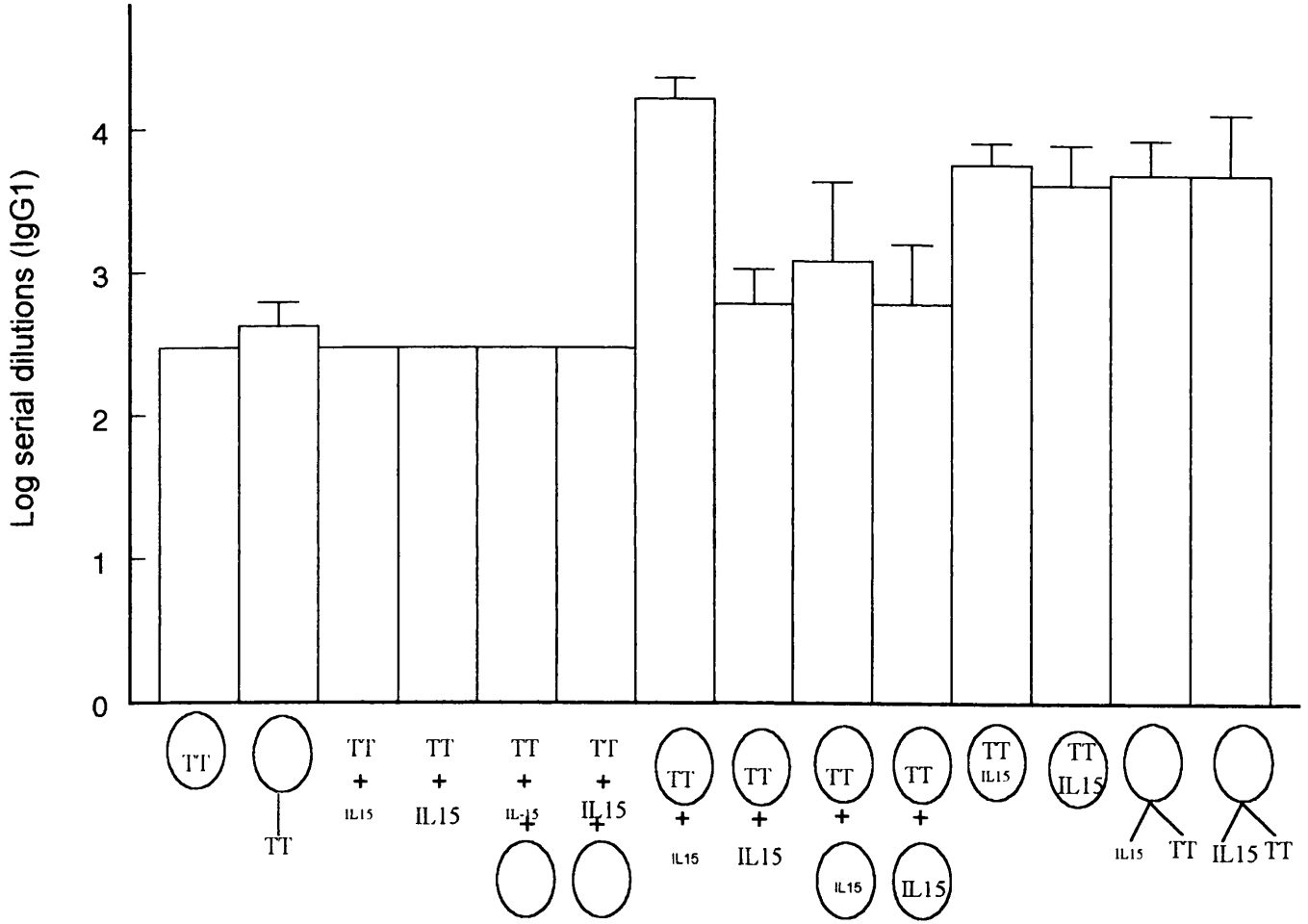
Tetanus toxoid and/or IL-15 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-15: low dose; IL-15: high dose.

Table 9.2. Comparison of primary responses

Preparation	IL-15 (L) + DRV(TT)	IL-15 (H) + DRV(TT)	CoEnt. (L)	Co-SL (H)		
	IgG_1	IgG_1	IgG_1	IgG_1	IgG_{2a}	IgG_{2b}
Ent. TT	**		*	*	*	*
SL TT	*		*	*	*	*
IL-15 (L)+TT	***	*	***	*	*	*
IL-15 (H)+TT	***	*	***	*		*
IL-15(L)+TT+DRV	***	*	***	*	*	
IL-15(H)+TT+DRV	***	*	***	*	*	
IL-15 (L)+DRV(TT)					*	
IL-15 (H)+DRV(TT)	*					
Sep.Ent. (L)						
Sep.Ent. (H)						
CoEnt. (L)						
CoEnt. (H)	*					
Co-SL (L)					*	
Co-SL (H)						

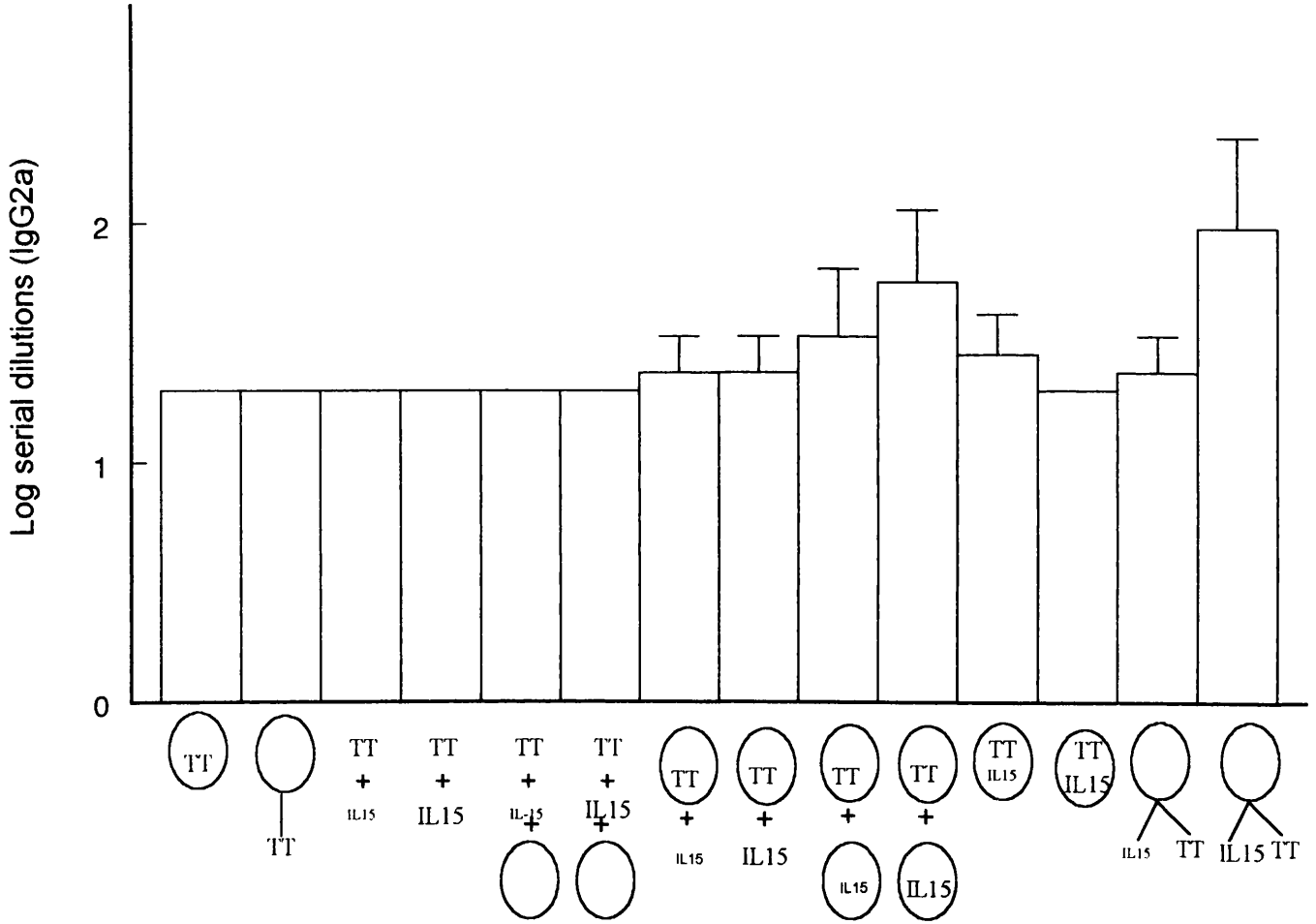
Tetanus toxoid and/or IL-15 were either entrapped alone or together in DRV composed of PC:cholesterol (1:1) or were covalently coupled on the surface of DRV. Only the formulations that improved the antibody titers are given in the table. *, $P<0.05$; **, $P<0.02$; ***, $P<0.01$. TT:tetanus toxoid; SepEnt.: separately entrapped; CoEnt.: coentrapped; Co-SL: co-surface linked; low:low IL-15 dose; high: high IL-15 dose.

Figure 9.5. Comparison of IgG_1 secondary responses to free or liposomal tetanus toxoid with or without IL-15



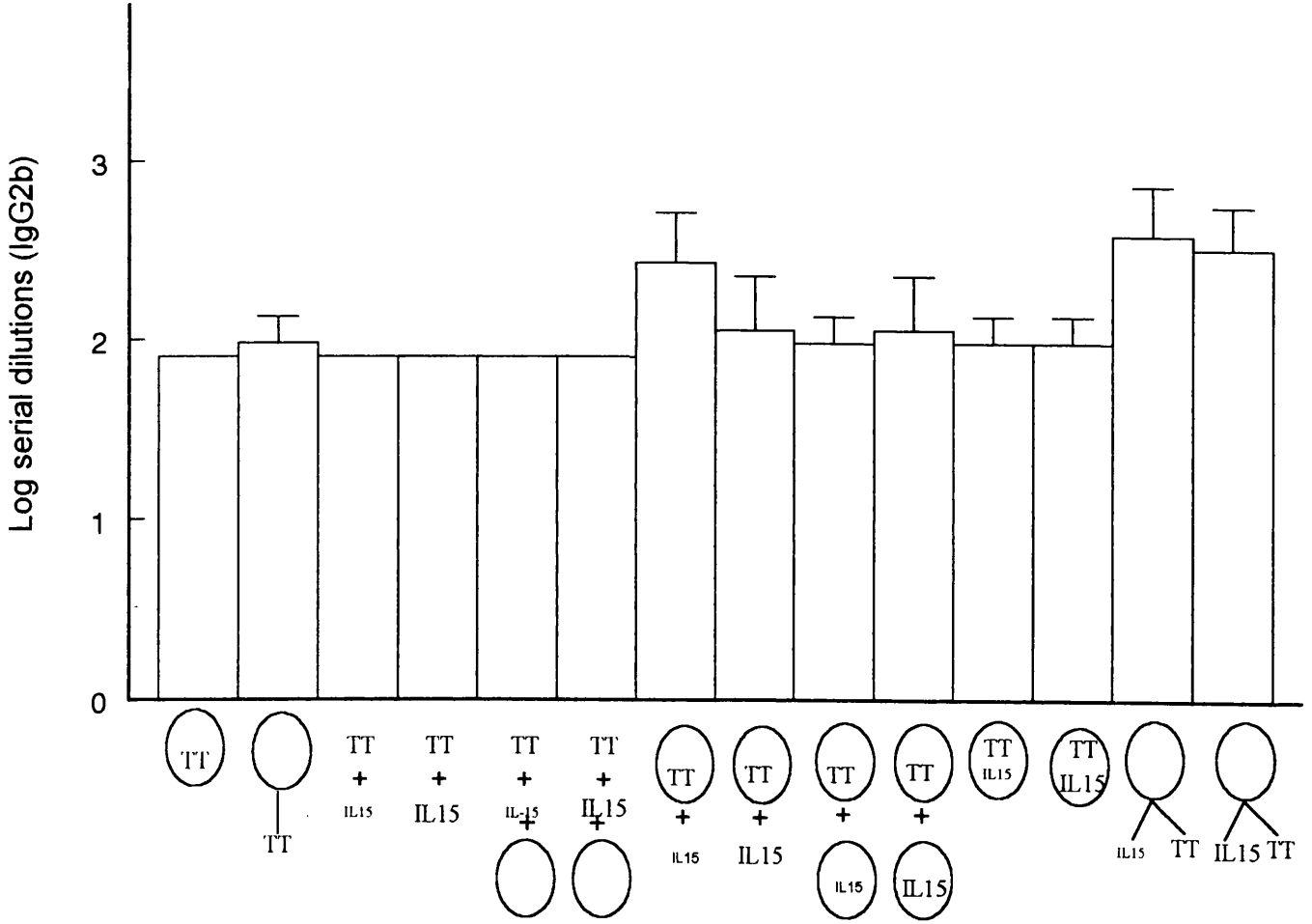
Tetanus toxoid and/or IL-15 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-15: low dose; IL-15: high dose.

Figure 9.6. Comparison of IgG_{2a} secondary responses to free or liposomal tetanus toxoid with or without IL-15



Tetanus toxoid and/or IL-15 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-15: low dose; IL-15: high dose.

Figure 9.7. Comparison of IgG_{2b} secondary responses to free or liposomal tetanus toxoid with or without IL-15



Tetanus toxoid and/or IL-15 were covalently coupled to or entrapped in DRV composed of PC:cholesterol (1:1 molar ratios). Protein in circles indicate entrapment while figures with attached protein represent covalent coupling. IL-15: low dose; IL-15: high dose.

Table 9.3. Comparison of secondary responses

Preparations	IL-15(L)+DRV(TT)		CoEnt. (L)	CoEnt. (H)	Co-SL (L)		Co-SL (H)		
	IgG_1	IgG_{2b}	IgG_1	IgG_1	IgG_1	IgG_{2b}	IgG_1	IgG_{2a}	IgG_{2b}
Ent. TT	****	*	****	***	***	**	**	*	**
SL TT	****		***	***	***	*	**	*	*
IL-15(L)+TT	****	*	****	***	***	**	**	*	**
IL-15(H)+TT	****	*	****	***	***	**	**	*	**
IL-15(L)+TT+DRV	****	*	****	***	***	**	**	*	**
IL-15(H)+TT+DRV	****	*	****	***	***	**	**	*	**
IL-15(L)+DRV(TT)									
IL-15(H)+DRV(TT)	***		***	*	**				
Sep.Ent. (L)	*					*	*		*
Sep.Ent. (H)	***		*	*	*				
CoEnt. (L)	*					*			*
CoEnt. (H)	*					*			*
Co-SL (L)	*								
Co-SL (H)									

Tetanus toxoid and/or IL-15 were either entrapped alone or together in DRV composed of PC:cholesterol (1:1) or were covalently coupled on the surface of DRV composed of PC:cholesterol:APSA (1:1:0.05). Only the formulations that improved the antibody titers are given in the table. Stars represent the significance levels (*, $P<0.05$; **, $P<0.02$; ***, $P<0.01$; ****, $P<0.001$). TT: tetanus toxoid; Ent.: entrapped; CoEnt.: coentrapped; Sep.Ent.: separately entrapped; Co-SL: co-surface linked; L: low IL-15 dose; H: high IL-15 dose.

groups except for the co-surface linked preparation (higher dose). This finding led us to investigate the possibility of an interaction of IL-15 with the liposomes, something which was not reported for IL-2 (Joffret et al, 1990). For this, empty DRV of the same composition as used in the immunization studies were incubated with IL-15 (17.5 μg) at 4°C for a week. DRV were pelleted and washed, and protein in the liposomal fraction and the supernatant was quantitated using the bicinchoninic acid assay. 45.92 % of the amount mixed was found to be adsorbed on the liposomal bilayers. The inefficiency of entrapped toxoid-free cytokine combination in boosting the anti-toxoid IgG_1 responses at a higher dose is somewhat surprising and needs to be investigated further. This same preparation (lower dose) and the co-linked formulations (both doses) boosted the secondary anti-toxoid IgG_{2b} titers significantly when compared to entrapped toxoid alone and to those where the antigen and the cytokine were in free forms. However, both of the co-linked formulations were more effective, being also superior to covalently coupled toxoid alone, separately entrapped (lower dose) and co-entrapped (both doses) preparations ($P<0.05$). With the IgG_{2a} subclass, only the co-linked formulation (higher dose) was effective in increasing the titers with respect to liposomal toxoid alone or to those where the antigen and IL-15 were either free or were not directly associated with the liposomes.

9.4. CONCLUSION

The immunization studies reported here using IL-15 as a co-adjuvant indicate that this cytokine is equally potent as IL-2 and that the spatial distribution of the antigen and the cytokine within the liposome may alter the extent and type of antibody production. As was observed with IL-2 (Chapter 6), for IL-15 to act as an adjuvant, its presence

together with the antigen in the same liposome is required. The inability of this cytokine to potentiate the immune response when it was separately entrapped from the antigen suggests a neccessity for the cytokine and the toxoid to contact the same antigen-presenting cell as supported by the findings of Heath and Playfair (1990) and explained earlier in Chapter 6. The high IgG_1 primary and secondary responses obtained in this study with entrapped toxoid-free IL-15 combination (lower dose) does not seem to contradict this hypothesis since ca. 50 % of the cytokine was shown to adsorb on the vesicle surface and this amount would still be targeted to the same antigen presenting cell together with the toxoid simultaneously. The inability of the same preparation to enhance the responses at a higher IL-15 doses might be explained by the fact that the higher amounts of free IL-15 (i.e.unadsorbed fraction) might act as a "spill over" to irrelevant immunocompetent cells and drive the responses in an unfavorable way thereafter. In fact, such a spill over is not present for the similar co-surface linked formulation (since all the cytokine is covalently coupled on the vesicle surface) and the higher dose acted as the most effective preparation, the only group that increased the IgG_{2a} secondary titers. All the cytokine and the antigen would be introduced to the same antigen presenting cell when they are present on the surface of the same vesicle. The same holds for the co-entrapped formulations but the differences observed with these groups might be attributed to the fact that surface-linked antigens might also be processed and presented with B-cells while there is only a single possible route of processing for the entrapped antigen, via the macrophage pathway (Shahum and Therien, 1994). Shahum and Therien in the same study demonstrated that the encapsulated antigen induces a short-lasting response dominated by IgG_1 while an increased and long-lasting production of IgM, IgG_3, IgG_{2a} and IgG_1 was observed with

the covalently coupled antigen. Also IL-15 might be able to interact directly with its receptor when it is in surface linked form, whereas it has to be released from the vesicles to interact with its receptor when it is entrapped.

The increased IgG_{2a} titers observed with the co-surface linked formulation in this study indicates a more effective stimulation of Th1 clones that represents the cell-mediated arm of the immune response and favour IgG_{2a} secretion from B-cells via the action of cytokines they secrete (Snapper and Mond, 1993).

Here we have also demonstarted that IL-15 adsorbs on the surface of liposomes (ca. 50 %) which might be an attractive approach in the production of vaccines due to the ease of the procedure and would provide control in adjusting the dose of the cytokine as required which unfortunately can not be done for a coentrapped or co-surface linked formulation. This property of IL-15 was not observed for IL-2 with liposomes (Joeffret et al, 1990) although and adsorption of ca. 20 % was found when this cytokine was incubated with erythrocytes (Kirch et al, 1994).

In summary the results in this chapter demonstrate that IL-15 acts as a potent adjuvant in vivo and that the extent and nature of the antibodies produced can be altered by the type of formulation they were administered with.

GENERAL DISCUSSION

With the development of biosynthetic and recombinant molecular biology techniques in recent years, use of purified subunit or synthetic vaccines was made possible. However, most of these vaccines proved to be poor immunogens and require adjuvants to evoke an immune response. Thus, there is a need for the development of well defined, potent adjuvant systems.

Since adjuvants are thought to improve immune responses to an antigen by creating a depot for the antigen, via targeting to antigen presenting cells and/or by providing T-cell help, here we have attempted to combine these three general mechanisms in one adjuvant system. Liposomes were our choice of vaccine carriers due to their ability to create an antigen depot and their potential for natural targeting to macrophages, one of the antigen presenting cells known. Based on the evidence from literature (Alving, 1991; Therien and Shahum, 1994) indicating a possibility of B-cell processing for antigens covalently linked on the surface of liposomes, we have analyzed immune responses to free, entrapped or surface linked tetanus toxoid and also established a dose response curve for this antigen in preliminary experiments. Although both liposomes types augmented the immune responses to the antigen significantly with respect to the free form, there were no significant differences between the two liposome types used contrary to several reports published (Aramaki et al, 1994; Shahum and Therien, 1994) showing the surface linked antigen being superior to the entrapped form. This controversy to liposome associated antigens might be due to different liposome

types, phospholipids and antigens used in different studies as well as the method of coupling the antigen on the surface of liposomes which might affect the extent of antigen exposed on the surface affecting interaction of these especially with B cells. In this respect, further studies focusing on different methods of antigen coupling and comparison of such vesicles in potentiating immune responses might prove beneficial.

Attempts to improve the targeting of liposomes to macrophages without the use of a foreign protein were made in experiments where the toxoid was mannosylated at a low and a high mannose:protein ratio and immune responses to these modified antigens in free or liposomally associated forms at two different doses were analyzed and compared to those obtained with the native antigen. However, the native toxoid proved to be superior to its modified counterparts. This was attributed to a possible change of antigenic epitopes during the mannosylation process, an enhanced uptake of the antigen containing liposomes Kupffer cells of the liver that which is thought to diminish antigenic properties of proteins processed in these cells (Murray et al, 1991) and/or an enhanced destabilization of mannose containing liposomes in blood as suggested by Funato et al (1994). Despite a lack of improvement in immune responses with the mannosylated toxoid here, the approach might still be applicable to other antigens or targeting to other antigen presenting cells by the use of appropriate ligands might shed light to the function of each cell type and the consequent immune responses they induce.

The applicability of giant vesicles as multiple vaccine carriers for soluble and particulate antigens was analyzed in experiments where animals were immunized with liposome ssociated tetanus toxoid and *B. subtilis* spores and the results were compared to those obtained with DRV liposomes. Despite better antibody titers obtained with DRV liposomes, giant vesicles were able to potentiate the responses to the antigens they entrapped and they might be especially valuable if there is a need to encapsulate attenuated bacteria which are not likely to be entrapped in DRV liposomes. Both vesicle types were able to protect their antigen content from binding of specific antibodies that might have practical implications in cases where problems associated with neutralization of the vaccine by maternal antibodies has to be avoided.

In the next part of the thesis, the ability of cytokines to act as co-adjuvants in liposomes was investigated. Of the four cytokines tested, only IL-1β (163-171) peptide proved to be ineffective probably due to the very low doses of the peptide used in immunization studies. However, IL-2, IL-12 and IL-15 in liposomes showed the general trend to alter the immune responses only when they were associated with the antigen in the same liposomes. This general trend indicates a neccessity for the cytokines and the antigen to contact the same antigen presenting cell simultaneously for the optimum effect to be observed. The type of liposomal formulation itself seemed to alter the responses obtained probably by modifying the biodistribution of the cytokine and affecting their interaction with cells of the immune response (such as ability to interact with its receptor directly for surface-linked cytokines). The liposome-cytokine-antigen combination might

prove valuable in certain vaccine applications due to the ability of creating an antigen depot, natural targeting to macrophages and/or B-cells in the case of surface-linkage as well as providing T-cell help in one formulation. The possibility of use of combinations of cytokines in the same liposomes together with the antigen might also prove to be beneficial while having the added advantage of abolishing the need of multiple injections and related toxicity problems observed with free-cytokine administration regimes. Finally, in this study we also demonstrated the ability of IL-15 to act as an adjuvant in liposomes for the first time.

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