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The applications of liposomes in immunoprophylaxis and immunotherapy

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Abstract

Liposomes have proven to be useful tools in numerous areas of biological research. The aims of this thesis are to further investigate their role and effectiveness in two different but well-established immunological applications, namely as adjuvants to bacterial and viral components and as antibody-mediated targeted carriers of entrapped drugs.

Dehydration-rehydration vesicles (DRV) were found to be capable of entrapping relatively large reconstituted influenza virus envelopes (RIVE) with high efficiency and reproducibility. Balb/c mice inoculated with DRV containing RIVE exhibited primary and secondary IgG_1 responses significantly higher as compared to mice inoculated with equal doses of unencapsulated RIVE. Similar responses were observed in the IgG_{2a}, IgG_{2b} and IgG_3 subclasses.

The adjuvanticity of liposomes on poorly immunogenic synthetic virus subunit peptides was demonstrated using DRV incorporating the peptides in their internal space and liposomes with covalently surface-linked peptides. It was found that liposome association boosted the primary and secondary IgG_1 responses against the peptides as compared to controls in which free peptides were administered. Surface-linked peptide gave an initially more rapid rise in antibody levels as compared to entrapped peptide but with no immunological memory whereas one of the encapsulated peptides elicited a milder primary response but later exhibited a strong anamnestic response.

Various strategies for enhancing the adjuvant effect of liposomes using tetanus toxoid as a model antigen were studied. 10,000 units of recombinant IL-2 co-entrapped with tetanus toxoid in DRV markedly increased antibody levels in both the primary and secondary responses as compared to control DRV containing only tetanus toxoid. A less pronounced effect was seen with 1,000 units of co-entrapped IL-2. IL-2, especially at the lower dose, administered in separate liposomes mildly suppressed antibody levels. A novel positively charged lipid, 1,2-Bis(hexadecylcycloxy)-3-trimethylaminopropane-HCl (BisHOP), when incorporated into the bilayers of PC and DSPC DRV, was shown to have a powerful effect in enhancing the primary and secondary immune responses to tetanus toxoid, with greater adjuvanticity at higher amounts of BisHOP incorporation. Plain DSPC DRV were found to have a greater adjuvant effect than plain PC DRV in the primary response but the opposite was true in the secondary response. The primary immune response to tetanus toxoid incorporated in PC DRV was examined after being administered as follows:- In one group of mice, the dose was divided into two equal portions and administered one-half intramuscularly and the other half intradermally in a relatively avascular site (the back). In the control group, the whole dose was given intramuscularly. Results showed that in both cases, peak IgG_1 responses were obtained 12 weeks after the injection. However, antibody levels in the group which received the same dose at two different sites was significantly greater. These results imply that liposome-based vaccines could be more potent and effective when incorporating lymphokines as co-adjuvants, by manipulation of membrane

characteristics and by varying the mode of administration.

The second part of the thesis relates to the behaviour of liposomes as targeted carriers of drugs. The influence of charge on the electrophoretic mobility of MLV in vitro and on the clearance from the circulation of SUV after intravenous administration was studied. It was found that positively-charged lipids (stearylamine and BisHOP) incorporated into DSPC SUV were cleared from the circulation in mice at the same rate as negatively-charged ones incorporating phosphatidic acid (PA), and in both groups, more rapidly than neutral SUV. However, stearylamine PC SUV were cleared more slowly than similarly positively -charged BisHOP PC SUV and negative PA PC SUV. This suggests that positively and negatively-charged SUV made by adding charged lipids to neutral phospholipids are cleared at the same rates if the charged lipid is confined in a rigid bilayer (eg. in DSPC SUV) or if the acyl chain of the charged lipid is saturated and long enough to prevent its removal from a more `fluid' bilayer (eg. in PC SUV). A study of the effect of pH on the electrophoretic mobility of charged MLV in vitro revealed that the net surface charge on liposomes is markedly influenced by changes in pH. These findings may have important implications for the clinical use of liposomes containing charged lipid, ligands or drugs and in states of acidosis or alkalosis.

For use as targeting ligands, monoclonal antibodies that bound to 3 human hepatocellular carcinoma cell lines and snap-frozen sections of a patient's tumour were raised by the novel method of immunizing mice with PLC/PRF/5 membrane preparations. The monoclonals designated RF-HCC 1 and

RF-HCC 2 were of IgG1 and IgM subclasses respectively.

A simple method for the coating of liposomes with proteins was also developed. Several proteins (eg. BSA, pig gammaglobulins and tetanus toxoid) could be made to adhere rapidly and efficiently to both MLV and SUV after treatment with HCl and $NaNO_2$. The reaction is complete within four hours and almost all of the protein is retained on the liposomes after storage for 2 weeks at $4^{o}C$.

CF-containing SUV coated with passively-adsorbed monoclonal antibody (RF-HCC1 and MOPC 21 IgG1) were used in in vitro studies for targeting to Mahlavu cells. Significantly greater amounts of the marker entrapped in antibody-coated SUV were associated with the target cells after incubation for one and a half hours at $4^{o}C$ as compared to plain SUV. These results demonstrate that passively adsorbed antibodies represent a rapid, convenient and effective method of ligand-mediated targeting of SUV to cells in vitro.

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List of Abbreviations

APSA	Aminophenyl stearylamide
BisHOP	1,2-Bis(hexadecylcycloxy)-3-trimethylaminopropane-HCl
BSA	Bovine serum albumin
CF	Carboxyfluorescein
Chol	Cholesterol
DCP	Dicetyl phosphate
DRV	Dehydration-rehydration vesicles
DSPC	Distearoyl phosphatidylcholine
EMIA	Enzyme membrane immunoassay
FCS	Foetal calf serum
HAT	Hypoxanthine Aminopterin Thioguanine
HDL	High density lipoprotein
HT	Hypoxanthine Thymidine
IL-2	Interleukin-2
ILA	Immunoliposome assay
LUV	Large unilamellar vesicles
MLV	Multilamellar vesicles
PA	Phosphatidic acid
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PE	Phosphatidyl ethanolamine
PEG	Polyethylene glycol
RES	Reticuloendothelial system
REV	Reverse-phase evaporation vesicles
RIVE	Reconstituted influenza virus envelopes
SA	Stearylamine

SAH	South African hepatoma
SUV	Small unilamellar vesicles
T_c	Transition temperature
TG	Thioguanine
TT	Tetanus toxoid

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General Introduction

Historical Perspective

During the period 1961-1963, shortly after Robertson had postulated his universal unit membrane theory, Bangham and co-workers in the course of an electron microscopy study, formulated the idea that isolated and purified phospholipids of cellular origin could spontaneously reform in the presence of water into a pattern of closed membrane systems (Bangham, 1980). Typical examples of membrane compounds which exhibit amphiphilic (i.e. oil-soluble at one end and water-soluble at the other) properties that cause them to form highly ordered phases in equilibrium with a water phase (liposomes) are the polar lipids eg. phosphatidylcholines, ethanolamines and serines, sphingomyelins, cardiolipins, plasmalogens, phosphatidic acids and cerebrosides. These compounds, when confronted with an encroaching aqueous environment, undergo a sequence of assemblages that reflect the thermodynamic perturbations of increasing water-water, water-oil and oil-oil interactions. These quasi-equilibrium structures referred to colloquially and in this thesis as `liposomes' are more accurately termed `smectic mesophases'. Smectic mesophases are layer lattices of alternating bimolecular lipid sheets intercalated by aqueous spaces. When formed from highly water-insoluble amphiphiles such as those mentioned above, they persist as stable structures in the presence of excess water. Their usefulness as a model system derives from the fact that, as the dry phospho- and/or other lipids of biological origin undergo their sequence of molecular rearrangements, there is an opportuni-

ty for an unrestricted entry of solutes (eg. isotopically labeled salts and proteins) between the planes of hydrophilic head groups before the unfavourable entropy situation of an oil-water interface intervenes. Subsequently, and because of the unfavourable entropy associated with immiscibility of hydrocarbon and an encroaching aqueous phase, a further arrangement of molecules takes place that yields a series of concentric closed membranes, each membrane representing an unbroken, bimolecular sheet of molecules. From this, it follows that every aqueous compartment is discrete and isolated from its neighbour by one such closed membrane and that the outermost aqueous compartment of the whole structure would be isolated from the continuous aqueous phase. The solutes and water originally entering the system are therefore sequestered and can only diffuse between compartments or into the bulk aqueous phase by crossing one or more bimolecular membranes. Thus, the concept of liposomes emerged and the early permeability data set out to show that phosphatidylcholine membranes were selectively permeable to ions.

In 1964, three researchers on a concurrent visit to Bangham's laboratory were to play a decisive role in the ultimate acceptance of the liposome model. Weissmann was the first to recognise and exploit the similarities between liposomes and cell lysosomes, using them to understand the pathology of certain conditions (eg. gout) and to attempt to remedy others. Papahadjopoulos' pioneering work which gave rise to a `school' devoted to the use of the liposome model in many branches of experimental biology and medicine, included studies on the ability of liposomes to induce cell

fusion and the mechanism of action of local anaesthetics on membrane structure and function. Chappell's work represented a landmark in an understanding of the ways in which ions cross hydrophobic barriers.

The permeating influence of liposomes on the fields of applied medicine has been due largely to the efforts of Gregoriadis who, in conjunction with various investigators, performed keystone studies which opened up new avenues in the use of liposomes as drug carriers, ligand-targeted vesicles, immunological adjuvants and for enzyme therapy. Relentless progress has culminated in the formation of commercial liposome-based companies and the appearance on the market of liposome-formulated products. Extensive clinical trials are presently being conducted to evaluate their ultimate applicability in man.

Morphology of liposomes and methods of production

Liposomes may have now become an almost generic formulation but they in fact, come in many different varieties. These encompass differences in chemical composition and physical characteristics, including size, number of bilayers and homogeneity which are largely determined by their mode of formation.

On the basis of morphology, liposomes may broadly be classified into:

1. Multilamellar vesicles (MLV) - these were the first type of liposomes described by Bangham, consisting of concentric aqueous and lipid layers. Lipids in an organic solvent (eg. chloroform) are deposited as a thin film on the walls of a container by evaporation of the solvent through a

vacuum or nitrogen insufflation. If several lipids are used, these are carefully mixed in organic solution before deposition. To hydrate the lipids, the aqueous solution to be added must be warmed to a temperature higher than the gel-liquid crystalline transition temperature (T_C) of the lipid or the highest T_C in the case of a mixture of lipids. [The T_C of a bilayer membrane is the temperature at which it undergoes a phase transition from a rigid (gel) state to a fluid (liquid crystalline) one. This phenomenon occurs rather abruptly as the temperature of the membrane sample is raised, with absorption of heat around the transition temperature, detectable by methods such as differential scanning calorimetry. The fluidity of liposomal membranes may be regulated by the incorporation of phopholipids of various transition temperatures, the latter governed by the lengths of their acyl chains and their degree of unsaturation. Phospholipids with longer acyl chains and lower degrees of unsaturation tend to form membranes with higher transition temperatures as the molecules are able to pack themselves more densely in the bilayer, thus rendering the liposomes which they form more `solid'. Phospholipids with shorter acyl chains and a higher degree of unsaturation form membranes with lower transition temperatures as the amount of kinking of individual molecules as well as their short length gives rise to a loosely packed bilayer, contributing to more `fluid' liposomes. The latter liposomes are subsequently more `leaky' to entrapped substances as compared to their densely packed `solid' counterparts. Membrane fluidity may also be modulated by the incorporation of cholesterol which inserts itself into bilayers with its long axis per-

pendicular to the plane of the membrane. Cholesterol prevents the crystallisation of fatty acid chains by fitting between them as well as sterically blocks large motions of fatty acyl chains, which makes membranes less fluid. The incorporation of cholesterol in liposomal bilayers composed of phospholipids with high T_c results in an exceptionally stable structure which exhibits minimal leakage of entrapped substances for prolonged periods.] Dispersion of the lipid is facilitated by agitation (eg. vortexing, shaking with glass beads or bath sonication.) Entrapped solutes may be associated with the lipid bilayers in the case of hydrophobic substances or be confined within the aqueous compartments in the case of hydrophilic ones. Encapsulation efficiency using the classical method of preparing MLV tends to be low, ranging from 5-15%. The size of MLV is heterogeneous, with vesicle diameters ranging from 0.5 um to several microns. Extrusion of MLV through polycarbonate membrane filters with pores of well-defined size permits greater uniformity and a reduction in size of the MLV preparation to be obtained.

A simple method reported recently by Kirby and Gregoriadis (1984) based on the fusion of pre-formed phospholipid vesicles by dehydration followed by rehydration produces structures termed dehydration-rehydration vesicles DRV. These also have the same morphology as MLV but with the difference that in the process of formation, up to 80% or more of the added solute may be entrapped without the involvement of potentially damaging conditions. Other advantages of the procedure are its amenability to scale-up for industrial use, ability to entrap particles as large as

viruses with high efficiency and reproducibility and ability to be stored in a freeze-dried form.

II. Small Unilamellar vesicles (SUV) - these consist of a single bimolecular layer enclosing a single aqueous cavity. Their diameter ranges from 20-60 nm. The following are common methods used to prepare SUV:-

a) Sonication.

This is by far the most widely used technique for preparing SUV. Initially described by Huang (1969), the method enables one to obtain vesicles surrounded by a single bilayer which are homogeneous in dimensions. Huang employed gel filtration to separate remaining multilamellar vesicles from unilamellar ones. As this procedure is used in work done for the thesis, it will be described in greater detail later on.

b) Injection of a solution of phospholipid in ethanol.

Another method proposed by Batzri and Korn (1973), which avoids sonication, consists of the rapid addition of a solution of phospholipid in ethanol to water or a buffered solution. The technique comprises four stages:

-the phospholipid and other constituents are first dissolved in ethanol; the concentration normally used is of the order of 20 to 40 μmoles of phospholipid per ml.

-the alcoholic solution is then rapidly injected with the aid of a syringe into a buffered solution (KCl 0.15M, for example) where small unilamellar vesicles form spontaneously

-the solution can then be concentrated by

ultrafiltration under nitrogen

-finally, the suspension is dialysed to eliminate all traces of alcohol.

c) Detergent dialysis

In this method, developed by Milsmann et al. (1978), mixed micelles are first formed by dispersing a phospholipid and a surfactant in an aqueous solution of the material to be encapsulated. This dispersion of mixed micelles is then dialysed extensively, when the surfactant is gradually removed and the phospholipid assumes a unilamellar liposome structure. Homogeneous dispersions of SUV are formed by this technique.

d) French Press extrusion

A suspension of multilamellar liposomes in a pressure-tight chamber are subjected to a constant pressure (20,000 psi) applied hydraulically via a piston (Lelkes, 1984). Upon opening of the pressure relief valve, the pressure gradient between inside and outside, created across the tiny outlet hole, generates shearing forces which are sufficient to disrupt MLV and convert them into a very homogenous population of SUV.

III. Large Unilamellar Vesicles (LUV) - these are a class of vesicles with similar dimensions to those of MLV but enclosed by a single lipid bilayer. Amongst the methods used to prepare LUV are the ether injection technique and by calcium-induced fusion. Reverse-phase evaporation vesicles (REV) are a mixture of oligolamellar vesicles and LUV-like structures but whose method of formation enables one to encapsulate substances in the aqueous phase to a high degree

of efficiency (Szoka and Papahadjopoulos, 1978).

Range of substances entrapped

One of the reasons why liposomes have insinuated themselves into a wide range of fields and a plethora of applications is that virtually any substance, regardless of solubility, electrical charge, size and other structural characteristics can be incorporated within their structure provided that they do not interfere with their formation (Gregoriadis, 1985). Detergent-like substances, for instance, will prevent liposomes from forming when present above a critical concentration. Further, because incorporation into either the aqueous or the lipid phase of liposomes is passive, there is usually no need to develop different techniques for individual drugs. This is clearly illustrated (Kirby and Gregoriadis, 1984) and further reinforced in this thesis for the case of DRV where salts, sugars, a variety of low molecular-weight drugs, proteins and nucleic acids, are all incorporated into liposomes by the same procedure. During the last 15 years many hundreds of drugs, including a wide range of anticancer and antimicrobial agents, proteins, enzymes, vaccines and genetic materials have been successfully entrapped in liposomes and tested under various experimental conditions. Some of the successful uses include cure of tumour-bearing animals with liposomes carrying agents activating tumouricidal macrophages, killing of microbes in bacterial and fungal infections, potentiation of immune responses to otherwise ineffective or weak vaccines and facilitation of adsorption of orally given insulin and blood clotting factors. Many of these agents (eg. antibiotics,

interferon, peptide hormones, factor VIII and vaccines) can now be made through recombinant DNA techniques and their availability and use (and the need for their delivery) are expected to increase considerably in the foreseeable future.

Stability and sterility of liposomes

Since liposome-encapsulated vaccines and drugs will eventually be used as pharmaceutical products, the final formulations must be stable for eighteen months to two years. The required temperature range over which the liposomes must be stable will be determined by the ultimate use of the product. For example, drugs that will primarily be used in hospital settings may be acceptably stored at $4^{o}C$ while drugs sold over the counter must be stable at least at room temperature. The extreme case involves many veterinary drugs which may be used in hot climates where they would be exposed to temperatures in excess of $40^{o}C$. Liposomal stability on storage is defined here as the extent to which a given preparation retains both its original vesicle size distribution and its drug load. Instability can occur, for example, when vesicle size increases spontaneously upon standing as a result of fusion of colliding vesicles; the larger vesicles will exhibit drastically different pharmacokinetics in vivo because their size determines their clearance rates and tissue distribution; for instance, large liposomes are removed from the circulation more rapidly than smaller ones (Abra and Hunt, 1981) and the latter, when of a diameter of about 100 nm or less can reach the hepatic parenchymal cells (Scherphof et al., 1983). A second important factor in instability is that drugs of low molecular

weight are likely to leak from stored liposomes.

Both aspects of liposomal stability have been investigated, especially by industrial workers and both have, to a large extent, been resolved. Fusion upon standing, for instance, is reduced in small vesicles bearing a surface electric charge. Other techniques, for instance the use of additives, have been routinely used in industry for the maintenance of colloid suspensions and, although no details have been disclosed, they no doubt have been applied in the case of liposomes.

Drug loss due to leakage can also be reduced considerably by the appropriate manipulation of lipid composition. Excess cholesterol in the liposomal membrane, for instance, packs the lipid molecules so that leakage of entrapped small molecules is minimised. Alternatively, fatty acid (or other lipid) derivatives of drugs can be formulated and can be incorporated into the liposomal structure in the same way as phospholipids and cholesterol. The advantage of this approach is that preparations can be lyophilized: liposomes reformed on rehydration retain the fatty acid derivative of the drug quantitatively. Its disadvantages include a lower drug to lipid ratio than could have been achieved with the water-soluble drug and the additional expense of synthesising the derivative.

Furthermore, newly developed efficient drying methods allow the production of liposome powders which, upon rehydration, do not lose integrity (Madden et al.,1985). Methods have also been devised to produce liposomes with an electric potentialacross the membrane (Bally et al.,1985; Mayer et al.,1985). When some charged drugs are added to

these liposomes, they are pulled through the membrane into the aqueous space/s with greater than 95% efficiency. This process effectively reduces stability considerations from two years to 24 h. That is to say, liposomes with an electric potential can be distributed to hospitals free of drug and only mixed with the drug immediately prior to use by the hospital pharmacist.

Owing to the small number of steps in the manufacture of drug-containing liposomes, there are no really insurmountable problems in maintaining sterility throughout the procedure. The common and not so common sterilization methods are listed below:

1. Filtration through 0.22 um filters. This method is quite promising in that liposomes are not only sterile but also reduced in size, exhibiting a mean diameter of approximately 0.2 um. In instances where liposomes of this size range are safe, stable and efficacious, this method can be used. If, however, larger multilayered liposomes are needed, other methods must be investigated.

2. Gamma-ray treatment. This method is not now commonly used in the pharmaceutical industry but may be ideal for sterilization of liposomes. However, caution should be taken since a published report (Ianzini et al.,1984) indicates that gamma-rays may disrupt liposome membranes.

3. Picosecond laser sterilization. This method (Ostro, 1988) involves a laser pulsing at a frequency that will disrupt DNA but not other molecules. This is still experimental and has not yet been applied to the sterilization of any commercial pharmaceutical.

4. Sterile processing. While this is probably the least desirable of the methods listed, it is currently the most commonly used. Simply stated, the component parts of the liposomes: lipid, buffer, drug and water are independently sterilized by autoclave or filtration and then mixed in a sterile environment.

Fate and behaviour of liposomes in vivo

Crucial to an understanding of how liposomes may best be administered to enable or optimise their efficacy as immunological adjuvants or as antibody-mediated drug carriers is knowledge of their fate in vivo. Administration of drug-containing liposomes of a wide range of sizes and lipid compositions to animals and man has been carried out through every conceivable route, including intravenous, intramuscular, intradermal, intrathecal, intraarticular, intratracheal, topical and enteral. It is now clear that encapsulated drugs exhibit pharmacodynamic and therapeutic properties markedly different from those of free drugs (Alving et al.,1978; Fidler et al.,1981; Forssen and Tokes,1982; Rahman et al.,1983; Proffitt et al.,1983; Lopez-Berenstein et al.,1984: Knight et al.,1985). For certain drugs, an enhancement of therapeutic index attributed to liposomes results from their ability to transport entrapped drug in the circulation in a slow-release form whilst shielding susceptible tissues from unwanted side-effects, carry it to organs containing large numbers of cells of the reticuloendothelial system such as the liver and spleen, and deposit substantially higher levels of drug in these organs relative to other tissues.

Most of the studies concerning the therapeutic and/or diagnostic effects of liposome-entrapped drugs have been conducted using the intravenous route of administration. Thus, it is important to characterise the fate of liposomes in vivo as a consequence of this particular route of administration. Pharmacokinetic parameters such as the rate of clearance from the blood, the rate of accumulation in various tissues, and the rate of release of encapsulated content must be defined and control strategies developed in order to realize the clinical potential of liposomes.

A vast array of experiments has revealed many aspects of liposomal behaviour in vivo which in turn have helped us to optimise liposomal drug action for particular needs. As the amount of data amassed is enormous, only facts central to the successful application of the system in immunology and drug delivery are reviewed here.

Physical and physiological considerations

After being introduced into the blood stream, some liposomes may be degraded due to interaction with serum proteins (Scherphof et al.,1984). The remaining intact liposomes may either distribute within the intravascular space or escape into the extravascular space, depending on the regional permeability of the vascular wall and the size of the vesicles. The passage of small liposomes with diameter less than 0.1 μm through the discontinuous capillaries of the liver has been well documented (Roerdink et al.,1981; Rahman et al.,1982; Scherphof et al.,1983). It was shown that after penetrating the discontinuous capillaries, the small liposomes were taken up effectively by the parenchymal

cells in the liver. Although the intracellular junctions in the discontinuous capillaries in the liver have a width ranging from 0.1 to 0.6 um, the mucopolysaccharide-rich interstitium in the spaces of Disse, which surrounds and lines the endothelium of the discontinuous capillaries in the liver restricts the passage of macromolecules larger than 36-50 nm in diameter (Granger et al.,1979). Therefore, there is virtually no transcapillary passage of liposomes larger than 0.2 um in diameter through the discontinuous capillaries of the liver (Roerdink et al.,1981; Poste et al.,1982; Scherphof et al.,1983).

Similarly, the narrow intercellular junctions (2-6 nm in width) in the continuous capillaries, prevalent in skeletal, cardiac, and smooth muscles, lung, skin, subcutaneous tissue, and serous and mucous membranes preclude the transcapillary passage of even the smallest liposomes (Poste et al., 1982). Information relating to the transcapillary passage of liposomes through the fenestrated capillary is not available. However, because of the dimensions of the open fenestrae (40-60 nm in diameter) and the presence of basal lamina, a layer of fine, fibrous networks of collagens, glycoproteins, laminin and fibronectin, surrounding the fenestrated capillary, the likelihood of liposomes larger than 60 nm in diameter penetrating the fenestrated capillary is small.

Therefore, under normal conditions, large liposomes (larger than 0.5 um diameter) are confined primarily in the intravascular space after intravenous injection. The tissues or organs involved in the uptake of large liposomes will be those containing the phagocytic cells, such as the

blood monocytes and the fixed tissue macrophages of the liver, spleen and bone marrow. On the other hand, small liposomes (smaller than 0.1 μm diameter) can have access to cells located in the interstitial space in addition to the phagocytic cells in the intravascular space.

Pharmacokinetic behaviour of large and small liposomes

It is well established that clearance from the circulation of liposomes of large size and negative surface charge is rapid (Gregoriadis and Neerunjun, 1974; Juliano and Stamp, 1975). Within minutes of intravenous injection, such liposomes are largely found in the fixed macrophages of the liver and spleen and (when made of appropriate lipids to ensure maximum drug retention in blood) constitute the vesicle of choice for the rapid delivery of drugs to these cells. Given certain liposomal lipid compositions coupled with the appropriate vesicle size (1-3 μm) and charge, there can also be significant retention of liposomes by the lungs. By making liposomes smaller (25 nm diameter being the smallest size possible) one can, for reasons that are still not clear, prolong their circulation time to some extent. Recent work (Senior and Gregoriadis, 1982) has shown that exceptionally long half-lives of over 20h can be achieved in the mouse if small liposomes having no net charge, a high cholesterol content and made of high-melting phospholipids are used. Such liposomes are exceedingly stable (in terms of solute retention in the presence of blood). Interestingly, the more stable liposomes are, the longer is their half-life, and it may be that plasma components (eg. opsonins) which adsorb onto liposomes and probably make them recogniz-

able by the RES, cannot do so efficiently when their membranes are packed (by cholesterol, for instance) or `solid' due to high-melting phospholipids (Gregoriadis at al., 1983).

The prolongation of the residence time of liposomes in the blood circulation resulting from the administration of a high lipid dose of liposomes was one of the first observations described in a pioneering paper dealing with the fate of liposomes in vivo (Gregoriadis and Ryman, 1972). Using large liposomes, several investigators found that the uptake of large liposomes by the liver and spleen can be saturated temporarily, thereby increasing the concentration of liposomes in the blood (Abra et al.,1980; Souhami et al.,1981; Kao and Juliano, 1981). In spite of a prolonged clearance half-life in the blood circulation, the uptake of large liposomes by the tissues in the non-reticuloendothelial system (RES) was not enhanced.

These observations appear to be consistent with a pharmacokinetic model in which the sites or tissues responsible for the uptake of large liposomes are saturable and are primarily confined in the intravascular space. Pharmacokinetically, the uptake of a heterogeneous size range of large liposomes by tissues or the elimination of these large liposomes in the blood is compatible with a non-linear, saturable kinetic process.

On the other hand, SUV (mean diameter 50 nm) can escape from the vascular compartment and reach tissues contiguous with the extravascular space. The kinetics of the elimination of SUV from the blood and the uptake of SUV by the liver appears to be compatible with a model proposed by

Beaumier et al. (1983) involving two parallel pathways. One pathway is a high `affinity', low capacity, capacity-limited Michaelis-Menten process mediated predominantly by the phagocytic Kupffer cells in the liver. The other pathway, which becomes significant at higher liposomal lipid doses is characterized as a high capacity, low `affinity' first-order process mediated predominantly by the parenchymal cells in the liver.

Large liposomes are degraded faster than small liposomes in the liver in vivo (Beaumier and Hwang, 1983). This observation may be related to the SUV's ability to escape into the interstitial compartment and partially avoid the efficient degradative machinery of specialised phagocytic cells, such as the Kupffer cells in the liver. Thus, the difference between the rates of degradation of large and small liposomes by the liver is a result of the difference in the sites or cells involved in the uptake of liposomes.

Conventional drug-containing phospholipid liposomes become unstable and leaky upon contact with blood. As a result of interactions with lipoproteins, liposomes can be disrupted with the consequence that their contents diffuse out rapidly into the circulation and the advantage of giving drugs through a delivery system is thus lost. When liposomal disruption does not occur, intact liposomes can acquire apoproteins, which may be recognised by cell surface receptors, thereby affecting the site and rate of delivery (Williams et al.,1986).

Creation of vesicular complexes of lipid and apoproteins

Amongst the factors implicated in liposomal disruption, when it occurs, are the lipoproteins, especially HDL (Tall and Small,1977). Two related mechanisms appear to operate. First, there can be transfer of substantial phospholipid mass from liposomes to pre-existing HDL particles (Tall, 1980). This process is facilitated by plasma lipid transfer proteins. Second, the insertion of apoproteins, particularly HDL apoproteins, into the liposomal lipid bilayer can cause it to break into discoidal bilayer fragments, thereby releasing any material trapped within the aqueous core (Chobanian et al., 1979).

Interference with these two mechanisms allows for liposomal stability in plasma. Liposomes given in high doses (200-800 mg phospholipid kg^{-1} body weight) can exceed the disruptive capacity of HDL and HDL apoproteins (Williams and Scanu,1986). Liposomes that contain unesterified cholesterol in their lipid bilayers do not readily lose phospholipid mass to HDL (Tall, 1980) although exchange of phospholipid between liposomes and HDL continues. In addition, unesterified cholesterol reduces the fluidity of the lipid bilayer making it less easily penetrated by apoproteins (Guo et al.,1980). Thus, liposomes that contain unesterified cholesterol are stable in plasma even at low doses (Kirby et al., 1980). Liposomes of large diameter acquire apoproteins less readily than do small liposomes and are therefore more stable (Scherphof and Morselt, 1984). Liposomes of saturated phospholipids at temperatures either much higher or much lower than the gel-to-liquid crystal phase transition

temperature are poorly penetrated by apoproteins and are resistant to disruption (Scherphof et al., 1979). Sphingomyelin is not lost from liposomes to HDL; thus, sphingomyelin liposomes are stable (Allen, 1981). Liposomes are more stable in whole blood than in serum, possibly because the cellular elements of blood donate cholesterol to the liposomes (Williams and Scanu,1986) and saturate phospholipid uptake by lipoproteins.

There is ample evidence that the liver is the major organ to catabolize circulating vesicular particles (reviewed by Williams et al., 1984). Intravenous injection of liposomes labeled in either the lipid bilayer or the aqueous core results in delivery of tracer mainly to the liver. Predominant clearance by hepatic parenchymal cells (Gregoriadis and Ryman, 1972) by Kupffer cells (Roerdink et al.,1981) and by both cell types (de Barsy et al.,1976) has been reported, and remains a matter of dispute. It has also been reported that SUV are preferentially cleared by hepatic parenchymal cells, whereas large multilamellar liposomes deliver their contents to Kupffer cells (Scherphof et al., 1983). The mechanism of hepatic clearance is believed to be via phagocytosis by the reticuloendothelial system as reticuloendothelial blockade has been shown to slightly inhibit (Souhami et al., 1981), and substantially inhibit (Kao and Juliano, 1981) hepatic uptake. The difference between these studies may have resulted from the use of liposomes of different sizes.

It was recently proposed that the formation of complexes between phospholipid vesicles and apoproteins promotes interactions with hepatic apoprotein receptors,

thereby enhancing hepatic clearance (Williams et al.,1984). Liposomes that contain asialoglycoproteins (Gregoriadis and Neerunjun, 1975; Spanjer and Scherphof,1983) or are attached to LDL (Vidal et al.,1985) have been shown to be rapidly cleared by the liver, presumably mediated by hepatic receptors for asialoglycoproteins and LDL.

<u>Interactions of liposomes with mononuclear phagocytic cells</u>

Regardless of the *in vivo* application to which liposomes are employed, the main group of cells which interact with them and have a profound influence on their fate are the mononuclear phagocytic cells of the reticuloendothelial system. However, the route of administration of liposomes affects the rate and extent of their uptake by the RES. Liposomes have been administered intracerebroventrically (Kimelberg et al., 1978), intra-articularly (Dingle et al., 1978), intragastrically (Desmukh et al.,1981a;), via the respiratory system (McCullough and Juliano, 1979) interstitially (Kaledin et al., 1981), intra-ocularly (Desmukh et al., 1981b) and subcutaneously (Patel et al., 1988). All these routes of administration reduce the rate of uptake of liposomes by the liver and spleen as compared to the intravenous route. The rate and extent of localization of liposomes in macrophages of the liver and spleen is also dependent on liposome composition. Liposomes composed primarily of egg PC and cholesterol are removed rapidly from circulation with half-lives dependent on liposome size. However, SUV made from a phospholipase-resistant dialkyl analog of PC (D-L-2-(tetradecyl)-octadec- cis11ene-1-oyl- (O)-

phosphorylcholine) and cholesterol had a three-fold longer circulation time than liposomes composed of egg PC and cholesterol (Deshmukh et al., 1978). Subsequent experiments showed that there is a relationship between liposomal stability in the presence of plasma and rate of removal from circulation of uncharged liposomes. Factors which decrease leakage of liposome contents in plasma also decrease the rate of uptake of liposomes by the MPS (Gregoriadis, 1988). The most important factor contributing to this appears to be bilayer rigidity, which renders liposomes more resistant to the destabilizing effects of serum components, in particular high-density lipoproteins (Scherphof et al., 1978). The rigidifying effect of cholesterol, therefore, appears to be the major contributor to the decreased rate of uptake by the MPS of fluid liposomes containing cholesterol. Solid liposomes such as those composed of DSPC or containing large amounts of sphingomyelin also show decreased permeability in the presence of plasma and decreased rate and extent of uptake into the liver. It has been reported that liposome size and surface charge override the state of membrane permeability in determining rates of liposome clearance (Senior and Gregoriadis, 1982).

As liposome dosage increases, the ability of the liver to remove liposomes from circulation becomes saturated. Saturation of liposome uptake into liver, or decrease in the rate of liver accumulation of liposomes almost always results in increased accumulation in spleen, which takes up liposomes more avidly than liver if they remain in circulation long enough. Saturation of splenic uptake of liposomes results in increased bone marrow accumulation (Poste, 1983).

Other than the accumulation of SUV in hepatocytes, no convincing evidence has yet been presented for accumulation of liposomes in cells other than those of the MPS. It is possible, however, by alteration of liposome dose, and composition to cause significant alterations in liposome accumulation within the different cell populations of the MPS. Liposomes with long half-lives not only accumulate in the liver and spleen at a much slower rate (an event which gives targeted liposomes the opportunity to interact with other tissues), but they also exhibit a different distribution within the RES, favouring the macrophages of the bone marrow; up to 30% of a dose of long-lived liposomes can end up in this tissue. In addition, liposomes smaller than approximately 100 nm can reach the parenchymal cells of the liver through the fenestrations. Further, in animal models bearing tumours, localization (up to 18% of the injected dose/g tumour tissue) of small liposomes in the tumour has been claimed (Proffit et al., 1983). However, evidence so far with normal animals suggests that even the smallest liposomes cannot cross capillaries to enter the extravascular space.

The fate of liposomes at the subcellular level includes their endocystosis by cells into lysosomes where the liposomal structure is broken down and entrapped drug contents are liberated. The contents either act within the lysosomes themselves or in other cell compartments, depending on the extent to which they remain active in the lysosomal milieu or their ability to diffuse through the lysosomal membranes. Current research suggests that it may be possible to interfere with the lysosomotropic process to enable

liposomes to present drugs to other cell areas without the hitherto obligatory step of lysosome localization (Allen, 1988).

Subcutaneously administered liposomes appear to be able to traverse the endothelial membrane and lymphatic channels and remain intact in the lymphatic circulation after which they become localized in the lymph nodes. The lymphatic uptake of liposomes depends on the charge, size and lipid composition of vesicles (Patel, 1988). Liposomes of a size range below the region of 100 nm are readily taken up by the lymphatics while those of a larger size may be retained at the site of injection (Bergqvist et al., 1983). Negative liposomes tend to be localized in lymph nodes more than positive, and positive liposomes more so than neutral SUV liposomes.

Immunological applications of liposomes in medicine

The ability of liposomes to encapsulate and then release markers or drugs and to interact with macrophages in vivo within the RES, the blood circulation or granulomata in a variety of tissues forms the basis for most of the medical applications of the system with some hope of early realization. In this respect, liposomes can operate in at least three ways: 1) by binding to specific receptors to enable their visualization or quantification by means of entrapped marker substances; 2) by introducing agents into cells to act on undesirable residents, be they micro-organisms or chemical agents; 3) by using drugs which activate pathways initiated by the cells leading to protection of the individual through elimination of the pathological agent.

Immunoliposome assays

In the past few years, a renewed interest has been placed on liposome-based immunoassays for detecting immune-related compounds or analytes of interest. This current trend is attributed to the intrinsic advantages in using immunoliposome assays (ILA) over other methods of immune detection such as radio-immunoassays (RIA) or enzyme linked immunosorbent assays (ELISA). For example, the aqueous space of liposomes can be made to carry a wide variety of reporter molecules thus avoiding the use of radioactive molecules whilst maintaining, or even improving the sensitivity of the immunoassay. Although the use of enzymes in ELISA-type assays evades the handling of radioisotopes, the intrinsic problems of elaborate time control requirement and extensive washing procedures remain a major drawback. In addition, well-trained technicians and relatively expensive instruments are required to achieve maximum efficiency of detection. Current understanding of membrane properties and their interaction with a wide variety of biological substances enables one to design ILA that are easily adaptable to various applications. Another advantage of ILA is their rapidity, with most assays being complete in minutes. Accordingly, ILA fits in with the growing demand for a simple immunoassay method that requires neither a skilled technician nor expensive instrument for home, physician's office or field applications.

Liposomes have been used in various types of immunoassays by several investigators (Axelsson et al., 1981; Freytag and Litchfield, 1984). The antigen is usually incorporated into the lipid bilayer membrane matrix of the

liposome. In the presence of specific antibody and complement, the membrane releases an entrapped enzyme marker which is then measured. A sensitive homogeneous assay for theophylline (Haga et al., 1981) was developed using a Clark-type oxygen electrode immunosensor and actively sensitized liposomes with entrapped horse-radish peroxidase enzyme. The liposome lysis is induced by specific anti-theophylline antibodies and complement. The reaction was monitored by the release of entrapped HRP enzyme. Theophylline was detectable at a concentration of 0.7 ng/ml.

A unique homogeneous immunoassay called enzyme membrane immunoassay (EMIA), based on the use of immunoreactive liposomes, has been developed by Collaborative Research Inc., Lexington, Mass. In this method, liposomes of a size ranging from 200-1000 nm in diameter are used to encapsulate alkaline phosphatase. The enzyme lies separated from its substrate, p-nitrophenyl phosphate solution which is located outside the vesicles. Antigen molecules are attached to the surface of each liposome. When specific antibody to the antigen is incubated with the antigen-coupled liposomes in the presence of complement, lysis occurs with release of entrapped enzymes. The released alkaline phosphatase cleaves the substrate and the resulting colour reaction product is monitored spectrophotometrically. Only one incubation step is necessary, even though there are two reactions. In the assay procedure, the reagent components are mixed with the test sample followed by incubation at 37°C for 30 min.

Assays have been reported for thyroxine with a sensitivity range of 20-200 ng/ml; and for chorionic gonadotrophin with a sensitivity range of 1-10 ng/ml. These assays

can be used for both small and large molecular weight analytes. The assay is sensitive and may be useful in screening procedures, but high levels of rheumatoid factor in sera may interfere with the precision of the assay.

The current status of ILA can be considered by comparing it with other established immunoassays. It is evident that at present, none of the ILA has been expanded to commercial scale although their sensitivities are comparable with commercially used immunoassays. In studies where ILA is used to detect HSV, ILA requires a significantly shorter time than the conventional plaque assay and includes no washing process. Similarly, in work on rheumatoid factor detection, the sensitivity of ILA is about tenfold higher than the clinical latex-agglutination assay. Thus, ILA do give a better overall performance than certain existing assays in addition to avoiding the use of radioisotopes. However, the lack of clinical ILA application is partly due to the instability of reagents such as complement components used in earlier designs of ILA. Recent advances in design such as the one based on target-sensitive immunoliposomes have removed some of the instability problems. With further improvement in sensitivity and stability, the commercial application of ILA looms in sight in the near future.

Liposomes as immunological adjuvants

Adjuvants are defined as agents which act in a non-specific manner to augment the immune response against a given antigen. The need for an effective and safe adjuvant for use in human immunization programs is well recognized

(Gregoriadis, 1985). Not only are many of the antigens costly or available in limited quantities (eg. products of recombinant DNA technology) but also, synthetic peptides for instance, can be weakly or non-immunogenic. Amongst the disadvantages of presently available adjuvants eg. complete and incomplete Freund's adjuvants, bacterial endotoxins, polyanions, mineral adsorbents, etc., are the induction of local and systemic toxicity, formation of unacceptable granulomas, lack of efficiency or the eliciting of only short term effects. Another possible hazard with some of these adjuvants is the production of allergic reactions to the associated vaccines in a minority of recipients, especially if antigens have been administered twice to boost immunity and in those already sensitized to the antigen. If adjuvants contain even traces of immunogenic materials such as proteins or glycolipids, the latter may themselves induce allergic or autoallergic reactions. On the other hand, live virus vaccines can potentially initiate persistent infections or even malignancy. Even the vaccinia strategy which employs the original smallpox virus vaccine as a genetically engineered vector poses considerable risk.

Liposome-mediated enhancement of antibody formation which spawned a burgeoning field of investigation on the possible role of liposomes as immunological adjuvants was first established when strong antibody responses to diphtheria toxoid entrapped in liposomes were observed after administration into mice (Allison and Gregoriadis, 1974). In contrast with other adjuvants, there were no granulomas at the site of injection and no hypersensitivity reactions were observed in pre-immunized animals when the antigen was given

in the entrapped form. In addition, liposomes composed of the appropriate phospholipid (eg. egg PC) do not develop antibodies against their phospholipid component nor have they produced any side effects in repeatedly injected patients (Zonneveld et al., 1988). Thus, the inherent advantages of the liposomal model encouraged workers to see if the adjuvant effect was reproducible for other bacterial, viral and cellular substances. This was fortunately found to be the case with subsequent work extending observable liposome adjuvanticity to a large variety of antigens. These include tetanus toxoid (Davis et al., 1987), hepatitis B surface antigen (Manesis et al., 1979), and polypeptides derived therefrom, *Herpes simplex* virus antigens (Naylor et al., 1982), *Plasmodium falciparum* antigens (Alving et al., 1980), *Streptococcus mutans* cell-wall antigens (oral route) (Wachsmann et al., 1985), *Streptococcus pneumoniae* serotype 3 (Snippe et al., 1983), *Salmonella typhimurium* lipopolysaccharide (Desiderio et al., 1985), foot-and-mouth disease virus peptides (Francis et al., 1985), Epstein-Barr virus gp340 protein (Epstein et al., 1985) and spermatozoal polypeptide fraction (Mettler et al,. 1983).

The exceptional structural versatility of liposomes in terms of vesicle size, lamellarity, surface properties and composition, and their ability to accommodate antigens in a variety of ways (eg. entrapped within the aqueous phase, embedded in the lipid bilayers and adsorbed or covalently linked to the liposomal surface) suggests variability in immunoadjuvant action which could be tailored to satisfy particular needs. On the other hand, such versatility in conjunction with variation in the type of anti-

gens, animal species and immunization protocols used, have contributed to conflicting results (Kinsky, 1978; Hedlund et al., 1984; van Rooijen and van Nieuwmegen, 1980; van Rooijen, 1988). Amongst these controversies is one concerning the role of antigen localization, with some workers supporting the view that antigens linked to the surface of liposomes give stronger responses compared to when the antigen is entrapped within the aqueous compartments and others claiming the exact opposite. Additional liposomal parameters thought to promote adjuvanticity include small vesicle size, unilam ellarity of vesicles and bilayer rigidity.

Although contrasting opinions with regard to the role of various liposomal characteristics may well be legitimate and merely reflect variations in experimental materials and protocols, there have been cases where experimental conditions have not taken into account influencing factors which have recently come to light. That liposomal lipid to antigen mass ratio is instrumental in inducing liposome adjuvanticity has been stressed by Davis et al. (1987). However, review of the literature reveals that an extreme range of such ratios has been employed by as many groups (even within experiments of a given study) and it may be that a ratio which is advantageous for one antigen or a particular variety of liposome is not so for others.

The general principles governing liposomal adjuvanticity, in view of the multiple parameters involved, were attempted to be systematically elucidated using immunopurified toxoid as a model bacterial antigen incorporated into multilamellar liposomes composed of equimolar phospholipid and cholesterol by Davis et al. (1987). Incorporation was

carried out either by passive entrapment (DRV liposomes), or by covalent linkage (using a diazotization procedure) to the surface of multilamellar liposomes (MLV) of comparable size and lamellarity but prepared by the classical procedure. It was interestingly found that the addition of the cryoprotectant trehalose during freeze-drying of formulated toxoid-containing DRV for storage purposes could markedly improve retention of entrapped antigen upon subsequent rehydration prior to use. Liposomes with entrapped or surface-linked toxoid were employed in a variety of immunization experiments in Balb/c mice. Antibody (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgM) responses to the antigen were monitored by an enzyme-linked immunosorbent assay (ELISA). Results indicated that liposomes increase the antibody response within an individual IgG subclass independently of their effect on other subclasses. When the responses of mice injected with high and low doses of toxoid were compared, it was found that as the dose of antigen was increased, there was an increase both in the antibody response within an individual subclass (whether the antigen was associated with liposomes or not) and the number of subclasses involved in the response. Thus, the antibody response within the IgG1 subclass showed an adjuvant effect following injection of low doses of toxoid. Effects with IgG_{2a} and IgG_{2b} could be seen at almost all doses whilst an adjuvant effect was seen within the IgG_3 subclass only at the highest dose studied. A significant adjuvant effect was also observed with the IgM response but only in one experiment. Therefore, liposomes in acting as adjuvants, do not lead to qualitative changes (shift) in the IgG subclass produced and can be considered as type 1

adjuvants. In other experiments in which mice were primed with liposomal surface-linked antigen and boosted with free, liposome-entrapped and liposomal surface-linked toxoid, it was found that the level of antibody (IgG_1 and IgG_{2b}) after the second injection was determined by the amount of injected antigen and was independent of its form (free or liposomal). Direct interactions between the antigen and its antibody on B cells with subsequent stimulation of the B cells and avoidance of antigen processing by antigen-presenting cells might be expected following the injection of the antigen. It was therefore postulated by Davis and Gregoriadis (1987) that presentation of an antigen on the surface of liposomes would enable this interaction with B cells to take place and offered the best opportunity to observe an adjuvant effect during a secondary immune response. The lack of such an adjuvant effect following a second injection of the antigen indicates that it is the events following the first injection of antigen which determine the adjuvanticity of liposomes.

The effect of liposomal membrane characteristics on the immune response was also studied. It is initially assumed that the immunoadjuvant action of liposomes would be favoured by an increased concentration of antigen in individual vesicles as this would allow presentation of sufficient quantities of antigen to immunocompetent cells. Contrary to such expectations, data suggested that the higher the liposomal lipid:toxoid mass ratio, the higher the immune response (IgG_1 and IgG_{2b}). Although a ratio of 48:1 was already established as sufficiently high for adjuvanticity to occur, Davis and Gregoriadis (1987) showed that adjuvan-

ticity is improved to a much greater extent when ratios as high as 3000:1 or more are used. It thus seems that liposomal adjuvanticity is related to the lipid dose, possibly in conjunction with a slow rate of degradation/removal of liposomes given in excessive amounts from the site of injection. There is therefore a fundamental difference between experiments designed to demonstrate the adjuvant effect of liposomes using free antigen as control and experiments to compare the adjuvant effect of various liposome preparations. In the latter case, it is not sufficient merely to compare the antibody response of mice injected with the same mass of antigen in different liposome preparations. The mass of the injected liposomal lipid must also be the same. Hitherto, these conditions have been difficult to fulfill since antigen entrapment values with the procedures used have been low and/or unpredictable or the significance of an optimal lipid to antigen mass ratio has not been appreciated. These problems are reflected in the diversity of views regarding liposomal adjuvanticity and its control.

Another liposomal characteristic examined was bilayer fluidity. Other workers have shown that liposomes composed of `high-melting' phospholipids (and incorporating membrane antigens) promote a higher immune response than liposomes composed of `low-melting' phospholipids. Results, however, with tetanus toxoid (incorporated in the aqueous phase of liposomes) suggest the opposite. Using a variety of liposomal phospholipids and equimolar cholesterol with a wide range of T_c, higher responses (IgG_1 and IgG_{2b}) were found with PC (T_c -8^oC) liposomes than with DRV liposomes composed of DSPC (T_c 54^oC). The former preparations are

expected to be `fluid' at body temperature whilst the latter remain `solid'. Liposome adjuvanticity can, therefore, be observed under two sets of conditions. Membrane antigens which show high antibody responses when presented in `solid' liposomes and antigens which require processing by antigen presenting cells and give higher antibody responses when presented in `fluid' liposomes. The difference in liposome adjuvanticity between these two conditions may be related to the events which follow the interaction of liposomes with the cell membranes. `Solid' liposomes would be expected to hold the antigen firmly and, in the case of membrane antigens, this rigid platform may help in the interaction of the plasma membrane of presenting cells and that of antigens requiring lymphocytes. However, the same rigidity may impede the release of antigens requiring processing intracellularly, particularly in antigen-processing cells which are deficient in phagocytic activity. Finally, examination of the mode of antigen localization in liposomes revealed no differences in immune responses (IgG_1 and IgG_{2b}) between liposomes incorporating the toxoid in their aqueous phase and liposomes bearing the toxoid on their surface. These results contrast with those of others (van Rooijen and van Nieuwmegen, 1980; Snyder and Vannier, 1984) who, however, employed widely different lipid to antigen ratios in the liposome-entrapped and surface-linked antigen preparations.

Activation of macrophages with liposome-entrapped immunomodulators

The selective targeting of immunomodulators to appropriate sites of action is an integral and important

component of immunotherapy for infectious diseases and cancer. Considerable attention has been focused on the potential use of liposomes to deliver different classes of drugs. However, most attempts to target liposomes to solid tumours have been disappointing because large phospholipid vesicles are unable to reach organ parenchyma and are rapidly bound to and endocytosed by fixed and circulating phagocytic cells.

This natural fate of liposomes has been taken advantage of to target, albeit passively, immunomodulators entrapped within liposomes to cells of the RES, which results in their activation to the tumouricidal and viricidal state (Fidler et al., 1988). In addition, the systemic administration of liposomes containing various immunomodulators has been shown to bring about regression of lymph node, lung and liver metastases in rodents and dramatic prophylaxis of viral infections in rodents. It has been proposed that the exploitation of this natural liposome-localization phenomenon can achieve the selective delivery of immunomodulators to phagocytic cells and thereby enhance host resistance to various infectious diseases and cancer. (Fidler et al., 1988).

Targeting of liposomes

In vitro studies

The early observation of quantitative interception of liposomes by the fixed macrophages of the RES has prompted workers interested in delivering drugs to other cells to develop means of endowing liposomes with target selectivity.

Cumulative evidence suggests that the following criteria are of particular importance for successful targeting (Gregoriadis, 1985). Firstly, the rate of uptake of liposomes by the RES must be diminished considerably so that liposomes can circulate in the blood long enough to ensure quantitative vesicle binding to the target. In this respect, small (about 64 nm diameter), neutral unilamellar liposomes composed of high-melting phospholipids and cholesterol appear suitable. Larger liposomes (accommodating much more drug than small ones) could also be targeted if interaction with the relevant cells is very rapid and occurs quantitatively before significant interference by the RES. This seems unlikely, however, in view of the avidity with which liver and spleen remove large liposomes from the circulation. Attempts to delay clearance of large vesicles, successfully tested in animals, include saturation of the RES with empty liposomes followed by the administration of vesicles containing the drug. Unfortunately, such an approach requiring the injection of large amounts of lipid may be too toxic for routine use in the clinic. Alternatively, it may be possible to coat the liposomal surface with agents which render liposomes less recognizable by the RES. One such agent, Poloxamer (a non-ionic surfactant of the polyoxyethylene-polyoxypropylene series), has been used with some success in reducing the rate of clearance of nanoparticles (Illum and Davis, 1984).

Secondly, liposomes must bear on their surface, appropriate molecules (ligands) which can bind onto receptors located on the surface of target cells. A variety of targeting ligands have been used for this purpose, including antibodies (raised against cell surface antigens) and glyco-

proteins or glycolipids with terminal sugars which recognize their respective cell-surface receptors. Grafting of ligands onto the liposomal surface can be carried out by various techniques. These are classified into those with anchoring hydrophobic regions enabling spontaneous insertion of the ligand into the lipid bilayer during the preparation of liposomes, and those which require a coupling reaction between ligand and liposomes.

IgG is an example of a hydrophobic ligand: up to 20-30% of the immunoglobulin can be incorporated into small liposomes formed by sonication in the presence of the immunoglobulin with the antigen-recognizing variable region available on the surface of the liposome. In the coupling methods, a heterobifunctional reagent is used to join appropriate groups of the ligand and of one of the liposomal lipid components. Regardless of how targeted liposomes are prepared, ligands are generally capable of mediating their uptake by the cell target in vitro. Uptake can vary from poor to quantitative depending on such parameters as the type of ligand, the curvature of liposomes and the line of cells used (eg.sufficient density of surface receptors).

Thirdly, where there is a need for pharmacological action, it is usually desirable that ligand-mediated binding of liposomes to cell receptors is followed by the interiorization of the drug-containing carrier and subsequent liberation of the drug in active form. A series of experiments have now demonstrated pharmacological action on cell targets following binding, presumably upon entry of liposomes into the cells. However, other workers have shown that liposome interiorization by cells cannot be taken for granted once

binding has occurred. Factors influencing liposome entry include the metabolic state of the cells, some of the liposomal structural characteristics (eg. size, surface charge) and the nature of binding.

<u>In vivo studies</u>

Although the ability of targeted liposomes to interact with relevant cells specifically has been demonstrated mostly in vitro, there is convincing evidence to show that targeting can also occur in vivo. A classic example is that of liposomes bearing molecules which bind to the galactose receptor in the liver. Upon injection, these liposomes are taken up selectively by all accessible tissue cells expressing the galactose receptor (Spanjer and Scherphof, 1983; Szoka and Mayhew, 1983; Gregoriadis and Senior, 1984), as shown by blocking the receptor with a free ligand such that specific uptake, but not non-specific uptake, of liposomes decreases. More recently, targeting in vivo within the vascular system was achieved using small liposomes coated with monoclonal antibodies (Wolff and Gregoriadis, 1984). AKR mice were injected intravenously first with AKR-A cells and soon after with liposomes coated with monoclonal IgG1, specific for the Thy.1 antigen expressed on the surface of these cells. Liposomes and cells still in the circulation interacted to some extent. The advent of hybridoma technology has now added further impetus to liposome targeting which could, under certain conditions, have advantages over the use of antibodies coupled to drugs directly. For instance, liposomes can accommodate large quantities of a wide range of drugs, keep them in isolation from the biolog-

ical milieu and bring them into contact with cells, potentially by a single immunoglobulin molecule per vesicle. In addition, liposomes with antibodies against more than one cell-surface antigenic determinant may exhibit a firmer and more specific association with the cells expressing the determinants. However, in contrast to antibodies, liposomes, like other particulate carriers, cannot undergo transcapillary passage and are not therefore ideal for targeting to extravascular sites, at least by the intravenous route.

Scope of the thesis

The aims of this thesis are to further investigate aspects of two rather widely differing but well established areas within the spectrum of an immunological approach to dealing with disease where liposomes have found a useful niche, and in which experimental foundations have been built to facilitate their eventual clinical use. Within this wide range of applications encompassing prevention, diagnosis and therapy, the possible utility of liposomes as carriers and adjuvants of viral and bacterial constituents in a new generation of vaccines is explored, together with possible alterations in the physical make-up and administration of the liposome-antigen complex which may render them more effective. These studies form the bulk of the thesis and constitute its first part. The second section examines the potential use of liposomes as antibody-mediated targeted carriers of drugs. It also attempts to evaluate the physical characteristics of liposomes and the conditions in the biological milieu into which they are released which may

influence their fate and efficacy.

As a development of the theme pursued by various investigators of using liposomes to entrap viral, bacteria and cellular products to enhance their immunogenicity and to initiate the section on liposomes as immunological adjuvants, the first chapter describes the encapsulation of reconstituted influenza virus envelopes (RIVE) in multilamellar dehydration-rehydration vesicles (DRV). These particles are of the same size as intact influenza virions and are the largest substances capable of being efficiently and reproducibly entrapped in liposomes to date. DRV markedly enhances the immune response against the entrapped agent and the complex may be a suitable candidate for a liposome-based influenza vaccine.

At the other extreme of size range exhibited by viral products, in contradistinction to the large reconstituted virions mentioned above, lies the class of oligopeptides synthesized using recently-developed technology which enables short immunogenic regions of whole portions to be predicted. Using two polio virus subunit peptides either encapsulated within or covalently-linked to the external surface of DRV via a novel ligand molecule intercalated in the bilayer membrane, the immune response against the two separate modes of presentation is studied in chapter 2. The difference in character of the response against each arrangement has important implications in considering the use of liposomes as a vehicle and adjuvant of subunit vaccines.

In addition to the `basic' structure of having a liposome entrapping or coated with an antigen, accessory substances such as biological response modifiers may be co-

entrapped with the antigen to form a `bivalent' liposome which would exhibit a desired effect. Chapter 3 deals with the co-entrapment of the lymphokine interleukin-2 (IL-2) together with tetanus toxoid as the model antigen and examines the possibility of using recombinant IL-2 as a supporting slow-release adjuvant in DRV. The positive and negative effects of IL-2 on the immune response to tetanus toxoid and their implications on the nature of IL-2 action are discussed.

The multicomponent character of liposomes enables one to tailor-make their constituents so as to study the variation in each of the parameters on the effectiveness of the complex as a whole. Chapter 4 examines the use of a novel positively-charged lipid, BisHOP, and phospholipids of varying acyl chain lengths on the adjuvant effect of liposomes containing tetanus toxoid. The beneficial effect of administering liposomes in two separate sites, one intramuscular and the other intradermal at a relatively avascular site, on the primary response is also described.

The second major section which deals with the use of liposomes as targeted drug carriers and the physical and physiological factors which may govern their fate and action begins by considering the properties which the positively-charged lipid, BisHOP confers on liposomes in which it forms a bilayer membrane constituent. Thus, using the same lipid, whose remarkable adjuvant properties were described above, in a totally different context but in which it plays no less important a role, chapter 5 details the effect of liposomal charge and lipid composition on the pharmacokinetic behaviour of small unilamellar liposomes after intravenous

injection in the mouse. A correlation between the microelectrophoretic behaviour of charged multilamellar liposomes in vitro is drawn but limits as to the direct inference to properties of two markedly different species of liposome in two dissimilar environments are pointed out.

The `magic bullet' concept of drug targeting conceived by Paul Ehrlich advanced a step closer to reality with the advent of monoclonal antibody technology. The latter approach was employed to develop a targeting ligand to be used in subsequent studies on the delivery of liposomes to hepatocellular carcinoma cells. Chapter 6 describes the novel approach of using membrane preparations derived from human hepatocellular carcinoma cells to immunize mice which are used in the production of monoclonal antibodies that bind to cell surface determinants present on three HCC cell lines as well as a patient's hepatoma tissue sections.

The next logical step in the construction of antibody-mediated targeted carriers would be to attach the antibodies to the liposomal structures. A simple and effective way of coating small and large liposomes with various proteins, including antibodies, was developed and is documented in chapter 7. The method makes use of the diazotization reagents NaNO2 and HCl/NaCl and is largely complete within four hours. Amongst the advantages of the technique are its amenability to control of the degree of binding by varying the molarity of the reagents employed, its rapidity and its applicability to a wide range of proteins.

The final chapter outlines the use of the monoclonal antibodies produced, in targeting small unilamellar liposomes containing the aqueous phase marker, carboxyfl-

uorescein to hepatocellular carcinoma cells in vitro. Antibodies were passively adsorbed to the liposomes as well as coated on liposomes by the method outlined in the preceding chapter. Results indicate that it is possible to deliver encapsulated material to target cells via this strategy.

Materials and Methods

Preparation of carboxyfluorescein (CF)

The aqueous phase marker CF was prepared as a 0.25M solution in distilled water to which NaOH was added to bring the pH to 7.4. The acid form of CF (as obtained from Kodak-Eastman Co., N.Y.) was only sparingly soluble in water so half the final volume of distilled water was added to the weighed orange powder, and NaOH pellets added gradually until a dark orange-brown solution was formed. The pH was adjusted to 7.4 with 1M NaOH and the final volume made up with distilled water.

Solubilized CF was purified on a Sephadex LH 20 column (40 cm x 2.5 cm) preswollen in distilled water. Elution with distilled water produced a series of coloured bands, with the bulk of material eluting some 20 ml after the void volume (approximately 80-100 ml from start of elution). Pure CF was a bright, clear medium orange colour. It was adjusted to a concentration of 0.1M by comparison with a standard concentration of CF using a fluorimeter, diluting samples appropriately.

Assay of liposomal stability

To assess stability of CF-containing liposomes, CF latency was determined. A fluorimeter (Perkin Elmer model 204) was set up with excitation and emission wavelengths of 490 and 520 nm respectively and standardized using 10^{-6} M CF. Free CF was measured in 5 ul of liposome suspension (about 100-200nmol lipid) diluted to 4 ml with PBS. Total CF was

measured by adding Triton X-100 (final concentration 1%) which disrupts lipid bilayers to release all entrapped CF. Complete disruption with Triton X-100 can be facilitated by warming the samples prior to reading at room temperature. With certain lipid compositions (eg. DSPC:Chol 1:1 molar ratio), MLV (but not SUV) exposed to a concentration of Triton X-100 well above 1% have failed to liberate CF fully unless samples are briefly boiled. Detergent disruption of liposomes with release of CF was seen when the slightly orange-coloured suspension of liposomes due to the presence of self-quenched CF became a bright fluorescent green solution of free CF. Samples were adequately diluted so that readings were below that of the standard and `drift' corrected for by restandardizing before each group of readings. Triton X-100 added neat (one to two drops) needs warming to dissolve with samples cooled to room temperature before reading. Alternatively, a suitable volume of 10% Triton X-100 in PBS may be added taking into account the dilution factor in comparing readings before and after detergent addition. Latency was calculated from:

$$\% \text{ CF latency} = \frac{dye_t - dye_f}{dye_t} \times 100$$

where t is total CF present measured after addition of Triton X-100, and f is free CF measured before the addition of detergent.

Preparation of Multilamellar Vesicles (MLV)

MLV were prepared from equimolar phospholipid (egg PC or DSPC) and cholesterol. 0.25 ml 100mg/ml (32 μmoles) egg PC (Lipoid) in chloroform or 0.5ml 10 mg/ml (32 μmoles) DSPC (Lipoid) was mixed with 0.625 ml 20mg/ml (32μmoles) cholesterol (British Drug Houses, U.K.) in a 50ml round-bottomed flask (Quickfit). For the preparation of charged liposomes, 5% and 10% positively- (stearylamine and BisHOP) or negatively-(PA) charged lipids were incorporated (on a molar basis) of the total amount of phospholipid used. The lipids were dried to a thin film by evaporation of the solvent at a low speed in a rotary evaporator (Büchi) connected to a running tap water pump. Once the lipid had dried to a thin film, the flask was left rotating at a high speed partially submerged in a water bath at about 35 C to 40 C to aid in driving off remaining solvent. Rotation for 10 min was adequate but since it is important to remove all traces of solvent, dry lipids were left under a stream of nitrogen for at least a further 10min.

2ml of pH adjusted PBS, or CF solution (for the subsequent formation of CF-containing SUV), or distilled water for the eventual formation of DRV) was added to the dried lipid film, and inert gas gently layered over the flask contents, especially if unsaturated lipids (including Chol) was used, to protect them from oxidation by atmospheric oxygen. Gentle shaking was sufficient to dislodge the lipid from the sides of the flask to form a cloudy emulsion but to expedite the process and render it more efficient, the flask was lowered into a bath sonicator (Kerry) and

subjected to bursts of sonication while being manually rotated. For phospholipids with a high liquid crystalline phase-transition temperature (T_c) eg. DSPC, some warming slightly above the T_c was required. The emulsion was allowed to anneal above the T_c of the phospholipid for at least 1h. Samples were stored at 4^oC under oxygen-free nitrogen until use.

Preparation of SUV

To convert multilamellar vesicles into small unilamellar vesicles, sonication of the MLV samples was performed using an MSE sonicator adjusted to maximum amplitude so that the sample was seen to vibrate vigourously. To obtain maximum entrapment, sonication was performed at a temperature suitable for the lipid used by partially submerging the flask in a beaker of water, at or slightly above, the transition temperature of the lipid. Since the sonication process itself produces heat, a 1-min burst of ultrasound is followed by 30-60 sec of cooling. Under the conditions described, a total sonication time of 10 min gives a clear preparation as the predominantly large vesicles in the original preparation become small. Sonication at temperatures below the transition temperature may still enable dye-containing SUV to form, especially for vesicles with Chol equimolar to phospholipid, but entrapment is substantially reduced. Typical sonication temperatures are 4^oC for egg PC and 60^oC for DSPC. After sonication, the preparation is left at the corresponding temperatures for another hour during which time clarity must be maintained. Occasionally, clear preparations composed of DSPC or DPPC and equimolar Chol became cloudy and were rejected.

For the preparation of CF-containing SUV to be used in in vivo clearance studies, large vesicles (together with titanium fragments liberated from the probe tip during sonication) were spun down at 100,000g in an ultracentrifuge, a typical run lasting for 40 min. Liposome suspensions sonicated below their T_c and/or left below it before or after sonication will produce large amounts of non-SUV lipid on centrifugation which may, on account of the relatively high density of 0.1M purified CF present, float. In all preparations where temperature criteria were met, a clear supernatant or, when the dense 0.1M CF was used, a clear infranatant was obtained.

For the preparation of protein- or peptide-containing SUV for the subsequent conversion to DRV, sonicated preparations were centrifuged at 500g for 15min to spin down titanium fragments but without the concomitant deposition of large vesicles, as this would constitute removing an unquantified amount of lipid whose presence would otherwise yield a higher entrapment value on controlled rehydration after lyophilization in the DRV-formation procedure.

CF-containing SUV together with unentrapped CF were passed through a Sepharose CL-6B (Pharmacia) column (1 cm x 20 cm) equilibrated with 0.1M sodium phosphate buffer containing 0.8% NaCl and 0.02% KCl, pH 7.4 (PBS) to separate the entrapped from free CF. Depending upon the lipid composition and the amount of Chol present, SUV eluted in PBS at the end of the void volume were collected in 1.5-2.0ml for 1.2ml of sample applied.

Chol-containing liposomes could be seen as a slightly opaque suspension even if `empty' (buffer only

entrapped). Free CF elutes about 15 ml after the void volume. The liposome suspension could be concentrated within dialysis tubing by exposing it to solid PEG 6000 which absorbs water. Sepharose CL-6B was re-used after extensive washing with distilled water, although caution was required as most materials (eg. CF, lipids and lipoproteins) stick to the column to some extent. Before being used, it was re-equilibrated with six times the volume of PBS compared to gel. Only very stable liposomes retained all associated marker on elution, so all preparations were used immediately or routinely dialysed against PBS at 4 C until use, usually overnight. This adjusts CF latency to 95-100%.

Preparation of Dehydration-rehydration vesicles (DRV)

1 ml SUV containing equimolar egg PC or DSPC Chol in distilled water prepared as described in the preceding section was mixed with 1ml of solution (in distilled water) of the material to be entrapped in a 50ml polycarbonate centrifuge tube. After freezing at $-20^{o}C$, the preparation was lyophilized in a freeze-dryer at a vacuum of 0.05mbar. Samples were left to freeze-dry overnight. The following day, the preparation was allowed to come to room temperature and rehydrated with distilled water using a volume equivalent to one-tenth of the total volume of SUV used (i.e. 0.1ml in the present case). During rehydration, the temperature of the water and lipids was maintained above the transition temperature of the phospholipid used to ensure adequate fluidity of the bilayers to enable liposomes to anneal. Larger volumes of distilled water used for rehydration often resulted in reduced levels of entrapment while smaller

ones were sometimes insufficient to completely wet the material. Rehydration of lyophilized material with one-tenth of the original solution volume results in a ten-fold increase in overall concentration of the solute, and this is reflected in the concentration of the material that is actually entrapped. Rehydration was generally aided by vortexing lightly and the preparation was left to stand for 30 min. 0.9ml PBS was then added to bring the volume of the sample to 1ml. The liposomes were separated from non-entrapped material by diluting with PBS to 20ml followed by centrifugation at 10,000g for 30 min.

Charged liposomes were prepared by incorporating BisHOP (positively-charged) as 10% and 20% (on a molar basis) of the total phospholipid. When preparing DSPC-containing DRV, the SUV were hydrated, sonicated and annealed at 50°C. After freeze-drying, the material was rehydrated at 50°C and maintained at this temperature for a further 30min.

Enzyme-linked immunosorbent assay (ELISA)

Antibody responses to liposome-associated antigens in immunised mice were monitored by a microplate ELISA as follows: A solution of the antigen in 0.05M sodium bicarbonate buffer (pH 9.6) was added into each of the wells of a plastic microelisa plate (Dynatech) and incubated at 4°C overnight. The wells were washed three times in 9.5mM sodium phosphate buffer containing 0.8% NaCl and 0.05% Tween 20, pH 7.4 (PBS-Tween buffer), allowed to dry and stored at room temperature for a maximum of four days. Sera were diluted PBS-Tween buffer with 0.5% BSA. Fifty ul portions of sera were dispensed into the microtitre plate wells, covered and

incubated at room temperature for 3h. Each well was washed three times with PBS-Tween buffer with a minimum of 3min between washes. Fifty ul of a horseradish peroxidase-labeled rabbit antimouse Ig serum diluted in PBS-Tween with 0.5% BSA and 5% fetal calf serum was dispensed into each well, covered and incubated at room temperature for 3h. The washing procedure was then repeated. Subsequently, 200ul of the substrate (40 mg O-phenylenediamine in 100ml 2.8mM Na phosphate/1.4mM citric acid buffer, pH 5.0, into which 40ul of 30% H_2O_2 had been added immediately before dispensing) was added to each well. After 30min, the reaction was stopped by the addition of 50ul 2.5M H_2SO_4 and the colour change read spectrophotometrically at 492nm. ELISA values were determined by subtracting the mean of two duplicate readings obtained with serum taken before injection of mice, from the mean of duplicate readings obtained with serum taken from the respective mice at various time intervals after the second injection.

Statistical analyses

Differences in absorbance readings, as determined by ELISA, between two groups of mice being compared were tested for significance by the Mann-Whitney non-parametric test for two samples. In this test, the statistic *t*, was taken to be the lower sum of ranks of all observation in the two groups being compared.

Where serum samples were titrated, comparison of antibody titres between two groups of mice was made using Student's *t* test.

Determination of liposomal (SUV) pharmacokinetics

Monitoring of latent CF blood levels has proven to be a convenient and accurate means to determine the half-lives of stable (non-leaky) SUV in the circulation since, in this way, intact vesicle clearance is measured.

a) Intravenous administration of SUV in mice : Male and female Balb/c mice, 20-25g in weight, 4-8 wks old, maintained at the Royal Free Hospital's Comparative Biology Unit were used in these experiments. 0.2ml CF-containing SUV of varying phospholipid composition and charge, prepared as described above, were injected via a 1-ml syringe (16mm length, 25G needle), taking care to expel all air bubbles, into the tail veins of the mice. To cause the blood vessels of the tail to dilate adequately for intravenous administration of the liposomes, animals were placed four at a time into a preheated `hot box' which consists of a metal box housing a high wattage light bulb upon which rests a heat-retaining mat enclosed by clear-sided walls and hinged lid (30cm x 30cm base, 25cm height). Animals were not left in the box for longer than about 10 min to prevent dehydration. The animals were restrained very securely in a specially constructed mouse restrainer which prevents the animal from jerking forward as the vein is penetrated. There are several veins prominent on the tail of the mouse into which injection can be satisfactorily made. In the dorsal view, the tail has three large veins clearly visible one on either side near the surface, and one in the centre of the tail which is deeper down. SUV were injected into one of the lateral veins approximately in the centre to upper half of the tail. Gentle pressure was applied to the site of injec-

tion to prevent bleeding as the needle was withdrawn.

b) Withdrawal of blood samples : Blood samples from the washed tail were taken from the lateral vein on the opposite side to that into which liposomes were injected. A first time interval of 2-3min after injection was allocated to allow time for the administered material to be mixed into the circulation. Bleeding was carried out by making a careful cut in the lower half of the tail where veins are narrow using a small blade (size no. 10 or 11) and taking care not to cut too deeply, but significantly to allow the blood to flow freely. Gentle massage of the tail helped to promote blood flow. Up to 100ul of blood can be collected from the warm tail of a mouse but 25ul samples were collected, with a disposable glass microcapillary tube at time intervals, to avoid removal of a significant proportion of the injected dose and for speed in sampling. Once the blood sample had been taken, bleeding was stopped by applying pressure to the cut and tightly wrapping a small piece of sterile tape around the cut. When another blood sample was to be taken, the tape was removed and the cut washed thoroughly in warm saline to stimulate fresh flow of blood so further cuts were unnecessary.

c) Processing of blood samples - Blood samples collected from the dry tail in microcapillaries wetted with anticoagulant (heparin at 5000 units/ml) were immediately diluted into 4ml of cold PBS. Diluted samples were kept on ice until ready for measurement of latent CF. Cellular components of blood were spun down at 250g for 10min. 3ml of the cell-free supernatant from each sample were transferred to another test tube. 0.5ml 5% Triton X-100 was added to

both the supernatant and the cell pellet to disrupt all SUV floating freely in the diluted plasma as well as the fraction adsorbed to the cellular elements of blood. The cell pellets were vortexed thoroughly to ensure that all red blood cells were lysed by the detergent until the liquid in the test-tube became a uniform bright red in colour. Disrupted cell pellets were heated at 62°C for 30min to coagulate the proteins present. At the end of the incubation period, the lysed cell fraction had changed from a bright red into a dark murky brown colour. After cooling to room temperature, 2.5ml cold PBS was added to bring the volume in each test-tube to 4ml and the coagulated protein suspension was mixed by inverting the test-tube several times. Proteins were precipitated by spinning down in a bench centrifuge at 1000g for 15min. 3ml of the supernatant from each coagulated protein sample were transferred to another test-tube for measurement of fluorescence in a fluorimeter. The fluorescence of the lysed non-cell associated SUV samples was also measured and the values corrected by multiplying by a dilution factor of 1.17 to take into account the added volume of detergent. Total fluorescence for each blood sample was obtained by adding the individual fluorescence readings of the cell-associated and freely-floating moieties together. This was expressed as a percentage of the fluorescence of the administered dose.

Measurement of electrophoretic mobility of liposomes in vitro

Microelectrophoretic mobility measurements of liposomes was carried out using a Rank Microelectrophoresis

Apparatus (Rank Brothers, Cambridge, U.K.). As sonicated SUV could not be observed, MLV of varying lipid composition and charge, prepared in buffered of graded pH were used instead.

Description of the apparatus

A diagram of the apparatus with its component parts is shown in Fig. 0.1.

The microelectrophoresis tube

This consists of a precision capillary of `Veridia' glass; internal diameter uniform to $\pm$0.01mm. The internal diameter lies in the range from 2.0-3.0mm as this is suitable for use with the x40 objective of the microscope. At less than 1.5mm diameter, the change of mobility of particles with depth in the tube is very large in the stationary level region, leading to large errors in mobility determinations for only small setting errors. Thermal convection effects are readily produced in tubes greater than 3.0mm in diameter because of the greater current flowing per unit field strength. The length of the capillary tube is about 150mm, this being convenient for leveling of the cell accurately. There is no risk of contamination by electrode products since a current flowing for five times as long is required for a mobility determination leads to contamination of approximately only the first 2.0mm of each end of the capillary.

Quickfit and Quartz B10 Pyrex sockets were fused to each end of the capillary tube and the viewing region formed by grinding and polishing a flat on the capillary wall. The capacity of the apparatus is approximately 5.0ml.

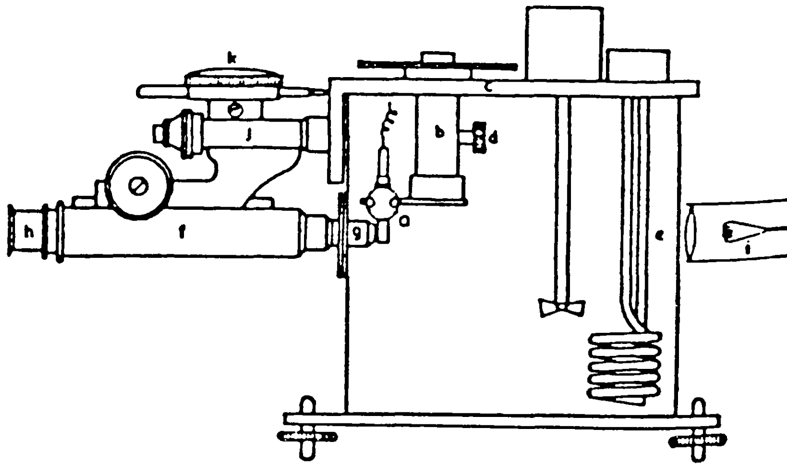


Fig. 0.1   Microelectrophoresis Apparatus

a) tube holder; b) calibrated vertical traverse; c) crossbar; d) locking screw; e) thermostat; f) microscope tube; g) objective; h)micrometer eyepiece; i) light source; j) microscope fine adjustment; k) dial test indicator

For the examination of very small quantities a similar tube fitted with B7 joints with a capacity less than 2.0ml is used. Electrodes of grey platinum foil of dimensions 12.5x20.0mm so that the current density is sufficiently low to obviate serious risk of polarization. The foil is formed into a cylinder and welded onto platinum wire which is inserted into a reservoir of mercury through a B10 soda glass or polyethylene plug. For currents less than 2.0m.amp, reliable results can be obtained from these irreversible electrodes.

The tube holder

The tube is fixed horizontally in a holder attached to a calibrated vertical traverse (reading to 0.01mm) mounted on a crossbar on the water bath. A locking device is incorporated in the adjustable traverse. Both tube and holder are immersed in a glass or Perspex water bath, the temperature controlled to +0.1o with an immersion heater (500kW) and a toluene regulator and `Sunvic' switch. The tube is set so that its capillary lies parallel to the surface of the water in the bath. Cleaning is effected in situ using a polyethylene cannula and a suction pump.

The microscope

The microscope tube, 160mm long attached horizontally to the crossbar is fitted with a x40 1/6in waterproofed objective numerical aperture 0.7, and a x10 micrometer eyepiece incorporating a chessboard graticule (5x5 squares of 0.25mm side). The optical system gives a magnification of 400 diameters and is calibrated against a 1.0mm

standard micrometer scale divided to 0.01mm standard micrometer scale divided to 0.01mm. The depth of focus is about 0.005mm.

A light source is conveniently mounted behind the water-bath at right angles to the tube and co-axial with the objective. The objective is inserted into the bath at right angles to the tube and co-axial with the objective. The objective is inserted into the bath through a water-tight rubber gasket and can be focused on the optical flat of the tube or on the stationary level using the horizontal fine adjustment of the microscope.

An adjustable zero dial test indicator reading to 0.002mm is mechanically linked to the fine adjustment of the microscope and bears on the crossbar to give rapid and accurate reading of the objective setting.

Circuit

A 150V radio battery supplies the potential through an on/off switch to the 25kOhm linear potentiometer which acts as a potential divider. A two-way double pole switch is wired for reversing the polarity of the electrophoresis cell, but not that of the meters in the circuit. The potential gradient was obtained directly using the applied potential and the distance between the electrodes.

Assembly of the apparatus

The glass capillary tube was clamped firmly in the cell holder. It was ensured that the electrode chambers were

supported from underneath by adjusting the screw-threaded supports. (This was important as the tube could have fractured when the electrodes were introduced). Before assembling the cell and microscope on the water-bath, the following alignments were carried out:

Vertical alignment - The capillary tube was filled with a dilute solution of methylene blue. The capillary tube holder was lowered by unscrewing the vertical adjustments until a blue/white interface bisected the microscopic field. A reading on the dial gauge indicator was taken at this point. The cell was then adjusted to a mid-point position but not yet secured.

Rotational alignment - The `optical flat' was focused on the microscope, using the deliberate imperfections on the surface as points of focus. The capillary tube holder was again raised and it was determined whether the optical flat was nearer or further away from the plane of focus. By trial and error, the capillary tube was rotated until the `flat' remained in more or less permanent focus over a full vertical translation. When this was achieved, the capillary was clamped and returned once more to the centre of the microscopic field.

The cell and microscope holder were assembled across the water bath. The microscope was slid into the sleeve and the tip of the objective manually positioned top within 1mm of the optical flat. The thermostat was fitted, the water bath filled and the microscope aligned to shine through the back of the bath onto the capillary tube. The d.c. source was connected and the platinum electrodes placed in the electrode holders at the back of the water bath.

Finding the stationary layer

Electrical end-osmosis along the sides of the glass capillary tubes gives rise to a flow of liquid in a particular direction depending upon the sign of magnitude of the zeta potential of the glass surface and the polarity of the applied potential. Since the capillary tube is a closed system, an equal volume of liquid must return symmetrically down the centre of the tube. Lamb's law predicts that there will be a theoretical plane of stationary liquid at 1000 um away from the side of the tube (internal radius of the capillary). The microscope was focused onto this plane and only those liposomes actually in focus were used for electrophoretic measurement. To adjust the microscope focus onto the stationary layer, the front (optical flat) face of the electrophoresis cell was identified. This was deliberately not a perfect finish so that the imperfections could be seen. The microscope was racked forward until the inside of the bore was identified, recognised by fine horizontal imperfections. The micrometer was set to zero and advanced a further 1000um. The objective was now focused on the (invisible layer).

Measurement of microelectrophoresis mobility values

The rate of movement of the liposomes was found by timing them (stop-watch reading 0.1sec) over a fixed number of graticule divisions selected so as to give a reading of about 10sec at an applied potential gradient of 3-5 Vcm^{-1}. The movement of ten individual liposomes was measured, reversing the direction of the current half-way

through each reading to minimize errors due to the effect of drift and polarization of the electrodes, ie. for each measurement, 20 timings were obtained. The mobility value was expressed in $\mu msec^{-1}V^{-1}cm^{-1}$.

Culture of hepatocellular carcinoma (HCC) cell lines

Three cell lines of human origin were maintained for the purpose of preparing cell membrane samples as well as for screening of hybridoma supernatants:-

1) PLC/PRF/5 (SAH or the Alexander cell line) was grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum albumin (Flow Laboratories), 2.9ug/ml L-glutamine, 50 I.U. penicillin/ml and 50ug streptomycin/ml.

2) Mahlavu and SK-SF cell lines were maintained in RPMI 1640, also supplemented with 10% fetal bovine serum albumin, 2.9ug/ml L-glutamine, 50 I.U. penicillin/ml and 50 ug streptomycin/ml.

Cells were grown to sub-confluence and split 1:4 every 4 days with 1:5000 versene solution (Wellcome).

Preparation of HCC cell membranes

Each of the cell lines PLC/PRF/5, Mahlavu and SK-SF was grown to sub-confluence and harvested with 1:5000 versene solution. At least 10^7 cells counted with an Improved Neubauer cytometer chamber were pelleted by centrifugation at 250g for 5 min. The pellet was resuspended in 0.5ml phosphate-buffered saline (PBS,pH 7.4) containing 0.02% sodium azide. The cell suspension was cooled on ice and 1ml 1% Nonidet P40 in PBS containing 1mM EDTA added

dropwise over 10min. The cell suspension was mixed very gently by slowly inverting the tube and incubated on ice for 30min. The mixture was centrifuged at 250g for 10min at $4^{o}C$. The supernatant was discarded. 1ml PBS was then added to the pellet. 0.46ml of 70% ice-cold sucrose was added and mixed so that the final concentration of sucrose was 48% (w/w).

After transferring the mixture to a 50ml ultracentrifuge tube, it was successively layered over carefully with 8 ml 45% sucrose, 10ml 41% sucrose and 4ml 37% sucrose. The layered mixture was spun for 2h at 65000g (25000rpm) at $4^{o}C$. When centrifugation was completed, the plasma membrane appeared at the interface between the 37% and 41% sucrose layers in a thin, compact sheet. The membrane layer was remove with a pasteur pipette, washed free of sucrose and suspended in 0.5ml PBS. Samples were stored at $-20^{o}C$.

Immunization of mice with HCC membrane preparations

Female Balb/c mice, 6-8wks old, were immunised by three intraperitoneal injections at six-week intervals. The priming injection consisted of cell-membrane preparations derived from $5x10^{6}$ HCC cells administered with 50ul Freund's Complete Adjuvant. The booster injections consisted of the same antigen dose admixed with 50µl incomplete Freund's adjuvant. 14 days after the last immunisation, mice received an intravenous booster of cell-membrane preparation in PBS and the spleens were removed 4 days later.

Preparation of cells for fusion

Spleen cells

Mice were killed by cervical dislocation and sterilized by immersion of the whole animal into 70% ethanol. The spleen was removed under sterile conditions and placed in a petri dish containing RPMI medium supplemented with 10% FCS. The spleen was teased apart with sterile forceps to obtain a single cell suspension. The white capsule of the spleen was discarded. As many spleen cells as possible were attemptedly recovered from the macerated tissue leaving behind the clumps, and transferred to a universal container containing 5ml RPMI medium. Cells were washed twice in serum-free RPMI by centrifugation at 250g for 7min. The number of viable lymphoid cells per ml was determined by counting in an Improved Neubauer cytometer chamber.

Myelomas

The myeloma cell line P3-NS1/1Ag4-1 (Flow Labs.) was grown in RPMI medium containing 10% FCS, 2mM glutamine, 100 I.U. penicillin/ml, 100ug streptomycin/ml and thioguanine. Cells were split 1:10 every 3-5days. Prior to fusion, cells were harvested with 5 times trypsin medium (trypsin:serum-free RPMI 1:1 v/v) and washed twice with serum-free RPMI medium. The number of viable cells was counted.

Fusion of spleen cells with myeloma cells

Myeloma cells and spleen cells were mixed in a 1:10 ratio eg. 10^7 myeloma cells to 10^8 spleen cells. The

cell mixture was centrifuged at 500g for 7min at room temperature. The supernatant was decanted carefully, finally inverting the tube to drain completely. The cell pellet was mixed by gently tapping the container an allowed to equilibrate to 37°C. The polyethylene glycol (PEG) 1500 solution 40% w/v in serum-free RPMI medium was similarly allowed to equilibrate to 37°C. 1ml PEG solution was added to the cells, mixing gently and incubated at 37°C for 7min exactly. After incubation, the PEG was diluted very slowly with 20ml serum-free RPMI over 5min, shaking gently between dropwise additions. The cells were spun down at 200g for 7min at room temperature. The supernatant was decanted away and the cell pellet resuspended thoroughly in 2 ml RPMI with 10% FCS. The cell suspension was incubated for 1-2h at 37°C and diluted in serum-containing medium to a concentration of $2x10^{7}$ cells per ml. 100ul of cell suspension were aliquoted into each well of a 24-well culture plate (Nunclon) and 1ml per well subsequently added to give a final concentration of $2x10^{6}$ cells per well. The plates were incubated for 24h in a humid 37°C incubator gassed with 5% CO_2 in air. The following day, 1ml of double-strength HAT medium (RPMI medium supplemented with 10% FCS, 2mM glutamine, 100 I.U. penicillin/ml, 100ug streptomycin/ml, Hypoxanthine 10^{-2}M, Aminopterin $4.0x10^{-5}$M and Thymidine $1.6x10^{-3}$M) was added to each well. The plates were examined 10days later for the presence of actively-growing colonies, indicated by a change in the colour of the medium from pink to yellow and by light microscopy.

Selection of Hybridomas

By 7-10 days after the fusion, the presence of clones of cells was evident. The supernatants from the wells in which growth is seen was tested as soon as possible to prevent the chance of highly active, antibody-negative clones overgrowing smaller, positive clones. The method used to screen for positive supernatants is outlined in the following section. Antibody-positive wells of cells were allowed to continue growing and when there were enough cells (2-3 weeks), the cells were subcultured into tissue culture flasks in HT medium (HAT medium without aminopterin). The positive cells were frozen down as soon as possible.

a) Screening assay

HCC cells (PLC/PRF/5, Mahlavu or SK-SF) were harvested and resuspended at a concentration of $1x10^6$/ml in PBS containing 1% BSA and 0.02% sodium azide. 50 ul of cell suspension was incubated with 50 ul supernatant from each well showing clone growth in a test-tube at room temperature for 2h. The incubation mixture was washed three times in PBS+1%BSA+0.02%azide by centrifugation for 5 min at 250g. 50 ul ^{125}I-labelled goat anti-mouse IgG (10000 cpm/50 ul) were added to the pellet in each test-tube and incubated for a further 2h at room temperature. The cells were again washed three times in PBS+1%BSA+0.02%azide. The radioactivity in the cell pellets was counted in a gamma-counter. As a positive control, supernatant from the K1 hybridoma (Wiedmann et al.) containing antibodies binding to HCC cell lines and tumours was used. HAT medium was used as a negative control.

b) Freezing and thawing of cells

Cells to be frozen were harvested and pelleted by centrifugation at 250g for 5min. The supernatant was discarded and cells resuspended in a known volume of serum-free RPMI medium. An aliquot of cell suspension was taken, mixed with an equal volume of 0.16% trypan blue in sodium chloride and counted. The range of numbers of cells frozen per cryotube was $1x10^6$ to $1x10^7$ in 0.5ml of serum-free RPMI medium. Equal parts (0.5ml each) of cell suspension and freezing mixture (2 parts FCS:2 parts RPMI:1 part DMSO, prepared in advance) both kept at 4^oC were mixed and transferred into a cryotube, keeping the mixture as near to 4^oC as possible. The cryotube was placed in the freezing cap of a liquid nitrogen cylinder for 2-3h before being lowered into the liquid nitrogen itself.

When required, cells were thawed by lowering the cryotube into warm water (about 37^oC) as quickly as possible, being careful not to submerge the vial. The cell suspension was allowed to thaw until completely liquid, with gentle shaking. It was diluted out slowly into about 10ml of warm medium and spun down at 250g for 5min. The pellet was resuspended in warm medium and cultured normally in a tissue culture flask.

Cloning of hybridomas by Limiting Dilution

Cloning was carried out as early after fusion as possible and three clonings at least were performed to ensure a monoclonal line.

Spleen cell feeder layers were prepared by removing the spleen from an unimmunized Balb/c mouse under asep-

tic conditions as described in the fusion protocol and resuspending the teased-out spleen cells in 10ml RPMI+10%FCS. Hybridoma cells growing in 50ml tissue culture flasks were harvested and resuspended at a concentration of $1x10^4$cells/ml HAT medium. This was further diluted a hundred-fold to give a concentration of 100 cells/ml HAT medium. To 0.7ml of the hybridoma cell suspension was added $7x10^6$ spleen cells and the volume of the cell mixture was made up to 7ml by adding HAT medium. 100ul of the spleen and hybridoma cell mixture was aliquoted into each of the 60 inner wells of a 96-well microtitre plate such that each well contained 1 hybridoma cell and $1x10^5$ spleen feeder cells. 100ul HAT medium was further added to each well. Excess hybridoma cells secreting specific antibody were frozen and stored.

In 10-14 days when colonies were macroscopically visible, hybridoma cells were resuspended in the wells and transferred to the larger wells of 24-well tissue culture plates. When these colonies were sizeable, they were transferred to 50ml tissue culture flasks and the cloning procedure repeated after screening of the supernatants.

Production of antibody-rich ascites

0.5ml Pristan was injected into the peritoneal cavity of each Balb/c mouse. After 7 days, 10^7 hybridoma cells were injected intraperitoneally into each mouse. In 2-3 weeks' time, mice were examined for ascites or solid tumour formation. Ascitic fluid was drained via a 19G needle into heparinized tubes and clarified by centrifugation at 500g for 15min. Ascites was drained and clarified for the

lifetime of the mouse.

Purification of Monoclonal Antibody

A protein fraction was obtained from clarified ascites by ammonium sulphate precipitation and the monoclonal antibody further purified by gel exclusion chromatography on Sepharose 4B.

a) Ammonium sulphate precipitation

4ml saturated ammonium sulphate solution (made by dissolving 11.5g NH_4SO_4 in 20ml distilled water, stirring continuously) was added dropwise to 4ml clarified ascites. The mixture was allowed to equilibrate at room temperature for 30min. The precipitate formed was spun down at 1000g for 15min. The supernatant was discarded and the precipitate resuspended in 50% NH_4SO_4 (made by diluting the stock saturated NH_4SO_4). The spinning down and resuspension of the precipitate in 50% NH_4SO_4 was repeated another two times before finally resuspending the antibody-rich protein pellet in 2ml PBS.

b) Elution on Sepharose 4B column

A 2.5x40cm column (Pharmacia) was packed with Sepharose 4B suspended in PBS+0.01M EDTA,pH 7.2+ 0.02% azide. 2ml of the protein fraction were layered on the gel and eluted at a rate of 2.5ml/min PBS+0.01M EDTA+0.02% azide. 1ml eluted fractions were collected and their absorbance at 280nm read on a spectrophotometer. The concentration of antibody in mg/ml was obtained by multiplying the extinction coefficient by a factor of 0.7.

Determination of monoclonal antibody subclass

Isotype specificity of monoclonal antibodies was determined by Ouchterlony double diffusion analysis. 2% agar in barbitone buffer was melted in a microwave oven and 3ml poured onto each horizontally leveled glass slide. When the agar had cooled and set, holes were with a gel puncher in the pattern of a central hole surrounded by six peripheral holes. The agar plugs were sucked out with a pasteur pipette attached to a water vacuum pump and 5ul of neat, half- and quarter-strength goat anti-mouse subclass antibodies pipetted into each well. Central wells were filled with 5ul of 10x concentrated hybridoma supernatant-containing dialysis tubing by solid PEG 6000 (British Drug Houses, U.K.) Slides were incubated for 48h at room temperature in a humidified chamber and subsequently examined for precipitin lines.

Growth of PLC/PRF/5 tumours in nude mice

Male and female random-inbred nu/nu (congenitally athymic) mice maintained at the Royal Free Hospital Comparative Biology Unit were isolated from other experimental animals in a laboratory maintained at >25°C, housed in sterile filter cages, fed irradiated diet and given acidified water to minimize risk of pseudomonas infection. The mice were rendered further immunodeficient by sublethal total body irradiation of 450rads (Cobalt 60 single field, 80 skin-source distance). 48h after irradiation, they were inoculated with 10^7 viable PLC/PRF/5 cells in 0.2ml PBS subcutaneously via a 19G needle into the neck region or intrahepatically for a separate experiment. Inoculation sites were examined weekly for obvious tumour growth. When

tumours were sizeable, animals were killed by cervical dislocation, tumours dissected free of surrounding non-cancerous tissue and tumours diameters measured and weighed.

Preparation of tissue sections

Hepatocellular carcinoma (HCC) samples were obtained from operatively excised tumours in patients with histologically proven HCC. Normal liver tissue was obtained from surgical specimens. PLC/PRF/5 solid tumours were grown in nude mice.

4-10mm wide pieces of tumour or normal liver tissue were cut with a sharp blade. Isopentane in a universal container was cooled in liquid nitrogen until the isopentane started to solidify. A drop of OCT compound (Ames Company) was placed on a slice of cork. A piece of tissue was put on the cork in the middle of the OCT and another drop of OCT was added on the top of the sample to envelope it completely. The whole arrangement was snap-frozen by dropping it in the cooled isopentane. Once solidly frozen, the cork and tissue were placed in cryotubes and stored at $-70^{o}C$ or immediately mounted on a microtome. Cryostat sections of about 6um in thickness were made and mounted on glass slides. The slides were air-dried, wrapped in Clingfilm and stored at $-20^{o}C$.

Indirect immunofluorescence staining of tissue sections

Slides containing tissue sections stored at $-20^{o}C$ were defrosted, unwrapped and rehydrated in a PBS bath with stirring for 10min. Circles were scored in the glass around each tissue section with a metal pen. The slides were dried with lint-free tissue leaving the circles and sections wet.

50ul of hybridoma supernatant were pipetted onto each circle to completely cover the section of tissue. The slides were incubated in a humidified tray at room temperature for 45min before washing off the unbound antibodies in a PBS bath for 30min with constant stirring. Slides were again dried leaving the circles wet. 10ul 1:10 diluted fluorescein-labeled goat anti-mouse antibody were pipetted over the sections. Slides were incubated in a humidified tray at room temperature for a further 45min before washing in a PBS bath for 30min. Slides were dried around the sections which were then mounted by inverting the slides over a coverslip containing fixative (50% PBS, 50% Glycerol, 0.3M Diazabicyclooctane). Tissue sections were examined under a fluorescence microscope and scored accordingly to the intensity of fluorescein staining of the tumour or liver cells.

Radiolabeling of protein by the Chloramine-T method

a) Theoretical basis of radioiodination

Iodination of protein antigens or ligands is accomplished either directly or indirectly by conjugation of a prelabeled tyrosine molecule to the protein. ^{125}I is substituted into other amino acids, including histidine, but not as efficiently. For large protein molecules, direct labeling is usually not a problem, since they will depend on the specific activity of the iodine used and the number and location of the tyrosine residues. For molecules that do not contain a tyrosine residue, that are too small to incorporate a large iodine atom, or where direct incorporation may affect immunoreactivity, iodination may be accomplished by

conjugation with a preiodinated molecule.

Originally marketed as a disinfectant, chloramine-T was found to oxidise ^{125}I-labeled sodium iodide for reaction with tyrosine residues. This method is useful for iodinating peptides and proteins. The chloramine-T method is inexpensive and simple, uses commonly available reagents, is quick, avoids pH extremes and produces minimal radiation exposure (Heal, 1985).

b) Preparation of the column

Dowex 1x8-200 (Sigma) was mixed to a slurry with an equal volume of 0.1% BSA in PBS. A 1ml plastic pasteur pipette (Alpha) was plugged with glass wool after cutting off the rounded bulb tip and the Dowex slurry poured in. After the column had packed down, it was washed through with 0.1% BSA to prevent it from running dry.

c) Reaction

10ug protein in about 10ul PBS was pipetted into an Eppendorf vial. 10µl (about 1mCi) ^{125}I or ^{131}I was added to the protein via a disposable glass micropipette. The reaction was activated with 10ul Chloramine-T (1mg/ml distilled water, prepared fresh) and allowed to proceed for 50sec at 4°C in a fume cupboard. The reaction was terminated with 50µl tyrosine (0.5mg/ml distilled water,prepared fresh). For radioiodination of monoclonal antibody, 5µl ^{125}I (about 0.5mCi) and 5ul 0.5mg/ml Chloramine-T in distilled water were used. The reaction was allowed to proceed for 30sec. All other reagents and conditions were identical as for other proteins.

The reaction mixture was layered on the top of the Dowex column clamped on a retort stand. Residual protein in the Eppendorf vial was washed out with 200ul 10% BSA. 0.5ml fractions were collected and monitored for radioactivity. Fractions >1MBq were pooled. Duolite beads were added to the radioiodinated protein which was stored at $4^{O}C$ until use.

Incorporation of reconstituted influenza viral envelopes into liposomes : studies of the immune response in mice

Introduction

Early observations that the entrapment of diphtheria toxoid in liposomes considerably enhances the immune response against the antigen in injected animals have been confirmed with a large number of liposome-associated bacterial and viral antigens relevant to human and veterinary immunization. This adjuvant effect of liposomes has been demonstrated for hepatitis B surface antigen and derived peptides (Manesis et al., 1979), *Plasmodium falciparum* antigens (Alving, 1980), Epstein-Barr virus gp340 antigen (Epstein et al., 1985), *Streptococcus mutans* cell wall antigens (Wachsmann et al., 1985), foot-and-mouth disease synthetic peptides (Francis et al., 1985) and rat spermatozoal polypeptide fraction (Mettler et al., 1983). In several experiments, protection of animal models was achieved by immunisation with the relevant liposome-incorporated antigens.

The dehydration-rehydration method for producing oligo- and multilamellar vesicles (DRV) has been found to be effective in encapsulating a wide range of substances with high efficiency. The procedure has therefore been adapted in this study to incorporate reconstituted influenza virus envelopes (RIVE), which retain their highly fusogenic surface polypeptide and glycoprotein structure, into liposomes

to serve as a model antigen for studies of the potential of DRV as an effective adjuvant vehicle and of the system as a vaccine.

Materials and methods

Entrapment of RIVE in DRV liposomes

DRV were prepared as described on page 61 using 16 µmol of equimolar phospholipid and cholesterol. The concentration of RIVE (gift from Prof. A Loyter, Institute of Life Sciences, Hebrrew University of Jerusalem, Israel; prepared according to Nussbaum et al., 1987) added to SUV before lyophilisation was 50 ug/ml water).

To measure the percentage of RIVE that could possibly be passively adsorbed to the surface of DRV, 1ml DRV in PBS was incubated with 1ml RIVE (50ug/ml PBS) overnight at room temperature. The amount of RIVE associated with the liposomes was also determined by assaying ^{125}I radioactivity.

Animal immunisation experiments

In studies designed to investigate the immunoadjuvant action of DRV liposomes and to reveal possible effects of liposomal phospholipid T_c on their adjuvanticity, Balb/c mice were primed intramuscularly in groups of five with 0.1ml of various amounts of RIVE free or entrapped in DRV.

Four weeks later they were boosted with the same amount of free or liposome-associated antigen. Blood samples were obtained from the tail veins 1 day before the priming injection, 14 days after priming and 10 days after the

booster injection. Serum samples were kept at -20°C until they were assayed for anti-RIVE IgG_1.

Enzyme-linked immunosorbent assay (ELISA)

Antibody responses to the toxoid in immunised mice were monitored by ELISA, as described on page 62, with the following variations: RIVE (50µl, 10µg/ml) in 0.05M sodium bicarbonate buffer (pH 9.6) was used to coat the wells of the plastic microelisa plates. Serum samples from immunised mice were diluted one hundredfold in PBS-Tween buffer.

Results and discussion

Entrapment of RIVE in DRV liposomes

In agreement with previous results obtained with a wide range of substances of varying molecular weight and already confirmed by others (Davis and Gregoriadis, 1987) entrapment of RIVE in DRV liposomes made from SUV was substantial (31.2 ± 4.8% of the amount used, 3 preparations) (Table 1.1). Entrapment values did not vary significantly between phospholipids of different acyl chain lengths and transition temperatures, the value for equimolar DSPC and Chol liposomes being 29.4 ± 7.0%, 3 preparations. This contrasts with results obtained for the entrapment of tetanus toxoid in DRV liposomes composed of equimolar DSPC and Chol which are nearly twice as great as that of equimolar PC and Chol liposomes (Gregoriadis et al., 1987). This is probably because RIVE are relatively large, being in the same size range as intact virions (80-120nm in diameter) and are equally difficult to constrain within the bilayers of both PC and DSPC liposomes, whereas tetanus toxoid (molecular weight 150,000) is a much smaller solute which is efficiently retained within and may even be interacting hydro-

Table 1.1 Incorporation of RIVE into liposomes

	RIVE incorporation		RIVE surface adsorption/fusion	
Liposomes	% ± s.d.	ng ± s.d. per umol phospholipid	% adsorbed	ng per μmol phospholipid
PC:CHOL, (DRV)	31.2 ± 4.8 (3)	975 ± 150	3.8	119
DSPC:CHOL (DRV)	29.4 ± 7.0 (3)	919 ± 219	3.2	110

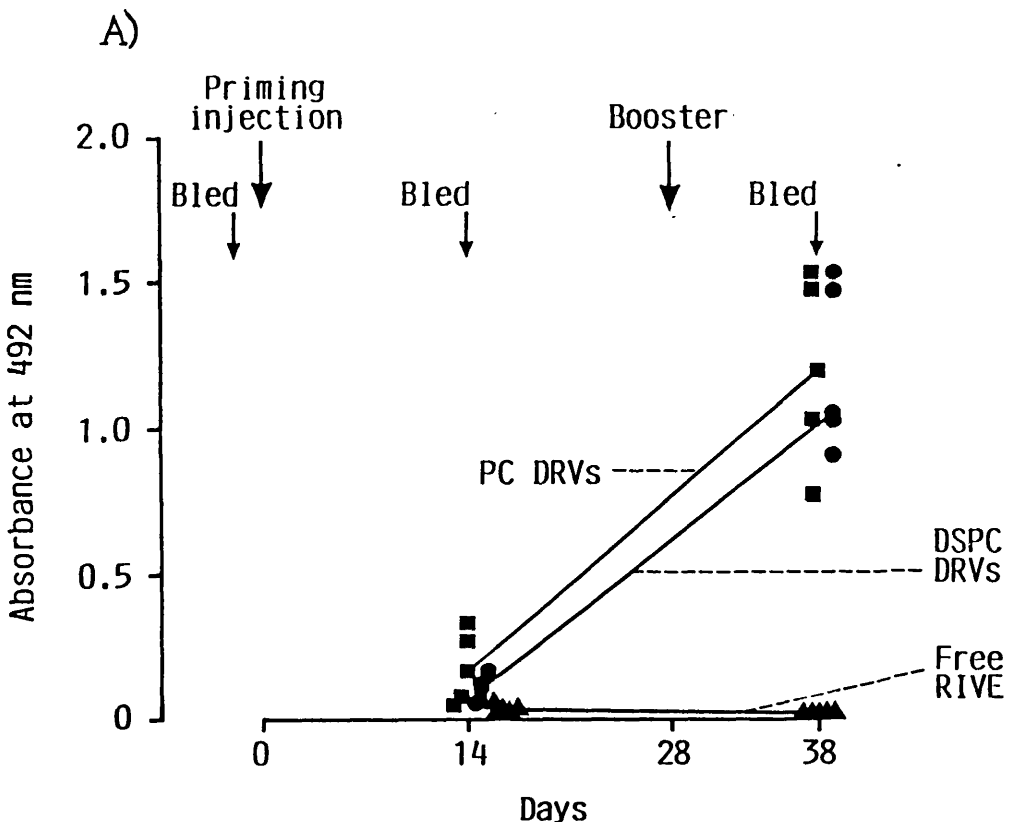
RIVE (50 μg) was entrapped in or incubated with DRV liposomes generated from SUV liposomes composed of equimolar phospholipid and cholesterol. s.d. denotes standard deviation and numbers in parentheses denote individual preparations.

phobically with the bilayers of DSPC liposomes. Indeed, RIVE adsorbs poorly to the surface of DRV on passive incubation at room temperature (3.8% of the amount used for PC:Chol DRV and 3.2% for DSPC:Chol DRV). This agrees with previous studies in which the extent of RIVE interaction with PC liposomes at 37°C was monitored by fluorescence dequenching (Nussbaum et al., 1987). Thus, it is assumed that more than 90% of RIVE associated with both PC and DSPC DRV is actually entrapped within (or perhaps sandwiched between, in view of their large size) the liposomal bilayers.

Immune response to liposome-incorporated RIVE

The adjuvanticity of DRV liposomes to entrapped RIVE was investigated in experiments in which groups of mice were immunised with two doses of the antigen, free or entrapped in DRV composed of equimolar PC or DSPC and Chol. For both doses of RIVE used (0.1 and 1.0µg), encapsulation in liposomes boosted primary and secondary IgG_1 responses to significantly higher levels compared to identical doses of the free antigen (Fig. 1.1).

In the primary response to the 1.0ug dose, antibody titres against liposome-encapsulated RIVE were about 10 times that of the free antigen ($0.02>p>0.01$) whereas for the 0.1µg dose, only PC DRV-entrapped RIVE gave a statistically significant ($0.02>p>0.01$) increase in mean antibody titres of nearly twofold (Fig. 1.2). In the secondary response against the 0.1µg dose, mean antibody titres were increased 37 times for PC and 24 times for DSPC DRV ($0.01>p>0.001$) over that of the free antigen; and against the 1.0µg dose, mean IgG_1 levels were boosted 224 times for PC and 138 times



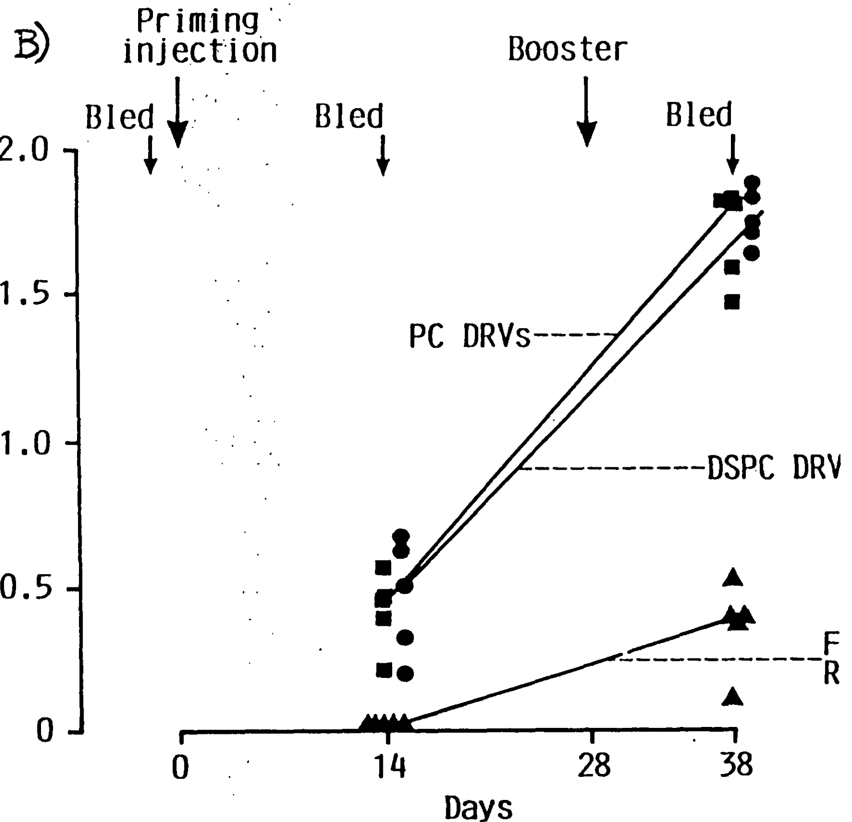


Fig. 1.1 Antibody responses to free and liposome entrapped RIVE. Balb/c mice in groups of five were injected intramuscularly twice (with a 28 day interval between injections) with 1 (A) and 0.1 μg (B) of RIVE, free or entrapped in PC or DSPC DRV. Animals were bled 14 days after the first injection and 10 days after the booster injection and anti-RIVE IgG_1 responses were measured in sera by ELISA.

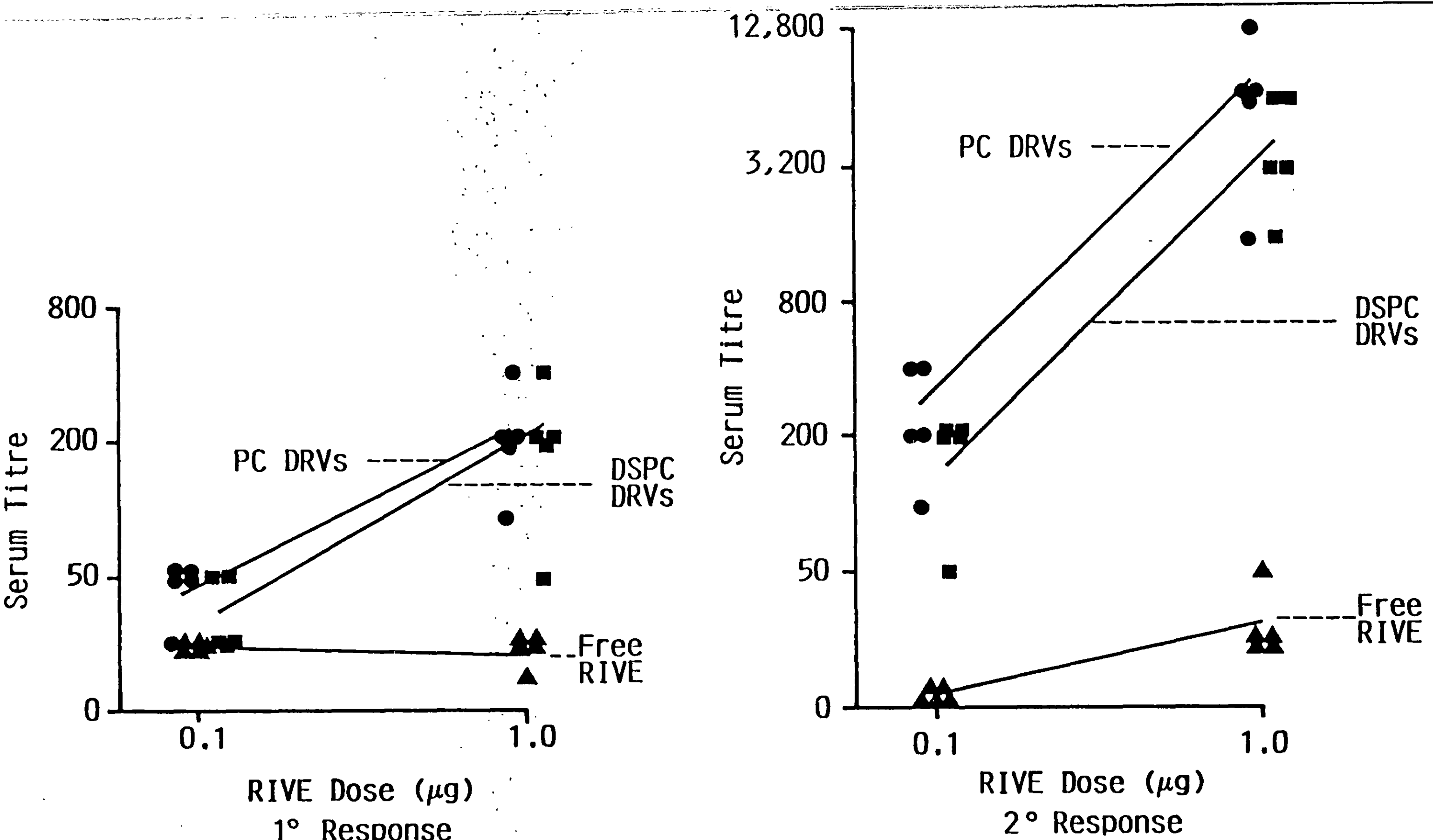


Fig. 1.2 Titration of antibody (IgG_1) responses against free and liposome-entrapped RIVE. Sera obtained from mice immunised as in the legend to fig. 1 were titrated by doubling dilutions. Results are expressed as the highest titre of serum that yielded an optical density of 0.200. The difference between antibody titres at either antigen dose in the primary and secondary responses were examined using the t-test.

for DSPC DRV (0.01>p>0.001) compared to those of the unentrapped RIVE. There was no significant difference in the primary or secondary IgG_1 responses to liposome-encapsulated RIVE (both doses) between PC and DSPC DRV).

Qualitatively similar results were observed for the secondary responses in the IgG_{2a} and IgG_{2b} subclasses (Fig. 1.3) when serum samples from the same groups of mice were tested for the presence of these anti-RIVE antibody isotypes. Enhancement of RIVE immunogenicity by liposome incorporation was less clearly demonstrated in the IgG_3 subclass. There was no observable potentiation of the IgM response for the 0.1 µg dose of liposome-encapsulated RIVE. However, for the higher 1.0 µg dose, a significantly higher secondary IgM response ($p<0.05$) was detected for PC DRV encapsulated RIVE as compared to the free antigen.

These results demonstrate the marked immunoadjuvant effect of liposome entrapment on IgG_1 responses against RIVE in keeping with observations from other studies. However, unlike studies on tetanus toxoid entrapped in DRV, there was no significant difference when phospholipids of different acyl chain lengths and transition temperatures were used, in this case PC (T_c -8°C) and DSPC (T_c 54°C). This is probably because RIVE consists of antigenic polypeptides HA_1 and HA_2 and glycoprotein NA already embedded in a viral phospholipid membrane (Nussbaum et al., 1987) unlike tetanus toxoid and other free protein antigens which are not surrounded by or contain any phospholipid component. Thus, the liposomes serve mainly as a depot for the antigens before engulfment and antigen processing by antigen-presenting cells such as macrophages, the microenvironment around

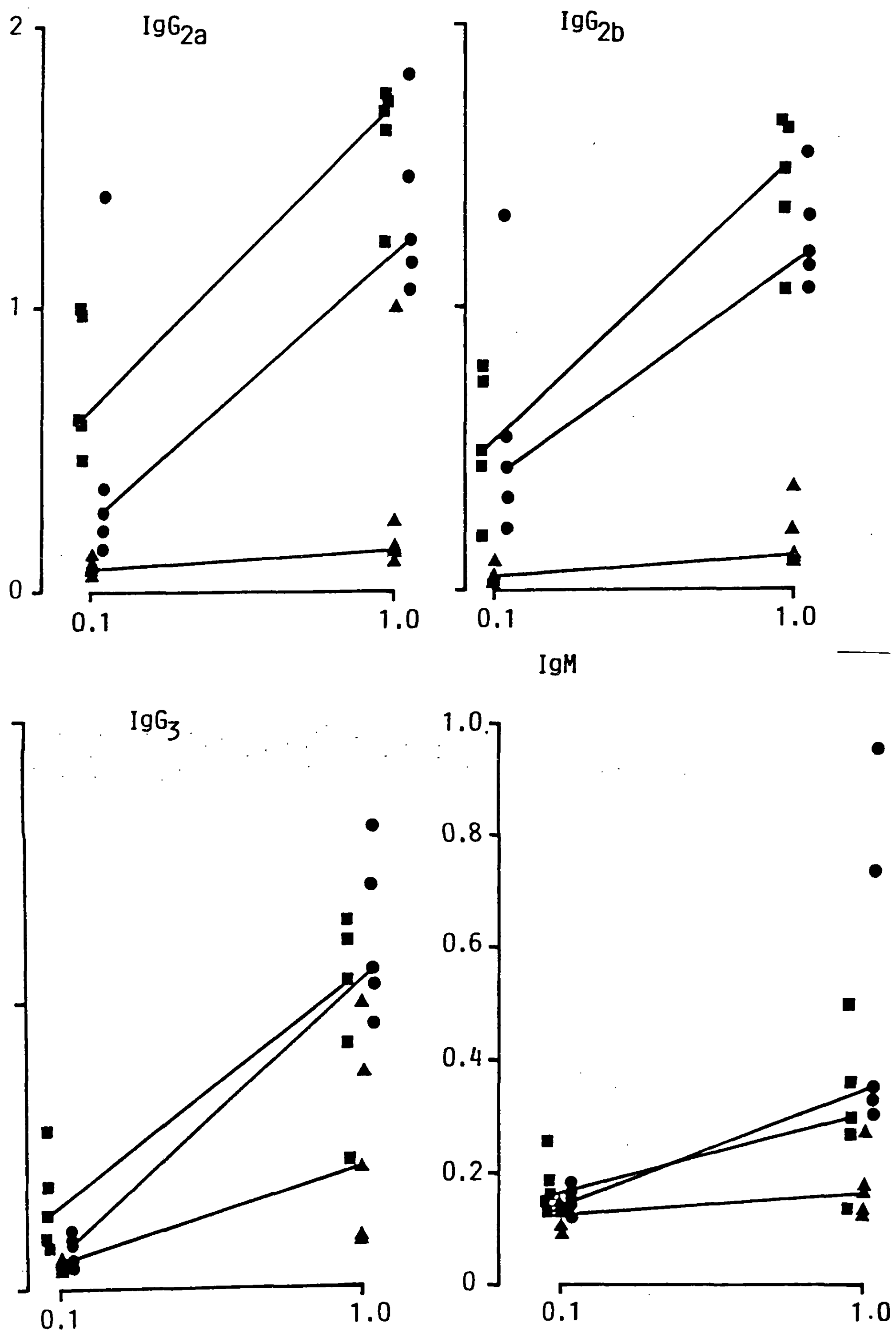


Fig. 1.3 Secondary antibody subclass responses to free and liposome-entrapped RIVE

Sera taken 10 days after the booster injection from mice immunised as described in fig. 1.1 were analysed for anti-RIVE IgG$_{2a}$, IgG$_{2b}$, IgG$_3$ and IgM antibody levels by ELISA.

each antigenic molecule being determined by the viral membrane instead of the liposomal bilayer. In this way, irrespective of whether DSPC (which gives rise to more rigid bilayers and may hinder antigen distribution and mobility) or the more fluid PC is used, the immediately surrounding matrix of the antigens still remains the viral membrane and which finally interacts with immunocompetent cells.

Influenza surface antigens, haemagglutinin (HA) and neuraminidase (NA), possess low immunogenic activity. The binding of HA and NA antigens of influenza virus with high molecular weight carriers, particularly liposomes, is one of the ways to increase their immunogenic and protective activity. The considerable increase in humoral immune response during animal immunisation with liposomes containing influenza virus antigens has been demonstrated in this study as well as by other workers. The effectiveness of liposomal presentations of influenza antigens in affording animals protection against a subsequent viral challenge has also been recently reported. (Torchilin et al., 1988).

Conclusions

Multilamellar liposomes can reproducibly incorporate substantial amounts of RIVE when formed by the dehydration-rehydration method. Balb/c mice inoculated with PC and DSPC DRV containing 0.1 µg and 1.0 µg of RIVE exhibited primary and secondary responses significantly higher as compared to mice inoculated with equal doses of unencapsulated RIVE. Similar responses were observed in IgG_{2a}, IgG_{2b}

and IgG_3 subclasses against the antigen.

Chapter 2

Comparison of the immune response against polio peptides covalently surface-linked to or internally entrapped in liposomes

Introduction

Synthetic antigens have been very useful in the study of various immunological phenomena, including the chemical basis of antigenicity and the genetic control of the immune response. It has been demonstrated that synthetic peptides can elicit antibodies with the capacity to inactivate the respective viruses and to protect against a viral challenge.

However, synthetic oligopeptides are non- or only weakly immunogenic and are presently used experimentally as haptens coupled to carrier proteins, often in mixture with Freund's adjuvant. The presentation of peptides associated with liposomes may represent a novel and clinically acceptable way of enhancing their immunogenicity.

In this chapter, the adjuvanticity of liposomes on the polio virus peptides type 3-VP2 (W1) and type 2-VP2 (W2) is described. A comparison is made between two different modes of presentation of the peptides:- internally entrapped and surface-linked. Entrapped peptides avoid conformational changes induced by coupling procedures and may give rise to improved immunological memory whilst linking peptides to the surface of liposomes results in more efficient antigen presentation in the primary response without the disadvantage of introducing an immunogenic carrier protein.

Materials and methods

Entrapment of polio peptides in DRV liposomes

Small unilamellar liposomes composed of equimolar egg PC or DSPC (16μmoles) and Chol were mixed with 1ml of polio peptides W1 or W2 (gift from M. Ferguson, National Institute for Biological Standards and Control, Hertfordshire, U.K.; amino acid sequences shown in fig. 2.1) at a concentration of 200 μg peptide/ml water and dehydrated overnight. The dry powder was then rehydrated with 0.1ml distilled water followed by 0.9ml PBS (pH 7.4). The suspension was diluted with 7ml PBS and centrifuged at 10,000g for 30min. The liposomal pellet was washed twice in 8 ml PBS by centrifugation. Polio peptide entrapment was estimated by measuring peptide concentrations in the supernatant using a protein assay from Bio-Rad (Watford, England).

Covalent coupling of polio peptides to DRV liposomes

To covalently link the polio peptides to liposomes, the hydrophobic `anchor', N-glutaryl phosphatidylethanolamine (structure shown in fig. 2.2) was used. The acyl chains of the anchor intercalate between phospholipid molecules of the liposomal bilayer whilst the carboxyl group at the opposite extremity,after activation, binds to the amino groups on lysine residues present in peptides or proteins. Dehydration-rehydration vesicles were generated from SUV prepared from 16 umoles egg PC, 16umoles Chol and 1.2-4.54umoles anchor mixed with tracer N-glutaryl(^{14}C) phosphatidylethanolamine (^{14}C-labeled anchor), both

W1 **C F N K D N A V T S P K R E F C**

(polio type 3)

W2 **C F T P D D N Q T S P A R R F C**

(polio type 1)

Fig. 2.1 Amino acid sequences of polio peptides W1 and W2

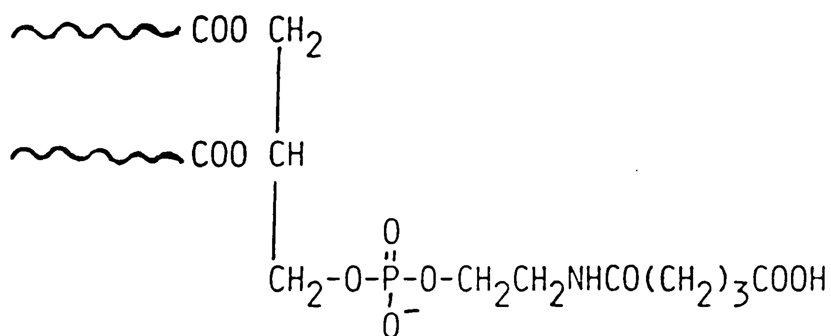


Fig. 2.2 Anchor: N-glutaryl-phosphatidylethanolamine

Table 2.1 PEPTIDE COUPLING TO LIPOSOMES

Molar ratios of lipids (PC:chol:anchor)	Peptide bound (% of used)	
	Polio type 3VP-2	Polio type 1VP-2
1:1:0.11	21-70 (5-13)	23-52 (8-9)
1:1:0.28	51-65 (7-8)	41-63 (6-7)

DRV incorporating anchor were interacted in the presence or absence (controls) of carbodiimide with 200 μg peptide. Peptide coupling estimated from amount measured in supernatant.

Numbers in parentheses denote control values.

synthesised according to Weissig at al.,(1986). Incorporation of the anchor into DRV was measured by assaying ^{14}C radioactivity.

DRV (1.0ml) incorporating various amounts of anchor were adjusted to pH 3.5 with 40ul 0.01HCl, and mixed with 8-15mg 1-ethyl-3(3-dimethylamine propyl) carbodiimide. The mixture was incubated at 22°C for 5min, supplemented with 1ml 0.1M $Na_2B_4O_7$, pH 8.5 (borate buffer) containing p-aminophenyl-D-mannopyranoside and incubated at 22°C overnight. DRV were separated from non-bound polio peptides by centrifugation at 100,000xg for 60min and the liposomal pellet washed once with borate buffer. Control DRV were incubated with the peptides in the absence of carbodiimide. (The methodology described in the current and preceding paragraph was performed by Dr. V. Weissig from the Institute of Biochemistry, Martin-Luther University, Halle, GDR. All subsequent work was done by the author of the thesis).

Animal immunisation experiments

Balb/c mice were primed intramuscularly in groups of five with 0.1ml containing 5µg polio peptide free, entrapped in or covalently linked to DRV. Four weeks later, they were boosted with the same amount of free or liposome-associated antigen. Blood samples were obtained from the tail veins 1 day before and 14 days after priming and 10 days after the booster injection. Serum samples were assayed for anti- peptide (W1 or W2) IgG_1 by ELISA.

Enzyme-linked immunosorbent assay (ELISA)

This was carried out according to the method described on page 61 with the following differences : polio peptide (W1 or W2, 30 μg/ml) in sodium bicarbonate buffer was used to coat the wells of the microelisa plates. Serum samples were diluted twenty-fold in PBS-Tween buffer for the assay.

Results and Discussion

Immune responses to free and liposome-associated W1

In a study designed to compare the effects of immunising mice with free, surface-linked and liposome-entrapped W1, Balb/c mice in group of five were injected twice with 5μg of the polio peptide in its various formulations with a 28-day interval between injections. Animals were bled on the days indicated in fig. 2.3 and serum IgG_1 levels were determined by ELISA.

Results show that liposome-associated W1 elicited higher IgG_1 responses as compared to free W1 for both the primary and secondary immune responses (Fig. 2.3). Antibody levels against free W1 peaked at 14 days after the priming injection and fell to lower levels thereafter. No characteristic rapid rise in antibody levels as would be seen in an anamnestic response was observed even 10 days after the booster injection of 5 μg free W1.
Comparing levels obtained for covalently surface-linked and internally-entrapped peptide, much higher levels were observed for the primary response when the antigen was surface-associated. IgG_1 levels against the latter rose steeply

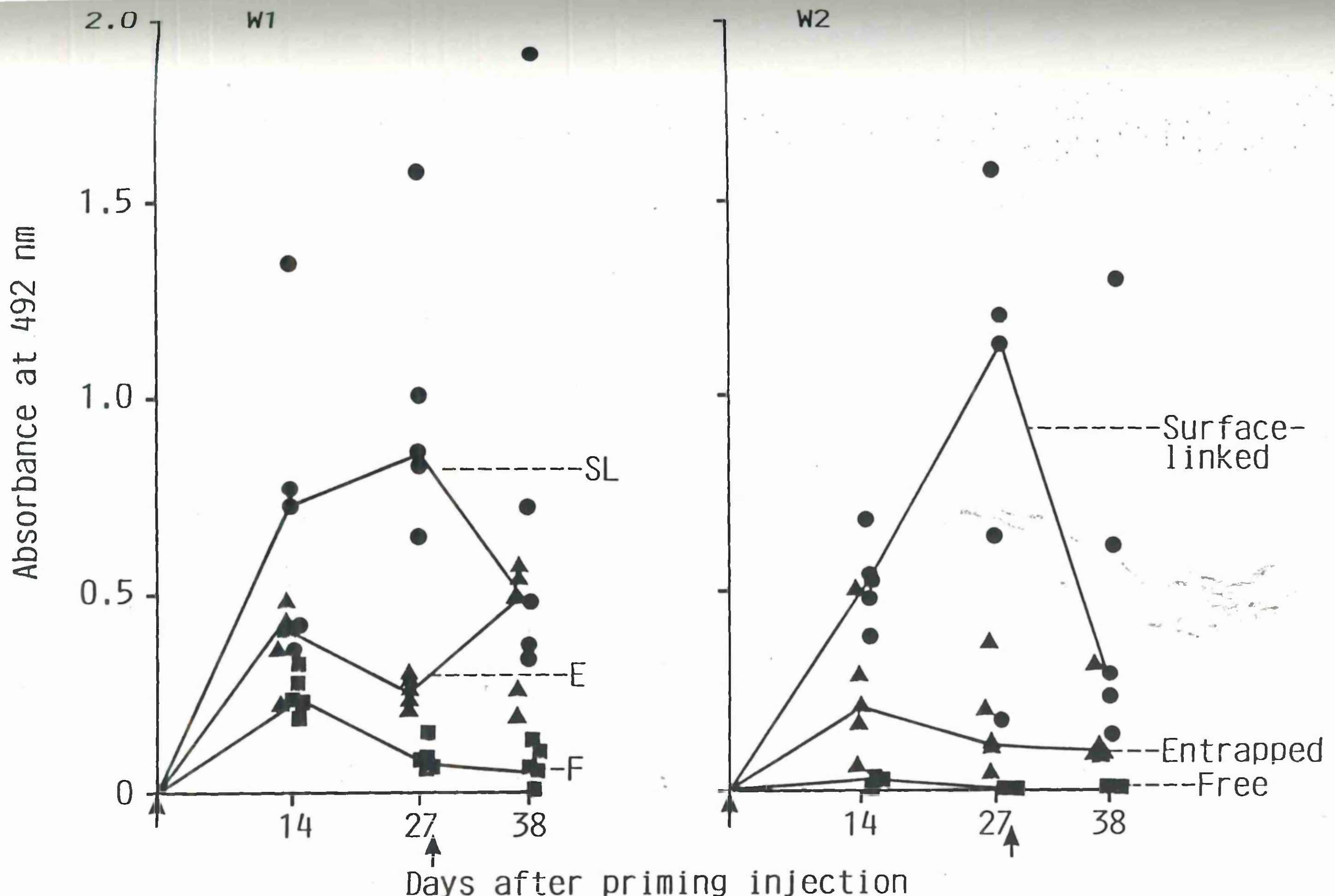


Fig. 2.3 Antibody reponses (IgG_1) to free and liposome-associated polio peptides

Balb/c mice in groups of five were injected intramuscularly twice (with a 28-day interval between injections) with 5 µg polio peptide :-free (■), covalently surface-linked to (●), or internally entrapped in (▲) PC DRV. Animals were bled one day before and 14, 27 and 38 days after the priming injection.

to attain a much higher level than that against entrapped W1 at day 14 and continued to rise and peak at day 27 before falling sharply thereafter even after the booster injection was given. Thus, it would appear that the immune response to surface-linked W1 was an exaggerated form of the response to the free peptide, probably induced by the carrier and adjuvant effect of the liposomes.

For the entrapped W1, even though IgG_1 levels in the primary response did not reach as high as those attained with surface-linked peptide, peaking at day 14 and declining in tandem with the response to free W1 to lower levels at day 27, a good anamnestic response was observed. 10 days after the booster injection, median IgG_1 levels rose to reach values higher than the peak at day 14.

Immune responses to free and liposome-associated W2

To determine the immune responses against various modes of presentation of the polio peptide W2, an identical experiment was performed using free, surface-linked and internally-entrapped W2 to immunise Balb/c mice.

Results for the primary response showed a similar pattern to that obtained for W1, with the notable exception that free W2 was intrinsically very poorly immunogenic, hardly eliciting any IgG_1 response at all under the assay conditions employed. As with W1, association of the peptide with liposomes resulted in a marked enhancement ($p<0.01$, $T=15$ where T is the lower sum of ranks of either surface-linked or entrapped W2 being compared to free W2 in the Mann-Whitney test) of immunogenicity with surface-linked W2 giving by far the highest response, with peak levels being

reached at 27 days and falling precipitously thereafter. A more sedate rise in IgG_1 levels is seen with entrapped W2, peaking at day 14 and decreasing gently after that.

An important difference between the secondary response to W2, compared to W1 is that no anamnestic response is observed, even 10 days after the booster injection of 5ug of the peptide. IgG_1 levels of all three modes of presentation of the antigen fall to below peak values observed during the primary response. This suggests that the dose used may be a tolerogenic one and that unlike the case of the peptide W1, association of W2 with liposomes does not alter the situation, apart from exaggerating the primary response.

Titration of IgG_1 responses to surface-linked and liposome-entrapped polio peptides

To determine the relative amounts by which IgG_1 levels differed when immunised with either surface-linked or entrapped polio sub-unit peptides, serum samples were titrated by doubling dilutions and results expressed as the highest titre of serum that yielded an optical density of 0.100 as measured by ELISA.

Since even undiluted serum from mice immunised with free W1 or W2 did not give absorbance values of over 0.100, serum titres could not be reliably compared with those of liposome-associated peptides. Suffice it to say that presentation of either of the peptides W1 or W2 in a form associated with liposomes, be it covalently surface-attached of internally entrapped results in an increase in IgG_1 levels of greater than an order of magnitude.

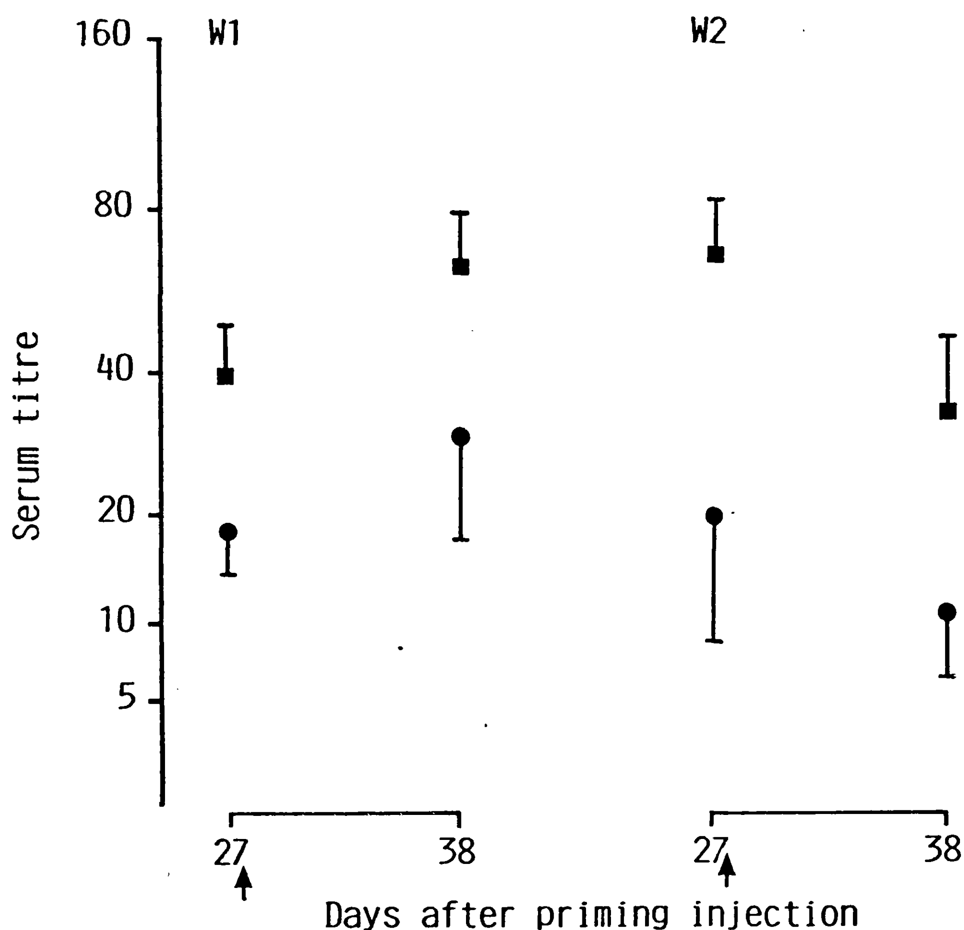


Fig. 2.4 Titration of antibody responses (IgG_1) to liposome-entrapped (●) and surface-linked (■) polio peptides

Sera obtained from mice immunised as described in the legend to fig. 2.1 were bled by doubling dilutions. Results are expressed as the highest titre of serum that yielded an optical density of 0.100 as determined by ELISA. (↑) indicates the time at which the booster injection was administered. Differences between liposome-entrapped and surface-linked groups on day 27 and day 38 for both W1 and W2 were significant ($0.01<p<0.05$ for all four comparisons made) as determined by Student's t test.

For W1, pre- and post-booster IgG_1 levels indicated that antibody levels elicited by surface-linked peptide are about twice as great as those elicited by entrapped peptide (Fig. 2.4). The mean serum titre observed 10 days (day 38) post-booster injection with surface-linked W1 appeared to be greater than the mean titre of entrapped W1 at day 38 whereas median values of untitrated sera (Fig. 2.3) demonstrate the opposite relationship to be the case. This discrepancy is due to the fact that a single mouse from the group of five immunised with surface-attached W1 showed an exceedingly high response at day 38, leading to a highly-skewed distribution. Serum titrations done 20 days post-booster reveal that IgG_1 levels in mice immunised with liposome-encapsulated W1 continue their ascent such that these become more than ten times greater than surface-linked W1 IgG_1 levels (results not shown). Thus, the relationship of median values of untitrated sera at day 38 give a more accurate reflection of the declining trend in surface-linked W1-elicited IgG_1 levels. In the case of W2, it is seen that pre- and post-booster levels of surface-linked peptide-elicited IgG_1 are about three times as great as those of entrapped peptide-elicited IgG_1. In contradistinction to the opposite movements of antibody levels obtained by the two different modes of presentation of W1, IgG_1 levels in both modes of W2 presentation exhibited a downward trend, indicating that no secondary response was being elicited.

There are several antigenic components in viruses, each of which consists of multiple antigenic regions. Some of these are relevant in inducing a protective immune re-

sponse in the host. Identification of such regions and synthesis form the basis in the design of the new generation of vaccines. Advantages of synthetic antiviral vaccines include avoidance of problems associated with conventional vaccines (eg. large-scale cultivation of viruses and related risks and cost of extraction of viral agents from infected donors), stability in freeze-drying and storage conditions and improved purity.

In designing oligopeptide vaccines, the following have been taken into consideration: Selection of the appropriate viral peptide sequence most likely to be the target of the immune response. Approaches for peptide selection are both theoretical and practical. Theoretical treatments have largely relied on the identification of structures (known to be targets of protective immunity) on the surface of viruses, expected to be readily mimicked by an oligopeptide. It is thought, for instance, that because terminal aminoacid sequences are relatively unconstrained conformationally and are thus likely to be more readily mimicked by freely mobile peptides, they would be good candidates for synthesis. On the other hand, rigid peptide sequences have a restricted conformation and this is likely to be retained in the synthetic oligopeptide representing the sequence. Alternatively, since antigenic sites on viruses are probably localised on their surface, such sites are likely to be rich in hydrophilic aminoacids. Selection of these regions is helped by the fact that protein molecules have only a few highly hydrophilic regions. One of the currently used approaches for peptide selection is based on the prediction of the protein's secondary structure. It has

been argued, for instance, that a peptide sequence of alpha-helix formation on the surface of a protein is likely to be retained in the peptide when in solution. Finally, a practical approach for peptide selection (applied to type 3 poliovirus) has been the isolation of mutant viruses which are resistant to antibodies neutralizing the parent virus. Since mutations would be confined to the areas which are targets for the neutralizing antibody, immunodominant regions of the viral protein can be identified.

Assuming that an immunogenic peptide sequence has been defined, peptides of varying sizes and incorporating the relevant sequence at different positions so as to select the most immunologically active species, can be synthesized. Identification of the dominant antigenic sites (and of immunologically active peptides) is being recently attempted by X-ray crystallography. The approach consists of identifying the more mobile antigen surface regions which would be expected to form a good fit for a wide range of binding sites for the antibody (than it would be expected for more rigid regions) and also be more easily mimicked by free, mobile peptides. An interesting argument supporting the validity of using such peptides has recently been put forward by Lerner (1983) who suggested that it is the very lack of constraints on shape that may give rise to the shape exhibited when the peptide forms part of the protein: a small peptide in the bloodstream will twist and bend and, as it encounters lymphocytes, it can trigger the secretion of antibodies against each shape in turn. It is possible that one of these shapes will be similar to the conformation the peptide adopts when part of a large protein.

Generally, synthetic oligopeptides are thought to be too small to induce antibodies and they are thus coupled, via a variety of reagents, as haptens to larger carrier molecules such as keyhole limpet haemocyanin, diphtheria toxoid and albumin. However, this approach is undesirable and will need to be replaced: peptides will assume a number of conformations upon coupling and preparations are difficult to standardise. Others, working on hepatitis B vaccines, have found that peptide cyclization by oxidation gives rise to immunogenic products, without the need of coupling to carriers. This, however, may be the result of polymerisations. It is, therefore, more likely that immunological adjuvants will be needed if enhancement of oligopeptide immunogenicity is to be achieved. As already discussed, powerful adjuvants such as Freund's complete adjuvant cannot be used in man and acceptable adjuvants such as alum (which has proven effective with some synthetic peptides) can only be a temporary measure. At present, muramyl dipeptide (MDP), especially some of its non-pyrogenic derivatives, appear to be promising. MDP with added acyl chains or covalently linked to antigens have been incorporated into liposomes for further improvement of adjuvanticity. Liposomes thus seem a reasonable alternative, especially since no conformational changes in the peptides are expected to occur upon passive entrapment.

Conclusions

The adjuvanticity of liposomes on poorly immunogenic virus subunit peptides was demonstrated by incorporating the synthetic polio virus peptides, W1 and W2, into the internal

space of and covalently-linked to the surface of DRV. It was found that for both peptides, liposome association boosted the primary and secondary IgG_1 responses against 5 ug peptide as compared to controls in which free peptide was administered. Surface-linked peptide gave an initially more rapid rise in antibody levels as compared to entrapped peptide but with no observable secondary response whereas encapsulated W1 showed a milder primary response but later elicited a strong secondary response.

The action of interleukin-2 on the primary and secondary immune responses to liposome-entrapped tetanus toxoid

Introduction

Interleukin-2 (IL-2) occupies a central position in the cascade of cellular events involved in the immune response. IL-2 supports the growth and proliferation of antigen-activated T cells and the generation of effector T cells, including helper, suppressor and cytotoxic T cells (Fauci, 1987). Proliferating T cells also produce other lymphokines that affect cells of B cell and macrophage lineages. Experiments using *Herpes simplex* (Weinberg and Merigan, 1988) and *Haemophilus pleuropneumoniae* (Anderson et al., 1987) vaccines suggest that IL-2 may function as a potent adjuvant to vaccination to increase the specific and durable response to vaccine immunogens.

In this chapter, the action of various doses of recombinant IL-2 on the immune response to liposome-encapsulated tetanus toxoid is studied. IL-2 was administered either co-entrapped with the model antigen in liposomes or separately entrapped. Results show that IL-2 co-entrapped with tetanus toxoid in DRV liposomes functions as a powerful co-adjuvant, further potentiating the primary and secondary responses to the toxoid. Therefore, the encapsulation of IL-2 in liposomes circumvents the requirement for its periodic systemic administration, as is the case with *Haemophilus*

pleuropneumoniae bacterin (Anderson et al., 1987), and potentially, its side effects as well.

Materials and Methods

Entrapment of rIL-2 and tetanus toxoid in DRV liposomes

Recombinant interleukin-2 (rIL-2)(des-ala$_1$-ser$_{125}$ mutein; $3x10^6$ Cetus units/mg) was a gift from Cetus Corporation, Emeryville, CA., USA. Immunopurified tetanus toxoid (Wellcome Biotech, Beckenham, Kent, U.K.) was radiolabeled with ^{131}I and rIL-2 with ^{125}I as described (p. 83).

Small unilamellar liposomes composed of equimolar egg PC or DSPC and Chol were mixed with 1ml of tetanus toxoid (50 µg) and/or rIL-2 ($2.5x10^4$ or $2.5x10^5$ units) (mixed with corresponding radiolabeled tracers) and used to generate DRV as already described (p. 61). Toxoid and rIL-2 entrapment were estimated by assaying ^{131}I and ^{125}I radioactivity respectively.

Animal immunisation experiments

Male Balb/c mice were injected intramuscularly, in groups of five, twice (on day 0 and day 42) with 0.1ml DRV containing toxoid (1µg) only or toxoid and rIL-2 (10^3 or 10^4 units) co-entrapped, or with a mixture of two DRV preparations containing the toxoid and rIL-2 respectively. Blood samples were obtained from the tail veins 1 day before and every 14 days after the priming injection. Serum samples were assayed for anti-toxoid antibodies by ELISA.

Enzyme-linked immunosorbent assay (ELISA)

This was carried out according to the method as described for assaying anti-RIVE antibodies (chapter 1), the only difference being that tetanus toxoid (15 μg/ml) in sodium bicarbonate buffer was used to coat the wells of the microelisa plates.

Results and Discussion

Entrapment of tetanus toxoid and interleukin-2 in bivalent DRV

Amongst the versatile features of liposomes lies their ability to encapsulate more than one substance at a time to yield bi- or multivalent liposomes. DRV, with their high entrapment efficiency, are especially suited for this purpose. Substances such as antigens and immunomodulators may be entrapped within their aqueous compartments or lipid bilayers such that the presence of one material may not necessarily interfere with the encapsulation of others, should they preferentially segregate in separate phases.

Using the DRV method to entrap tetanus toxoid as a model antigen and the lymphokine IL-2 as a co-adjuvant, it was found that high and reproducible entrapment values could be obtained for both these substances at the same time within the same liposomes (Table 3.1). Attempting to encapsulate IL-2 alone at two different doses with a ten-fold difference between them (250,000 and 25,000 units in total per 16uMol phospholipid), it was seen that high entrapment values (68.4 ± 0.4% for the higher dose and 67.0 ± 0.2% for the lower dose) were obtained.

Table 3.1 Incorporation of tetanus toxoid and IL-2 into liposomes

Radiolabelled tetanus toxoid and recombinant IL-2 were co- or separately entrapped in DRV liposomes generated from SUV liposomes composed of equimolar egg phosphatidycholine and cholesterol. S.D. denotes standard deviation of two individual preparations.

Total amount added		Toxoid incorporation		rIL-2 incorporation	
Tetanus Toxoid (μg)	rIL-2 (Cetus units)	% ± S.D.	μg ± S.D./uMol phospholipid	% ± S.D.	units ± S.D./uMol phospholipid
50	250,000	42.6 ± 0.4	2.66 ± 0.02	77.4 ± 0.3	24187.5 ± 93.8
50	25,000	35.8 ± 0.1	2.24 ± 0.01	75.5 ± 0.3	2359 ± 9.4
50	-	42.8 ± 0.4	2.67 ± 0.02	-	-
-	250,000	-	-	68.4 ± 0.4	21353 ±109.7
-	25,000	-	-	67.0 ± 0.2	2102 ± 7.4

The fact that the percentage entrapment of IL-2 is considerably higher than that of tetanus toxoid (42.8 ± 0.4%) which is probably entrapped in the aqueous compartments along with many other hydrophilic proteins that also exhibit similar entrapment values, indicates that IL-2 interacts strongly with the bilamellar leaflets of the liposomes. The absence of any marked difference in entrapment values despite a ten-fold difference in initial total concentration suggest that at the doses used, the capacity of the lipid bilayers to interact with and become saturated with the IL-2 molecules is far from saturated. Moreover, the relatively constant entrapment values between the two doses indicates that observed proportions entrapped are more a function of the amount of lipid used in a given volume of water, as long as the saturation capacity of the lipid bilayers is not exceeded, rather than the total initial amount of IL-2 employed.

Co-entrapment of tetanus toxoid and IL-2 in the same DRV resulted in no reduction of percentage entrapment values of either of the substances. This further supports the assumption that they may be preferentially segregated into different compartments and do not mutually interfere in each other's process of encapsulation. In fact, slightly higher entrapment values for both doses of IL-2 were observed when they were co-entrapped with tetanus toxoid, suggesting that there may be some attractive interaction between the toxoid and the hydrophilic portions of the IL-2 molecules to attract more of the latter into the aqueous compartments of the liposomes.

The effect of liposomal IL-2 on the primary immune response against tetanus toxoid

To investigate the action of two doses of IL-2 (1,000 and 10,000 units) when administered in separate and in the same liposomes as DRV-encapsulated tetanus toxoid as a model antigen, Balb/c mice in groups of five were given a single intramuscular injection of 1ug liposomal tetanus toxoid alone as a control and the same dose of toxoid given with the various preparations of IL-2. IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgM antibody subclass responses in sera from mice bled every two weeks were determined by ELISA.

The most graphic example of IL-2 action is seen in the majority IgG_1 subclass (Fig. 3.1). Antibody levels rise steadily in the control group in keeping with the concept of slow release of antigen from the liposomal depot. With both doses of IL-2 entrapped in separate liposomes, suppression of IgG_1 levels is seen with the effect most marked at day 28 and occurring more strongly with the 1000 unit dose than with the ten-fold greater dose. Some recovery from the suppressive effect is seen after day 28 with antibody levels in both groups rising gently although still remaining markedly lower than the control group at 42 days post-immunisation. The situation is initially exactly the opposite for both doses of IL-2 co-entrapped with tetanus toxoid, where there is a large increase in IgG_1 levels peaking at day 14. The rise is however, not sustained and levels actually fall below control values at day 28 indicating that there is some suppressive effect at this point, again more marked with the 1000 unit than with the 10000 unit dose. As with separately-

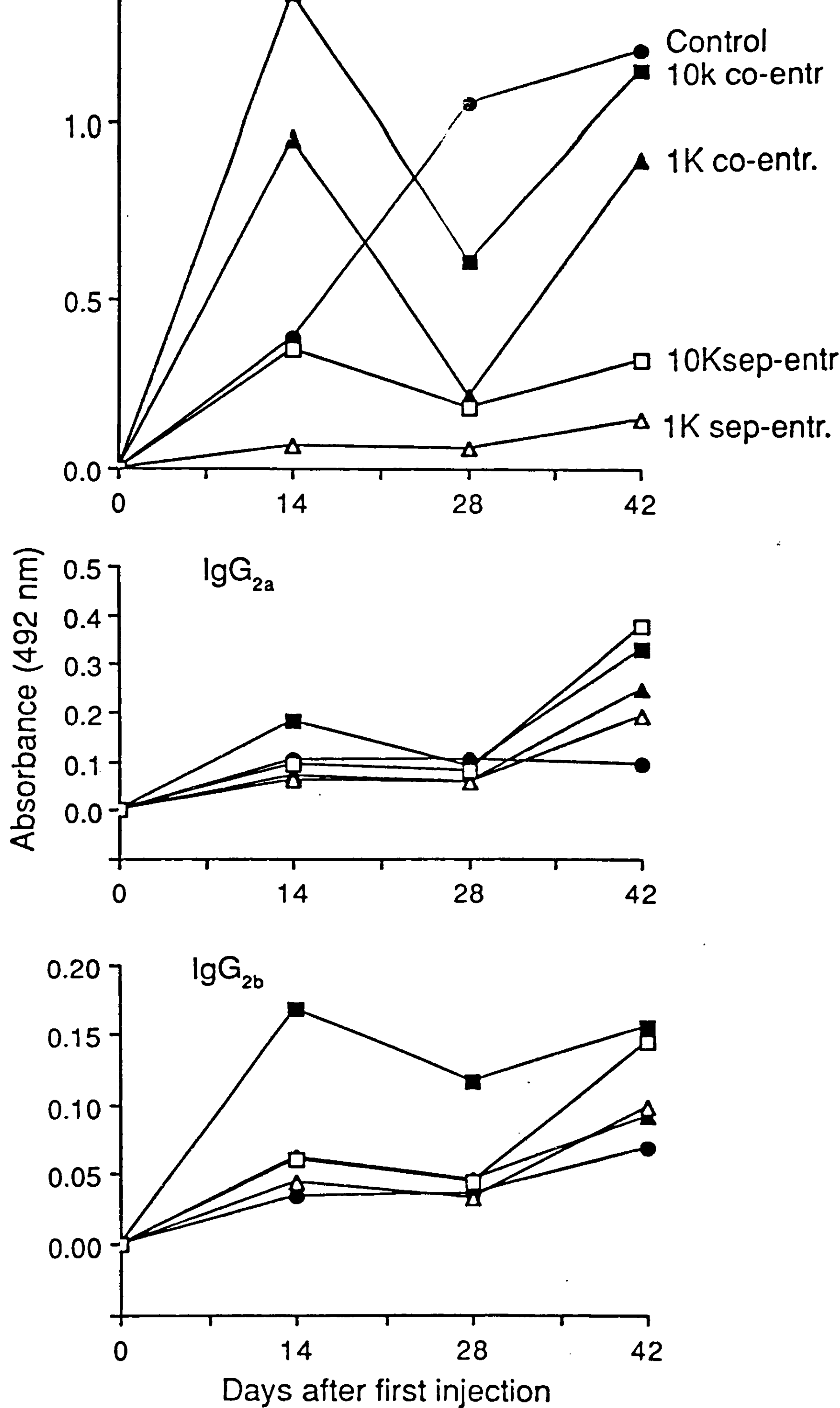


Fig. 3.1 The effect of liposomal rIL-2 on the primary immune response to liposomal tetanus toxoid

Balb/c mice in groups of five were injected intramuscularly with 1 μg tetanus toxoid entrapped in DRV liposomes without (●) or with rIL-2 (co-entrapped) (▲,■) or entrapped alone in DRV which were mixed with similar DRV containing rIL-2 (separately entrapped) (△,□). Animals were bled every two weeks and anti-toxoid IgG_1 responses were measured in sera by ELISA.

entrapped IL-2, IgG_1 levels recover to almost catch up with the control group by day 42 suggesting that the opposing IL-2 effects have worn out by this time and that the normal adjuvanticity of the liposomes is regaining predominance.

The effects of IL-2 on the primary response to liposomal tetanus toxoid are not as dramatic in the other subclasses, probably because very low levels of these isotypes are elicited in the primary response. What seems to be the general trend is that the 10000 unit doses of IL-2 either co- or separately entrapped seems to increase IgG_{2a}, IgG_{2b} and IgG_3 levels above control values with the effect being most obvious at day 42. Since antibody levels of these isotypes are already very low as to be hardly detectable, no suppressive effect by IL-2 is apparent. IgM levels show a very gradual and low-level rise with time and are not at all affected by IL-2.

The effect of liposomal IL-2 on the secondary response against tetanus toxoid

To investigate how the primary response of the various antibody subclasses was further modulated by a booster injection of IL-2, identical liposomal formulations of tetanus toxoid and IL-2 were again administered intramuscularly in the same groups of mice on day 42 and antibody levels monitored by ELISA.

The secondary IgG_1 response was seen to be most effectively potentiated by rIL-2 when it was entrapped together with tetanus toxoid in the same liposomes. It is seen that a tenfold greater increase in the dose of IL-2 employed did not proportionally further augment the IgG_1

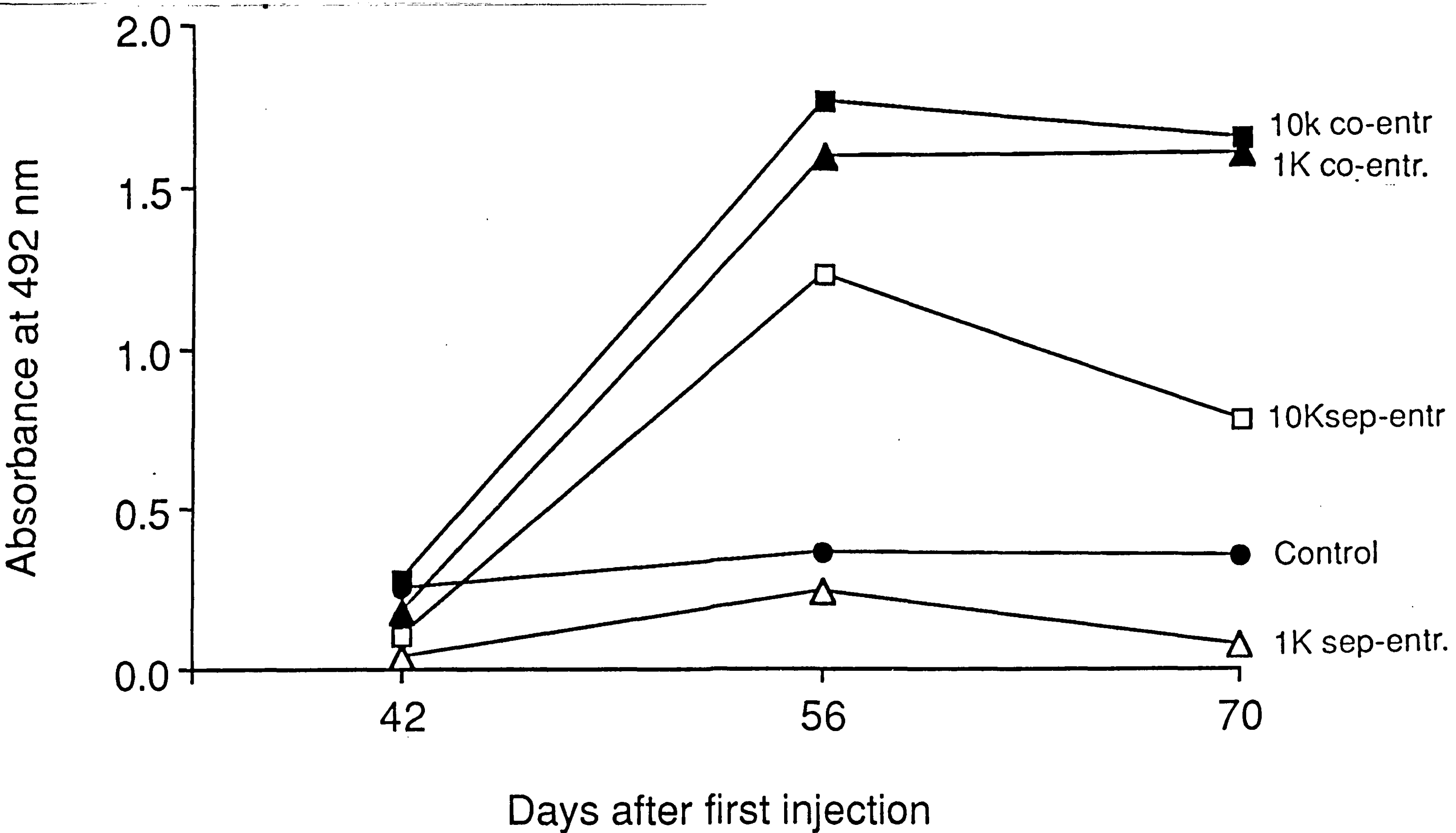


Fig. 3.2 The effect of liposomal rIL-2 on the secondary IgG_1 response to liposomal tetanus toxoid

Balb/c mice in groups of five were injected intramuscularly twice (on day 0 and day 42) with the liposomal preparations described in fig. 3.1. Animals were bled at two-week intervals and anti-toxoid IgG_1 responses were measured in sera by ELISA.

response as compared to merely incorporating the lower dose of 1,000 units. This may be due to the possibility that 1,000 units of co-entrapped IL-2 already boost the IgG_1 response to near maximal levels and that feedback mechanisms operate to ensure that overproduction of this antibody isotype does not occur. 10,000 units of IL-2 entrapped in separate liposomes was also able to increase IgG_1 levels over and above those seen in the control group but not as effectively as even the tenfold lower dose encapsulated in separate liposomes. Moreover, antibody levels fell to near-control values four weeks after the booster injection. In all groups, antibody levels rose steeply to attain maximal amounts in serum 2 weeks after the booster injection. The only group to show IgG_1 levels below those of the control was the one in which 1,000 units of IL-2 was entrapped in separate liposomes. This phenomenon is consistent with the occurrences seen in the primary response as well as the secondary response in the IgG_2 subclasses.

With the IgG_{2a} subclass, a clearly dissimilar and demonstrable effect of the two doses of IL-2 is observed (Fig 3.3). 10,000 units of IL-2 administered either co- or separately entrapped with tetanus toxoid in DRV, both greatly potentiated the immune response as compared to the control group. However, in the separately entrapped IL-2 group, IgG_{2a} levels fell off more rapidly from peak values at day 56 than for the co-entrapped group. This implies that some suppressive effect is at play in the 10,000 unit separately entrapped group which exerts itself after the stimulating effect has worn out, and that the stimulant effect persists much longer in the co-entrapped group. For the 1,000 unit

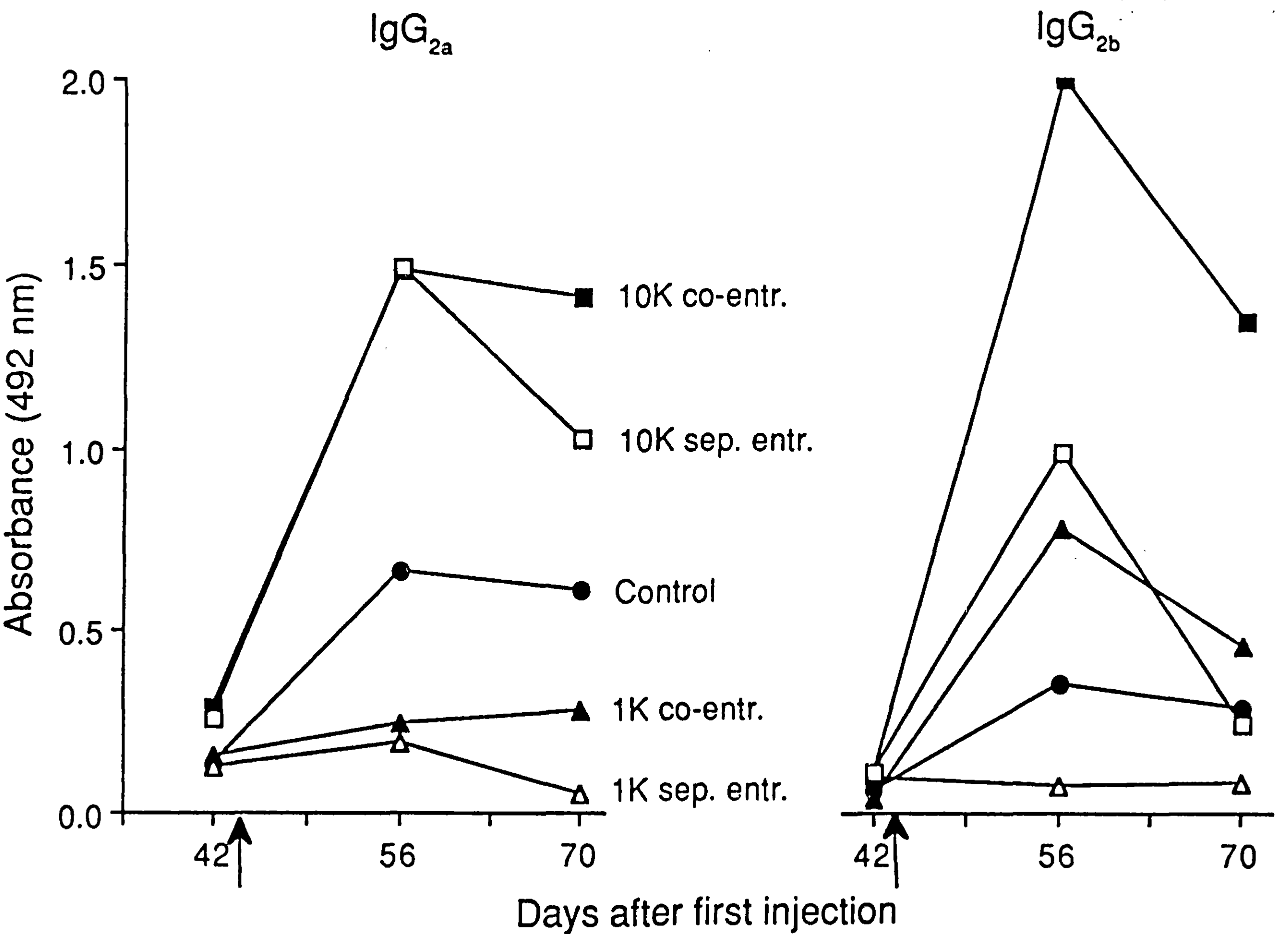


Fig. 3.3 The effect of liposomal rIL-2 on the secondary IgG_2 responses to liposomal tetanus toxoid

Balb/c mice in groups of five were injected intramuscularly twice (on day 0 and day 42) with the preparations described in Fig. 3.1. Animals were bled at two week intervals and anti-toxoid IgG_{2a} and IgG_{2b} responses were measured in sera by ELISA.

IL-2 dose, it is seen that in both co- and separately entrapped groups, IgG_{2a} levels are reduced well below control values. As with the 10,000 unit groups, IL-2 entrapped in separate liposomes led to a tailing-off of IgG_{2a} levels after the peak at day 56.

For the IgG_{2b} subclass, the relative increase in antibody levels over those in the control group elicited by 10,000 units IL-2 co-entrapped with tetanus toxoid was greater than in all other IgG subclasses. Peak levels observed two weeks post-booster injection were much higher than the control group but considerable reduction in IgG_{2b} levels occurred after this period. Significant increases in isotype formation were also seen with 10,000 units separately-entrapped and 1,000 units co-entrapped IL-2 although these were well below the levels attained in the 10,000 unit co-entrapped group. In keeping with the effect seen with the IgG_{2a} isotype, IgG_{2b} levels elicited by the 10,000 unit separately-entrapped IL-2 group fall steeply after day 56, in this case, to about control values. As consistently observed in the IgG subclasses hitherto discussed, 1,000 units separately-entrapped IL-2 kept IgG_{2b} levels suppressed to very low levels with no observable increase seen throughout the four-week period after the booster.

Akin to the primary isotype responses, IgG_3 and IgM levels after the booster injection were barely detectable with no rise to indicate that an anamnestic response was occurring in these subclasses. Moreover, there were no significant differences in the low antibody levels seen in all groups of IL-2 doses and formulations, suggesting that IL-2 has no adjuvant or suppressive effects in these sub-

classes or that control antibody levels have to be above a certain threshold for the effect of IL-2 to become apparent.

Many investigators have attempted to enhance the immunoadjuvant activity of liposomes further by the incorporation of immunomodulators. Studies carried out with endotoxin and lipid A show an enhanced adjuvant effect to the antigen in association with liposomes (van Rooijen and van Nieuwmegen, 1980b). However, further increase in the adjuvant activity is observed when the antigen and the immunomodulator are incorporated in the same liposomes. Another compound possessing immune potentiating activity is N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide <MDP>), the minimal structural unit that can replace the immunoadjuvant activity of mycobacteria in CFA. Encapsulation of MDP in liposomes enhances its effectiveness in producing an immune response against antigen compared to the unencapsulated MDP (Phillips et al., 1988). Liposome-encapsulated MDP has been found to activate macrophages more efficiently in vitro than free MDP (Sone and Fidler, 1981). This property has been further extended in vivo for the destruction of spontaneous lymph node and visceral metastases (Fidler et al., 1988).

Interleukin-2 is a glycoprotein, secreted by activated T helper cells that is able to enhance cytotoxic defenses, to elicit interferon gamma production and to stimulate B cells. McDermott et al (1985) have shown that human recombinant IL-2 is a potent activator of the guinea pig immune defenses and in vivo administration of rIL-2 has a protective effect against acute and chronic HSV-2 infection in these animals. Weinberg et al.,1988 demonstrated

that the association of rIL-2 with crude or purified HSV subunit vaccine improves immunisation results in guinea pigs. The protective effect achieved by co-administration of rIL-2 with HSV glycoproteins appeared to be mediated principally by HSV-specific T cell-dependent cytotoxicity. Anderson et al. (1987), examined the effects of IL-2 on the ability of *Haemophilus pleuropneumoniae* bacterin to protect swine against pleuropneumonia caused by the microorganism. It was found that IL-2, administered systemically in conjunction with bacterin, enhances protection upon subsequent challenge. Daily administration of IL-2 was more effective than a single injection with vaccination. Continued systemic administration of IL-2 was also able to enhance markedly the potency of inactivated rabies virus vaccine. At optimal IL-2 doses, a greater than 100-fold increase in vaccine potency was observed. However, inactivated rabies virus vaccine potency was not increased when the IL-2 was administered as an oil-in-water emulsion with the vaccine.

In view of the potential of IL-2 in enhancing vaccine potency, its mandatory periodic systemic administration, short circulatory half-life, pyrogenicity and toxicity, it would make sense to entrap it in liposomes which would presumably circumvent its side effects while giving full rein to its adjuvanticity by confining it to the immediate vicinity of the co- or separately- entrapped antigen. Data presented in this chapter demonstrate the feasibility of this approach, suggesting that high doses of co-entrapped IL-2, being in the immediate vicinity of the antigen possibly at the time of antigen presentation as well, acts as a powerful stimulus in causing T-helper and B-cells specifi-

cally recognising tetanus toxoid to proliferate. The exogenously administered IL-2 probably augments the effect of endogenous IL-2 secreted by the T-helper cells.

Observations in this study also raise further questions as to why low levels of IL-2 should suppress the primary and secondary immune response. There could be the possibility that IL-2 that is not in the immediate vicinity of the antigen presenting cell at the time of antigen presentation (as would be the case when it is entrapped in separate liposomes), pre-empts surrounding IL-2 receptor sites, thus preventing the locally available T-helper cells from responding to the antigen being presented later.

Conclusions

The immune response to bivalent and separate liposomes containing the lymphokine Interleukin-2 and tetanus toxoid was studied. 10,000 units of IL-2 co-entrapped in tetanus toxoid containing DRV markedly increased antibody levels 14 days post-injection as compared to control DRV containing only tetanus toxoid. A less pronounced effect was seen with 1,000 units of co-entrapped IL-2. IL-2 administered in separate liposomes mildly suppressed antibody levels, with the lower dose causing more inhibition than the higher one. A similar effect was observed on the secondary response in the major subclasses, with co-entrapped high-dose IL-2 exhibiting the greatest adjuvanticity and low-dose separately-entrapped IL-2, the most suppression.

Chapter 4

Enhancement of the immune response against liposome-entrapped tetanus toxoid by manipulation of membrane characteristics and mode of administration

Introduction

One of the reasons for the widespread applicability of liposomes in diverse fields of biological research is the eminent amenability to alteration of their bilayer characteristics, enabling them to be tailored for specific needs. Parameters such as hydrophobicity, fluidity, charge, size, surface-associated molecules and encapsulated material may be varied to form a truly protean complex.

In this chapter, the effect of varying lipid composition, surface charge and route of administration of DRV on the immune response to encapsulated tetanus toxoid are investigated. Results indicate that antibody responses to the entrapped antigen may be greatly enhanced by optimal manipulation of liposomal characteristics and mode of administration. It is proposed that this represents an effective strategy in maximising the immunogenicity of an antigen in a liposome-based vaccine.

Materials and methods

Entrapment of tetanus toxoid in neutral and positively-charged DRV liposomes

Small unilamellar liposomes composed of equimolar

phospholipid (16μmoles egg PC or DSPC), or phospholipid incorporating 5 and 10% (molar basis) BisHOP, and Chol were mixed with 1ml of ^{125}I-labelled tetanus toxoid at a concentration of 50μg/ml water to generate neutral and positively-charged DRV (p. 61).

Animal immunisation experiments

Balb/c mice were primed intramuscularly in groups of five with the same amount of free or liposome-associated tetanus toxoid. Blood samples were obtained from the tail veins 1 day before and every 14 days after the priming injection. Serum samples were assayed for anti-toxoid antibodies by ELISA (chapter 3).

Results and discussion

Encapsulation of tetanus toxoid in BisHOP-containing DRV

To immunise mice with various formulations of tetanus toxoid in liposomes composed of phospholipids of widely differing transition temperatures and amounts of a novel positively-charged lipid, equimolar phospholipid (egg PC or DSPC containing 0%, 10% and 20% of the charged lipid BisHOP as a percentage of the total phospholipid used) and cholesterol were used to form liposomes entrapping tetanus toxoid by the DRV method.

In keeping with previously established data, entrapment values in PC and DSPC DRV were high and reproducible (40.0±0.4% and 42.6±1.2% respectively, Table 4.1). However, it was found that incorporation of increasing

DRV composition	% ± S.D. TT entrapped	µg TT ± S.D./umole phospholipid
PC:Chol	40.0 ± 0.4	12.5 ± 0.1
10% Bis:PC:Chol	32.6 ± 0.2	10.2 ± 0.1
20% Bis:PC:Chol	28.7 ± 2.3	8.9 ± 0.7
DSPC:Chol	42.6 ± 1.2	13.3 ± 0.4
10% Bis:DSPC:Chol	35.6 ± 2.2	11.2 ± 0.6
20% Bis:DSPC:Chol	27.4 ± 2.2	8.6 ± 0.6

Table 4.1 Incorporation of tetanus toxoid into neutral and charged liposomes

Tetanus toxoid (50 µg) was entrapped in DRV liposomes generated from SUV liposomes composed of equimolar phospholipid and cholesterol. s.d. denotes standard deviation of two individual preparations.

amounts of the novel positively-charged lipid BisHOP into the structure of liposomes led to progressively diminishing values, falling to 32.6±0.2% for 10% BisHOP:PC DRV and 28.7±2.3% for 20% BisHOP:PC DRV. For DSPC DRV, tetanus toxoid entrapment values also declined slightly to 35.6±2.2% for BisHOP:DSPC DRV and to 27.4±2.2% for 20% BisHOP:DSPC DRV.

These results contrast with previously published data which showed that for PC:Chol DRV incorporating the positively-charged lipid stearylamine SA or the negatively-charged phosphatidic acid, entrapment values for tetanus toxoid were higher than for control neutral liposomes. However, results are not strictly comparable as much less of the total phospholipid was made up of charged molecules (slightly greater than 1%) as compared to the present study in which 10% and 20% of the total were composed of positively-charged BisHOP. Increased entrapment values in the former case was attributed to the increased width of the aqueous spaces between the bilayers. With PC and DSPC liposomes incorporating much higher proportions of charged lipid as in the present study on BisHOP liposomes, it was found that the higher the proportion of charged lipid used, the more difficult it was to obtain clear SUV in the course of the DRV procedure. This is probably due to the mutual repulsion between the positively-charged lipid molecules which pack the MLV lamellae or the more `strained' SUV bilayer in relatively high density. This presumably leads to difficulty in forming stable SUV even when temperature criteria are met, their subsequent lysis or reversion to the MLV state causing the liberation of entrapped tetanus toxoid or re-

duced entrapment after dehydration and controlled rehydration. In spite of the marginally reduced entrapment compared to neutral DRV, encapsulation values are still much higher than those obtained with basic MLV formation. In any case, if amounts to the immunising antigen are not in short supply, this does not present any problem whatsoever.

<u>Primary immune responses to tetanus toxoid encapsulated in BisHOP-containing DRV</u>

In an experiment designed to compare the effect of adding increasing amounts of the positively-charged lipid BisHOP to PC and DSPC DRV on the primary immune response to 1 ug encapsulated tetanus toxoid, Balb/c mice in groups of 5 were administered a single intramuscular injection of various liposomal formulations of tetanus toxoid and bled every two weeks for 56 days. IgG_1 levels in appropriate dilutions of sera were monitored by ELISA.

Results observed for the control neutral PC and DSPC DRV were surprising in that DSPC liposomes showed a greater adjuvant effect than PC liposomes with IgG_1 levels in the DSPC DRV group rising steadily and more steeply than for the PC DRV group (Fig. 4.1). This is in direct contrast with previously established data (Davis and Gregoriadis, 1987) comparing the secondary responses obtained against tetanus toxoid encapsulated in `fluid' (PC) and `solid' (DSPC) liposomes, which showed that PC DRV had a much greater adjuvant effect than DSPC DRV where the effect was negligible, in mice bled 10 days after the booster injection. However, as will be shown later, the adjuvanticity of PC DRV

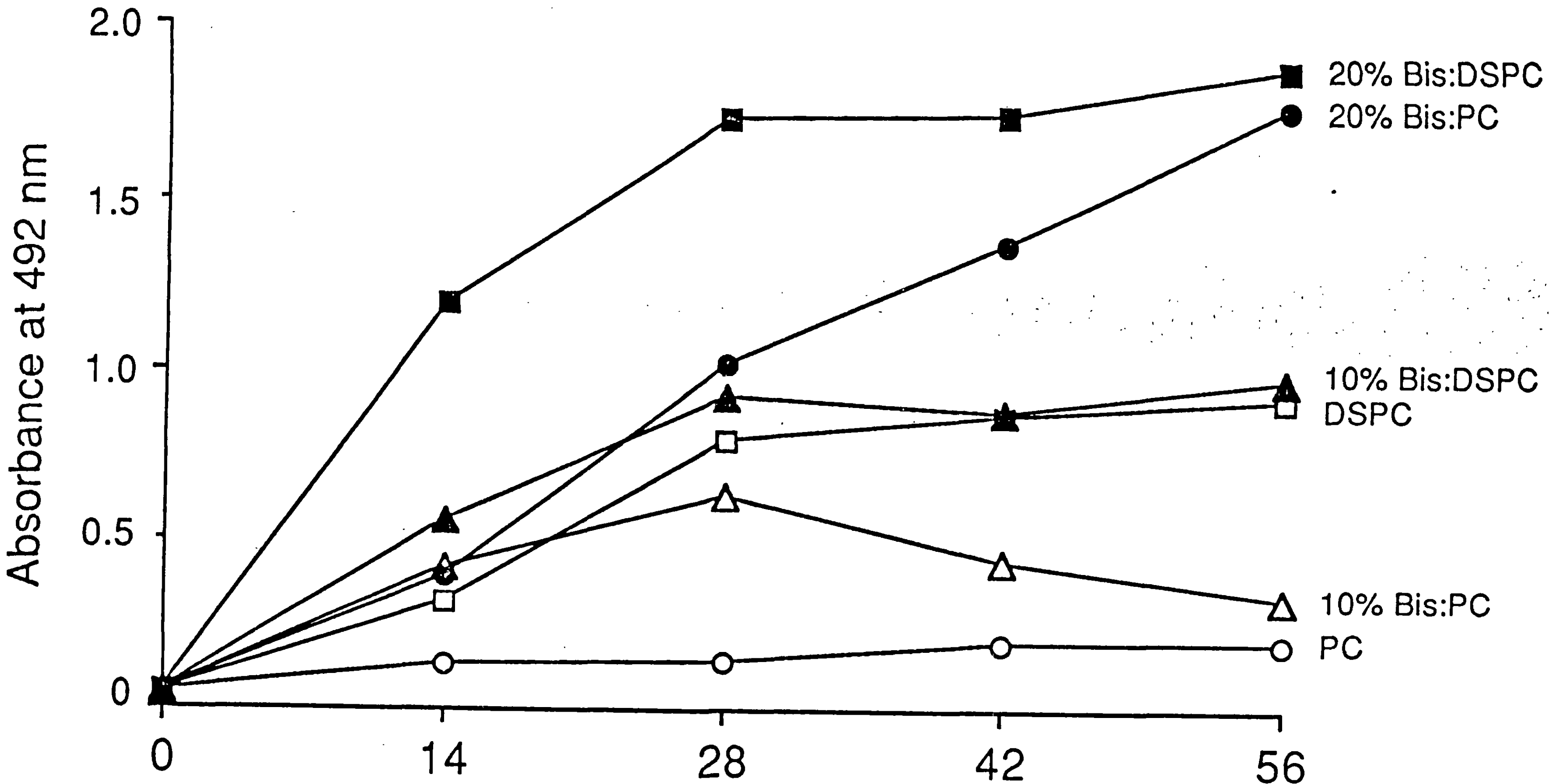


Fig. 4.1 Primary antibody responses to tetanus toxoid entrapped in neutral and positively-charged liposomes

Balb/c mice in groups of five were injected intramuscularly with 1 µg of tetanus toxoid entrapped in DRV composed of PC:Chol(○), 10% BisHOP:PC:Chol(△), 20% BisHOP:PC:Chol(●), DSPC:Chol(□), 10% BisHOP:DSPC:Chol(▲), 20% BisHOP:DSPC:Chol(■). Animals were bled just before and every 14 days after the injection. Anti-toxoid IgG_1 responses were measured in sera by ELISA.

is exerted much more strongly after the booster injection whilst their effect in boosting the primary response is not as great as that of DSPC DRV.

It is seen that irrespective of the transition temperature of the lipid used to construct the liposomes, the incorporation of increasing amounts of positively-charged BisHOP leads to marked enhancement of the adjuvant effect in the primary response. When DRV are composed of 20% BisHOP as a percentage of the total phospholipid used, IgG_1 levels attained similar values for both PC and DSPC liposomes which were considerably higher than neutral DSPC DRV and very much greater than for neutral PC DRV. However, incorporation of 10% charged lipid did not lead to much increase in adjuvant activity over neutral liposomes. This implies that there may be a threshold of positive charge above which liposomal adjuvanticity is greatly increased. The initial rate of rise of IgG_1 levels is seen to be the greatest for 20% BisHOP:DSPC DRV, whilst although the initial increase in antibody levels induced by 20% BisHOP:PC DRV is more sedate, it continues its rise relentlessly after IgG_1 levels of the former liposomes have reached a plateau and effectively `catch up' with them by day 56. Antibody levels for 10% BisHOP:PC DRV showed a good initial rise to peak at day 28 but fell off after this period to almost PC DRV levels by day 56.

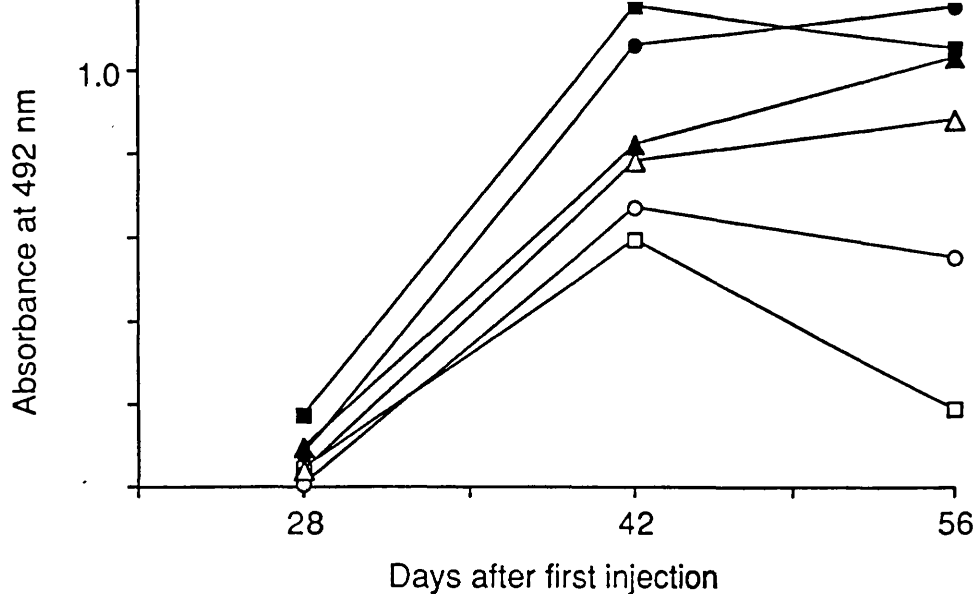
Primary antibody responses of the other IgG isotypes were much weaker, but a numerical comparison of the relationship of median serum absorbance readings on day 28 (pre-booster) in fig. 4.2, shows a tendency also for PC DRV to elicit lower antibody levels than DSPC DRV in the primary

(pre-booster) response for the IgG_{2a} and IgG_{2b} subclasses. Incorporation of higher amounts of BisHOP also generally led to increasing adjuvanticity in these two subclasses. IgM responses were not detectable, in keeping with findings from the previous chapters.

Secondary immune responses to tetanus toxoid encapsulated in BisHOP-containing DRV

To determine if a booster injection of identical formulations of liposomes entrapping 1ug tetanus toxoid would qualitatively alter the patterns seen in the primary antibody response, a separate batch of Balb/c mice in groups of five were immunised with two doses of BisHOP-containing DRV at 28-day intervals and bled every two weeks. Antibody subclass levels were again determined by ELISA.

In the secondary IgG_1 response, it is seen that irrespective of the differential levels of antibody enhanced by the various liposomal formulations, a booster injection of the same dose and formulation caused IgG_1 to attain roughly similar levels (Fig. 4.2). The only exception was for neutral DSPC DRV whose IgG_1 levels were somewhat less than in all other groups at the day 42 peak and which tailed off slightly more rapidly than the others as well after this period. Pre-booster IgG_1 levels in the PC DRV group rose dramatically from the lowest position to catch up and overtake the DSPC DRV group. Thus, these findings are in keeping with previous reports and is further substantiated by secondary responses seen in the other IgG subclasses. It would appear that the adjuvant effect of neutral PC liposomes in the secondary response is already so great that further



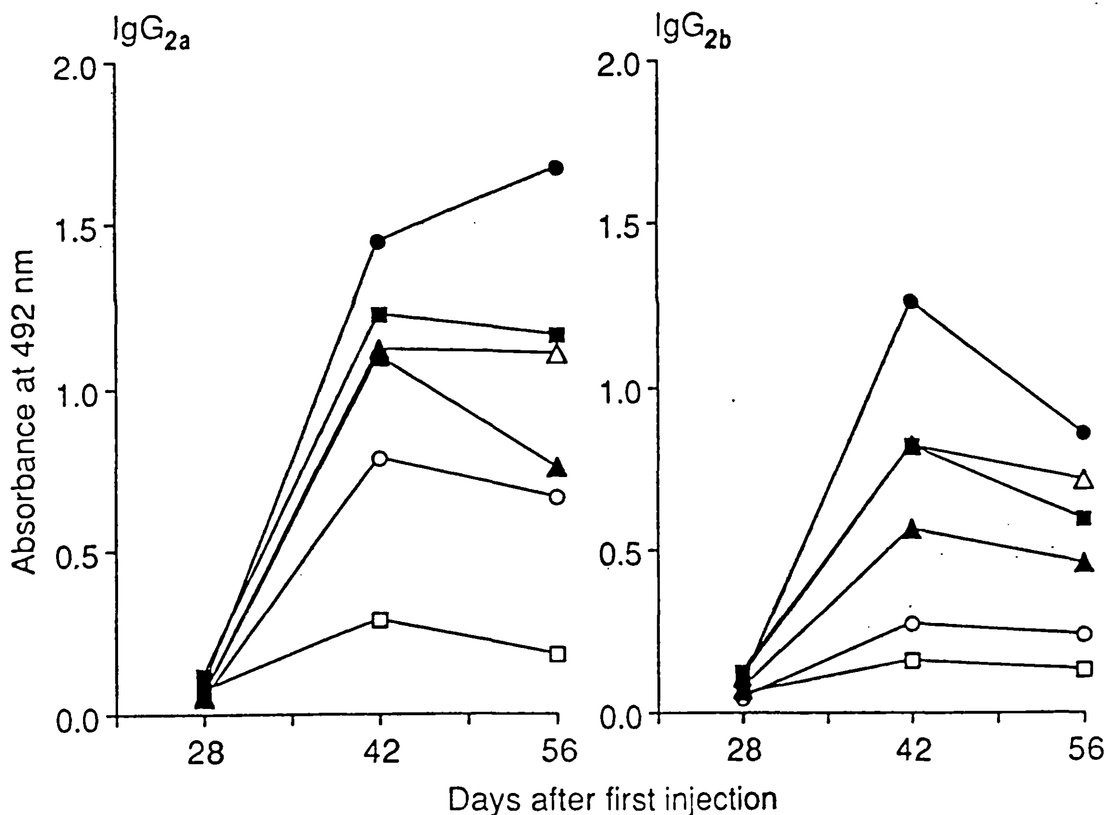


Fig. 4.2 Secondary antibody responses to tetanus toxoid entrapped in neutral and positively charged liposomes

Balb/c mice in groups of five were injected intramuscularly twice (with a 28-day interval between injections) with 1 ug of tetanus toxoid entrapped in DRV composed of PC:Chol(○), 10% BisHOP:PC:Chol(△), 20% BisHOP:PC:Chol(●), DSPC:Chol(□), 10% BisHOP:DSPC:Chol(▲), 20% BisHOP:DSPC:Chol(■). Animals were bled just before and every 14 days after the booster injection. Anti-toxoid IgG subclass responses were measured in sera by ELISA.

incorporation of positively- charged lipid does not additionally enhance the high IgG_1 levels attained, which are presumably near their `maximum' as far as liposomal structural adjuvanticity is concerned. However, for the less marked effect of DSPC DRV on the secondary response, incorporation of BisHOP into the liposomes does further increase IgG_1 levels although there is no significant difference between 10% and 20% BisHOP incorporation as levels are already very high for the former DSPC DRV.

For antibody isotypes which have been increased by a lesser degree compared to IgG_1 in the primary immune response, the adjuvant effect of various phospholipids and charge on the secondary response to entrapped tetanus toxoid is much more striking. As with the secondary response in the IgG_1 subclass, PC DRV proved to be more powerful adjuvants than DSPC DRV, essentially reversing the situation found at the pre-booster stage. With both `fluid' and `solid' liposomes, incorporation of increasing amounts of BisHOP led to progressively increasing amounts of BisHOP led to progressively increasing enhancement of adjuvanticity. The most impressive effect was demonstrated by 20% BisHOP:PC DRV where IgG_{2a} attained levels higher than all other groups 2wks after the booster injection and kept rising thereafter, whilst the rest remained static or declined.

The qualitative effects of the various liposomal formulations on the secondary IgG_{2b} response to tetanus toxoid were remarkably similar to that of IgG_{2a}. Again, PC DRV proved to be better adjuvants than DSPC DRV and increasing amounts of positively charged lipid further potentiated the `basic' adjuvanticity of neutral liposomes in the IgG_{2b}

subclass. The relative enhancement of the adjuvant effect by the incorporation of BisHOP in DRV over that of the neutral control liposomes was the greatest of all the IgG subclasses. For all formulations, and for 20% BisHOP:PC DRV in particular (in contrast to the situation observed in the IgG_{2a} subclass), antibody levels peaked at day 42 and declined sharply after that. The secondary IgG_3 response generally showed a similar trend as in the IgG_2 subclasses but the effect of adding increasing proportions of BisHOP was not so apparent. As with the primary response, no increase in IgM levels was detected.

<u>Primary immune responses to liposomal tetanus toxoid administered at one and at two different sites</u>

One of the proposed mechanisms by which liposomes, in common with many other adjuvants (eg. alum and mineral oils) act is by the retention of antigen at a depot, preventing its rapid dispersal from the site of injection and subsequently releasing small amounts of the antigen slowly over a prolonged period.

To test the hypothesis that this mechanism of action would be more effective if liposomes were administered at two sites, one intramuscular (well vascularised) and the other intradermal (relatively poorly vascularised) compared to the same dose of liposomal antigen all injected intramuscularly at one site, Balb/c mice in groups of five were given either a single dose of 1 ug tetanus toxoid intramuscularly in the hindlimb, or an identical dose separated into two equal portions at two sites, one half as in the control group and the other half intradermally into the

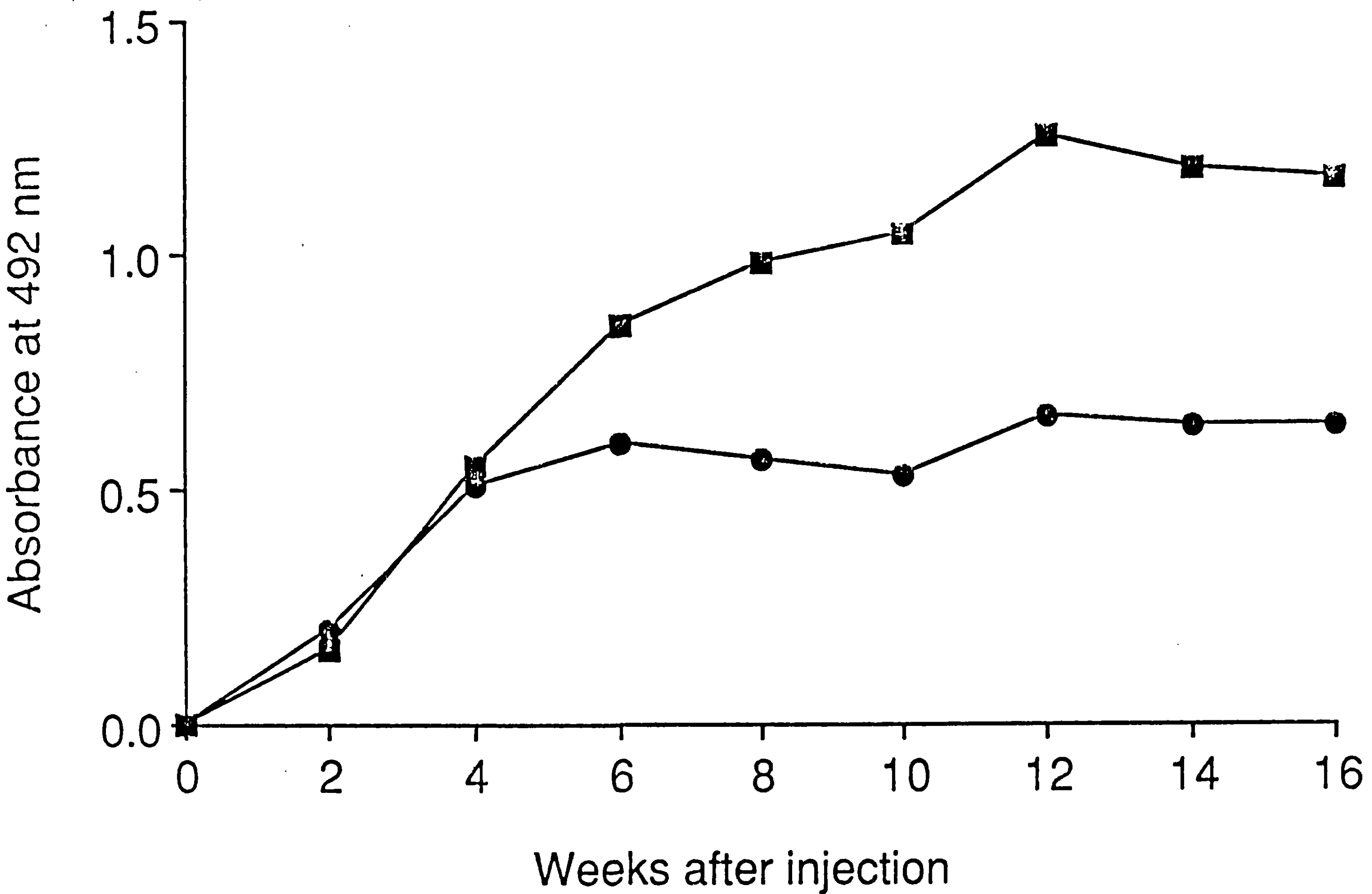


Fig. 4.3 Primary antibody responses to liposomal tetanus toxoid administered at one and at two separate sites

Balb/c mice in groups of five were injected with 1 µg of tetanus toxoid entrapped in PC:Chol DRV injected entirely intramuscularly into the hind limb(●) and divided into two equal portions, one injected intramuscularly into the hind limb and the other intradermally on the back(■) Animals were bled just before and every 14 days after the injection. Anti-toxoid IgG_1 responses were measured in sera by ELISA.

dorsal trunk area. Animals were bled at two-weekly intervals and IgG_1 levels in sera were measured by ELISA.

Results show that in both groups, there was an equal steady rise in antibody levels for the first four weeks (Fig. 4.3). Subsequent to this period, IgG_1 levels in the control group began to plateau off whilst those of the group which received the injection at two sites continued to increase and attained a peak at 12 weeks after which antibody levels also reached a plateau. Peak values in the control group were seen at 12 weeks even though the leveling off occurred much earlier. Median absorbance readings of sera for the group receiving the dose at two separate sites were significantly greater ($0.001<p<0.05$, $T=17$ where T is the lower sum of ranks of the two groups being compared) than those in the control group at and after peaks levels had been reached.

Liposomes may be neutral, negative or positively charged depending on the lipid used in the liposome preparation. The effect of surface charge on the immunopotentiating activity of liposomes was first observed by Allison and Gregoriadis (1974). It was found that inoculation of diphtheria toxoid in negatively charged liposomes elicited significantly higher antibody levels than when entrapped in neutral or positively charged liposomes. A later report showed that although negatively-charged liposomes could act as adjuvants, positively charged liposomes too could do the same (Heath et al., 1976). Similar results were obtained by van Rooijen and van Nieuwmegen (1980) showing that positively charged and neutral liposomes have the same adjuvant activity as negatively charged liposomes. Still most of the

workers studying the adjuvant properties of liposomes to antigens made use of negative liposomes. Recently, the effectiveness of positively charged liposomes in producing significant levels of antibodies has been demonstrated (Latif and Bachhawat, 1984). The antibody titres obtained with lysozyme entrapped on positively charged liposomes was found to be higher than neutral, negatively charged liposomes and even CFA. The authors suggest the possibility that the positive charge may hamper the fusion of the vesicles with the lysosomes thus allowing protection and prolonged exposure of the entrapped antigen to the immune system.

Most of the liposomal vesicles for immunological studies have been prepared from egg PC, Chol and a negatively charged phospholipid, dicetyl phosphate or phosphatidic acid. In order to introduce a positive charge, stearylamine has been used. The molar ratio of the lipids used in the preparation of liposomes which promotes effective antibody production (Allison and Gregoriadis, 1974) is usually 7:2:1 of egg PC, Chol and charged lipid, respectively. However, whether this ratio is indeed optimum for immune enhancement has not been established.

Egg lecithin or PC is the most common ingredient of liposomes used for adjuvant activity. It is biodegradable and a harmless compound when administered as liposomes (van Rooijen and van Nieuwmegen,1980) although some exchange may occur with the phospholipid of cells (Gregoriadis et al., 1977). The most important advantage of phosphatidylcholine liposomes as adjuvants is that, in contrast to phosphatidyl inositol, phosphatidyl glycerol and phosphatidic acid, phosphatidyl choline is a very poor antigen (Alving et al.,

1980). Liposomes prepared with phosphatidylcholine by themselves do not evoke an immune response in rabbits even when incorporated in IFA. However, an immune response against phosphatidylcholine is induced when lipid A is incorporated in the liposomes.

Liposomes prepared from sphingomyelin have been reported to be more effective in eliciting an immune response to incorporated antigen than liposomes made from phosphatidylcholine (Yasuda et al., 1977). There is a striking difference in the T_c of these lipids (PC -8° to 15°C, sphingomyelin 42°C) and it is suggested that high immunological response by sphingomyelin liposomes may be due to their greater stability. In contrast to these observations van Rooijen and van Nieuwmegen (1980) reported lower adjuvant activity of sphingomyelin liposomes than phosphatidylcholine liposomes to the associated protein antigen.

Liposomes composed of dipalmitoyl phosphatidylcholine (DPPC) and distearoyl phosphatidylcholine (DSPC) (T_c 41.4^o and 54.9^{o}C respectively) have also been reported to be more effective immunogens than those prepared from egg PC (Kinsky, 1978; Hedlund et al., 1984). It is postulated that these liposomes may have greater bilayer stability at physiological temperature and may thus persist longer in vivo than egg PC. In contrast, it has been found that although DPPC and DSPC liposomes are strong immunopotentiators of the entrapped antigens, liposomes prepared from egg PC are better adjuvants (Latif and Bachhawat, 1984). This study has shown that for tetanus toxoid, at least, DSPC functions as a better adjuvant than egg PC during the primary response and that the reverse is true during the secondary response.

Thus, the differing efficacies of adjuvanticity afforded by different phospholipids depends not only on the size and other physical properties of the antigen causing their unique and individual modes of interaction with the liposomal bilayers/aqueous compartments, but also on the time point at which antigen-specific antibody levels are assayed. It has also been pointed out in the General Introduction that the ratio of lipid to antigen administered is of crucial importance.

Conclusions

Various strategies for enhancing the adjuvant effect of liposomes using tetanus toxoid as the model antigen were investigated. A novel positively charged lipid, BisHOP, when incorporated into the bilayers of PC and DSPC DRV, were shown to have a powerful effect in enhancing the primary and secondary immune responses to tetanus toxoid. The adjuvant effect was significantly greater when 20% BisHOP was incorporated as compared to 10% incorporation and to control PC and DSPC DRV. Plain DSPC DRV were found to have a greater adjuvant effect than plain PC DRV on tetanus toxoid in the primary response but the opposite was true in the secondary response. Even though antibody levels at 8 weeks post- injection were similar for 20% BisHOP PC and DSPC DRV, the rate of rise of antibody titres was more rapid for 20% BisHOP DSPC than for 20% BisHOP PC DRV. These results suggest that faster and higher titres of antibodies may be obtained by optimal manipulation of the charged and non-charged lipid components of liposomes.

The primary immune response to 1 ug of tetanus

toxoid incorporated in PC DRV was examined after being administered as follows:- In one group of mice, the dose was divided into two equal portions and administered one-half intramuscularly and the other half intradermally in a relatively avascular site (the back). In the control group, the whole dose was given intramuscularly. Results showed that in both cases, peak IgG responses were obtained 12 weeks after the injection. However, the antibody levels (assayed by ELISA) in the group which received the same dose at two different sites was significantly greater. This implies that liposome-based vaccines could be more potent and effective when administered at two different sites, one vascular and the other, relatively avascular.

Chapter 5

Clearance of charged liposomes from the circulation - the influence of lipid composition and pH

Introduction

The duration of circulatory half-life of liposomes has important implications in the targeting of liposomes to specific cells within or immediately outside of the cardiovascular system and for liposome-controlled drug release. Incorporating lipids of different characteristics into the liposomal bilayer markedly alters their clearance rates from the circulation. Stable neutral liposomes made of long saturated acyl-chains such as DSPC remain for relatively prolonged periods in the bloodstream compared to shorter-chain PC liposomes. Incorporation of charged lipids reduces the half-life of liposomes considerably, with negatively-charged PC liposomes being cleared more rapidly than positively-charged ones. In this paper, we further explore the effect of incorporating charged lipids into DSPC liposomes on their clearance from the circulation and the influence of pH on the electrophoretic mobility of charged liposomes in vitro.

Materials and methods

Preparation of neutral and charged SUV liposomes

32 µmoles phospholipid (PC, DSPC or either including 5% of 10% PA, SA or BisHOP on a molar basis) and

32μmoles Chol were used to formulate SUV as described on page 60.

Measurement of CF clearance from the circulation in mice

Balb/c mice in groups of four were injected intravenously into the tail veins with 0.2ml CF-containing SUV (about 1.5mg phospholipid), bled at time intervals and plasma assayed for total CF using a fluorimeter (Kirby et al., 1980). CF clearance rates from the circulation were taken to represent those of intact SUV (Gregoriadis, 1988).

Measurement of electrophoretic mobility of liposomes

MLV prepared in PBS of varying pH values were used instead of SUV, as sonicated liposomes could not be visualised. Microelectrophoretic mobility measurements were carried out as described on page 67.

Results and Discussion

Clearance of DSPC SUV from the circulation

To study the rates of clearance of neutral DSPC SUV and DSPC SUV incorporating 10% charged components of the total amount of phospholipids, Balb/c mice in groups of four were intravenously injected with the liposomal preparations and blood samples withdrawn at time intervals to determine remaining CF fluorescence in the serum after liposome lysis by Triton X-100. In agreement with previous observations using similar quantities of phospholipid (Senior and Gregoriadis, 1982) the half-life of neutral DSPC SUV was about

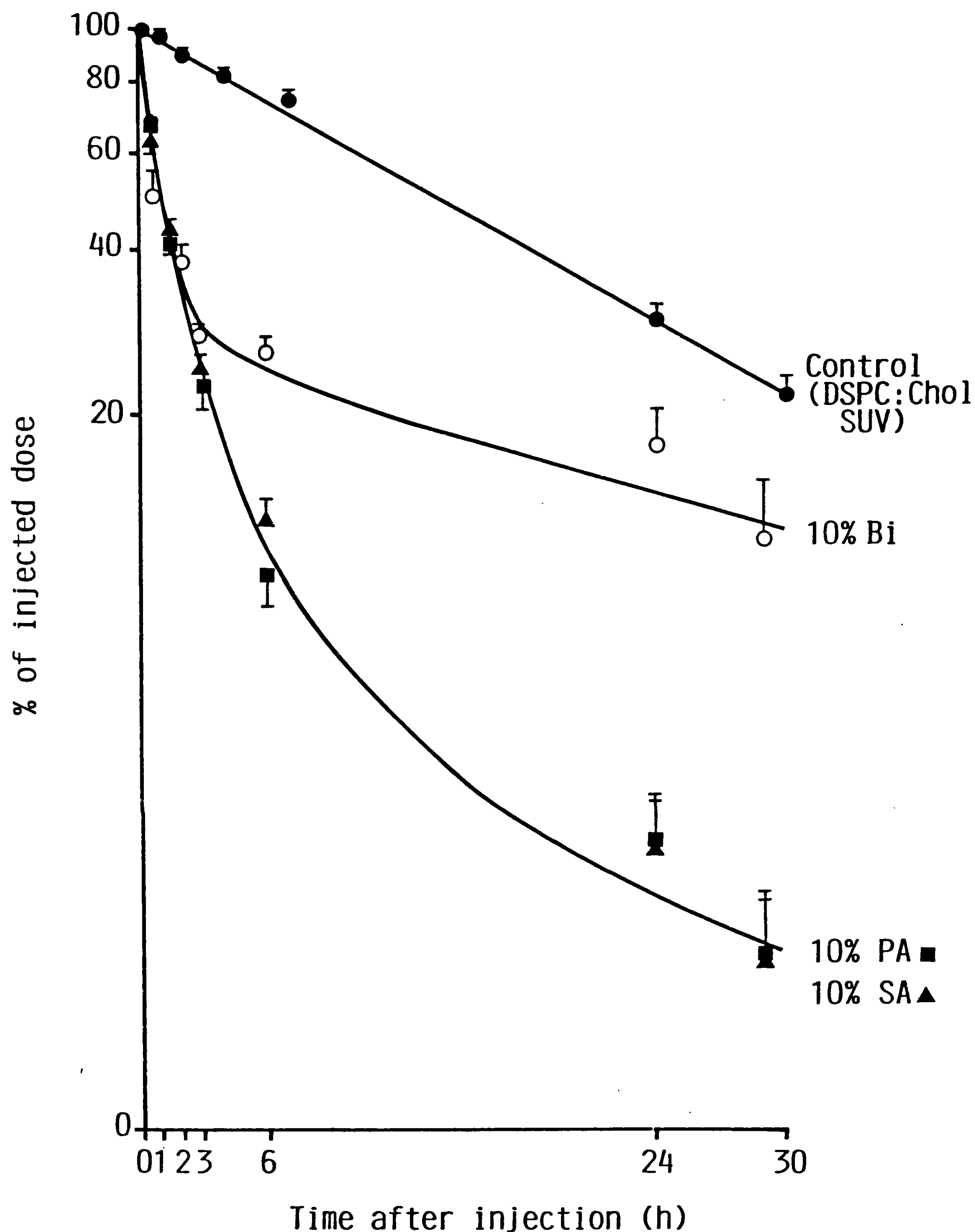


Fig. 5.1 Clearance of DSPC SUV after intravenous injection

Balb/c mice (in groups of four) were injected with CF-containing SUV composed of equimolar DSPC and cholesterol (●) or with similar SUV also incorporating (10% molar ratio with DSPC) PA (■), SA (▲) or BisHOP (o). Animals were bled at time intervals and total CF measured in blood plasma. Values are means ± S.D.

14h, while that of negatively-charged PA:DSPC SUV was markedly reduced to just over 1h (Fig. 5.1). However, a surprising finding was that positively-charged SA:DSPC SUV were cleared at the same rate as negatively-charged PA:DSPC SUV in contrast to previous data (Gregoriadis and Neerunjun, 1974; Kirby et al., 1980) which showed that positively-charged SA:PC SUV were cleared much slower than negative PA:PC SUV and at about the same rate as neutral PC SUV. It must be emphasised that in the latter case, the neutral phospholipid used to form SUV had a lower liquid-gel transition temperature and average chain length. As with SA:DSPC SUV, positively-charged BisHOP:DSPC SUV also exhibited similarly rapid initial clearance rates from the circulation. The pattern of clearance of DSPC SUV containing 10% BisHOP seems to be biphasic with a later slowing down of the initially rapid clearance to attain a half-life akin to that of neutral DSPC SUV in the second phase.

Clearance of PC SUV

To see if results obtained with charged DSPC SUV were related to charged PC SUV and to confirm established data, similar experiments as described were performed with PC SUV incorporating on a molar basis, 5% and 10% BisHOP, 10% SA and 10% PA. The observed pharmacokinetic behaviour of neutral, PA- and SA-containing PC SUV was in agreement with previous findings. However, as noted with BisHOP:DSPC SUV and in contrast to positively-charged SA:PC SUV, BisHOP:PC SUV were cleared more rapidly than neutral PC SUV with the rates of clearance being faster, the higher the percentage of incorporated BisHOP (Fig. 5.2). Thus, the half-life of 5%

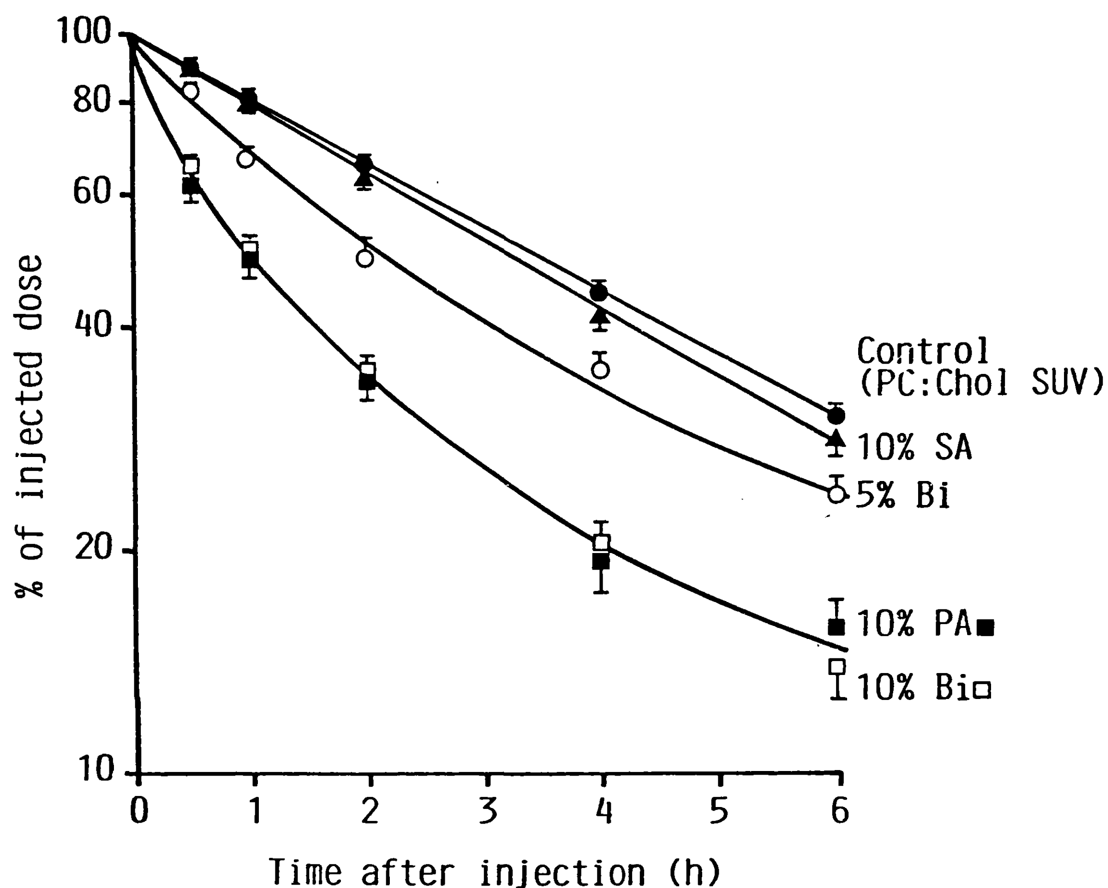


Fig. 5.2 Clearance of PC SUV after intravenous injection

Balb/c mice (in groups of four) were injected with CF-containing SUV composed of equimolar egg PC and cholesterol(●) or with similar SUV also incorporating (% molar ratio with PC) 10% PA (■), 10% SA (▲), 5% BisHOP (○) and 10% BisHOP(□). Animals were bled at time intervals and total CF measured in blood plasma. Values are means ± S.D.

BisHOP:PC SUV was just over 2h and that of 10% BisHOP:PC SUV about 1h (similar to negative PA:PC SUV), both being shorter than the half-life of neutral PC SUV (over 3h).

These findings imply that the enhanced clearance rates imparted upon neutral SUV by incorporated charged components are dependent upon the acyl chain lengths and transition temperatures of the majority neutral phospholipid molecules.

It is conceivable that with the more `solid' DSPC SUV, where packing of the phospholipid molecules is tighter and the liposomal structure more rigid, the removal of charged lipids sandwiched between the molecules by plasma components such as HDL is prevented or diminished. Thus, all or most of the charge would be retained on the SUV in the plasma and oppositely-charged opsonins attracted and adsorbed to the surfaces of these liposomes would markedly shorten half-lives as compared to neutral SUV which do not attract charged proteins. An important observation described here is the fact that irrespective of whether initial charge is positive or negative, as long as it is not removed from the liposomes in vivo, clearance rates will be identical. In the case of PC SUV, there is evidence to show that the looser packing of the `fluid' liposomes allows the removal of lipids like SA by plasma components such as HDL, causing the initially positive SUV to gradually become neutral in the circulation. The difference in pharmacokinetic behaviour of BisHOP:PC SUV as compared to SA:PC SUV even though they are initially both positively-charged may be attributed to the fact that BisHOP contains two long acyl chains per molecule as compared to SA's single stearyl chain, thus

rendering it more difficult for BisHOP molecules to be removed from PC SUV particles. Hence, as in the case of DSPC SUV, both PA and BisHOP:SUV are cleared at identical rates in spite of their being of opposite charges.

Electrophoretic mobility of charged liposomes in vitro

To investigate the electrophoretic mobility of liposomes composed of various charged constituents added to neutral phospholipids of different transition temperatures and to see if any relationship existed between their electrophoretic behaviour in vitro and their pharmacokinetic behaviour in vivo, a microelectrophoresis apparatus was employed for the measurements.

Owing to the near impossibility of visualising unstained SUV through low power light microscopy, MLV of varying charge and lipid composition were used instead. It is also undesirable to entrap dyes in SUV for visualisation as the presence of charged groups on the encapsulated dye molecules would affect the overall mobility of their enveloping vesicles giving rise to difficulty in interpreting results. Results showed that pH and plasma incubation influenced the overall charge on the vesicles to a marked degree, at times even overriding the charge imparted to the liposomes by their integral membrane constituents (Table 5.1).

Neutral PC MLV, intrinsically possessing no net charge, did not show any detectable electrophoretic movement at low pH (below 6), as would be expected for uncharged particles. However, surprisingly, as the pH increases to 7.4 and above, they behave as if they possessed a net positive charge, migrating to the cathode. A wide range of pH did not

MLV compn. \ pH	4	6	7.4	mouse plasma	after plasma washed off	8	10
DSPC	(+) 1.03 ±0.05	(+) 0.31 ±0.05	(+) 0.26 ±0.05	(+) 0.26 ±0.05	(+) 0.26 ±0.05	0.00	0.00
10% PA: DSPC	(-) 4.27 ±0.09	(-) 3.24 ±0.10	(+) 4.38 ±0.08	(+) 2.47 ±0.15	(+) 4.12 ±0.21	(+) 3.24 ±0.10	(+) 1.70 ±0.06
10% SA: DSPC	(+) 3.97 ±0.21	(+) 4.84 ±0.41	(+) 4.74 ±0.21	(+) 2.88 ±0.10	(+) 4.33 ±0.10	(+) 5.10 ±0.41	(+) 2.32 ±0.10
10% Bis: DSPC	(+) 3.19 ±0.10	(+) 4.79 ±0.41	(+) 4.69 ±0.21	(+) 3.40 ±0.21	(+) 2.32 ±2.32	(+) 4.58 ±4.58	(+) 3.45 ±3.45
PC	0.00	0.00	(+) 1.49 ±0.10	(-) 1.85 ±0.10	(+) 1.18 ±0.10	(+) 2.32 ±0.05	(+) 1.85 ±0.05
10% PA: PC	(-) 3.76 ±0.10	(-) 3.24 ±0.15	(-) 2.94 ±0.10	(-) 1.80 ±0.10	(-) 2.32 ±0.15	(-) 3.19 ±0.15	(-) 3.86 ±0.15
10% SA: PC	(+) 2.27 ±0.10	(+) 3.24 ±0.10	(+) 2.78 ±0.10	(-) 1.75 ±0.10	(+) 2.58 ±0.21	(+) 3.24 ±0.21	(+) 2.16 ±0.10
10% Bis: PC	(+) 3.55 ±0.28	(+) 3.19 ±0.15	(+) 2.83 ±0.10	(-) 1.70 ±0.05	(+) 2.42 ±0.15	(+) 3.14 ±0.15	(+) 2.27 ±0.10

Table 5.1 Electrophoretic mobility of liposomes in vitro

Electrophoretic mobility ($\mu m.s^{-1}.V^{-1}.cm^{-1}$ + S.D.) of 10 individual liposomes was measured in each experiment. (+) or (-) indicates that the net charge on the liposomes was positive or negative respectively.

alter the overall charge on 10% PA, SA or BisHOP MLV which retained their negative, positive and positive charges respectively. As previously reported (Black and Gregoriadis, 1976), incubation of neutral, positively- and negatively-charged PC MLV in plasma resulted in all of them acquiring a net negative charge, presumably through the adsorption of negatively-charged plasma proteins in the case of neutral and positive MLV. Charged behaviour as indicated by electrophoretic mobility values for both positively-charged SA and BisHOP MLV indicate that they are identical in behaviour in vitro, even on incubation in plasma where both acquire negative charges. This would seemingly be in contrast to their markedly different rates of clearance from the circulation, where SA:PC SUV are cleared more slowly (at the same rate as neutral PC SUV) compared to BisHOP SUV. However, caution must be used in extrapolating the behaviour of MLV in vitro to that of SUV in vivo as the morphology of these two species of liposome are strikingly different. MLV are of much larger diameter and gentler surface curvature whilst SUV are smaller with a unilamellar, highly `strained' surface. Thus, while SA may be removed from SUV in vivo by blood components such as HDL with relative ease owing to the nature of the SUV surface causing the pharmacokinetic behaviour of SA:PC SUV to approximate that of neutral PC SUV, it is envisaged that with MLV, the tighter packing of the lipid bilayers prevents this phenomenon from occurring to any marked degree. This would explain the identical electrophoretic behaviour of SA and BisHOP:PC MLV over a wide pH range and even on incubation with plasma but the difference in clearance patterns of SA and BisHOP:PC SUV in the circula-

tion.

The microelectrophoretic mobility values of DSPC MLV in vitro have important contrasts to those of PC MLV. It is seen that plain DSPC MLV with no charged components show no net electrophoretic movement only at high pH (8-10). At pH values below this, they acquire a net positive charge and moved towards the cathode. DSPC MLV containing 10% PA are only negatively-charged at low pH (4-6) but surprisingly acquire a net positive charge at pH 7.4 or higher. SA and BisHOP:DSPC MLV exhibit an overall positive charge over the wide range of pH used.

In marked contradistinction to PC MLV, DSPC MLV on incubation in plasma all acquire a net positive charge which is seen even with neutral and initially negatively charged PA:DSPC MLV. However, the magnitudes of mobility values varies greatly, being very low (0.26 ± 0.05) for neutral DSPC liposomes to 3.40 ± 0.21 for BisHOP DSPC MLV. The low values observed for neutral DSPC MLV may correlate with the prolonged half-life of DSPC SUV in vivo compared to the charged liposomes. The similarity of charge and magnitude of electrophoretic mobility values of the charged DSPC MLV probably accounts for their identical and rapid initial clearance. Thus, the relation between in vitro electrophoretic behaviour and in vivo pharmacokinetic behaviour is more apparent for DSPC liposomes than for PC liposomes. This may be due to the fact that DSPC has a longer pair of acyl groups and a higher transition temperature, causing it to pack and retain charged molecules with greater efficiency, even in the `strained' SUV structure. This would make it difficult for HDL to remove charged components from SUV in vivo. All

positively charged SA and BisHOP would then be retained and the SUV exhibit identical clearance patterns. Negative PA DSPC liposomes presumably attract and adsorb positively-charged opsonins from the plasma and behave in a similar fashion to SA and BisHOP DSPC SUV in vivo.

Incorporation of appropriately charged lipids into the liposomal bilayer can confer an overall neutral, negative, or positive charge, with the amount of charged lipid incorporated controlling the charge density. Lipids commonly used to confer a negative charge are DCP, phosphatidic acids with various fatty acid side chains, eg. egg PA, DSPA, gangliosides, PS, PG, cardiolipin, and phosphoglycerolipids other than PC or PE derivatives, which have a positive moiety as well as the negative charge of the phosphate group at physiological pH. Positive charge is usually conferred by SA but highly positive liposomes are reported prepared from PG.

Reasons for providing liposome bilayers with a net charge include use of negatively charged lipids to improve entrapment of solutes especially enzymes (Gregoriadis, 1977); in use of liposomes as adjuvants, negatively charged PS-containing liposomes are used in targeting to the lung after i.v. administration of vesicles (Abra et al., 1984); incorporation of gangliosides can reduce leakage of aqueous space markers for liposomes in plasma (Allen et al., 1985). Positively charged lipids have also been used for purposes of targeting in vivo, but evidence that positively charged liposomes containing SA may be toxic in vivo (Adams et al., 1977) has curtailed their use. Negatively charged lipids can, depending on their proportion, dramatically alter in

vivo clearance patterns of small liposomes when neutral vesicles of similar composition exhibit slow rates (Senior et al., 1985).

The interaction of neutral liposomes and those with a net charge, with plasma components was investigated by Black and Gregoriadis (1976) who showed, using the same electrophoresis technique employed in this study, that both neutral and positively charged liposomes are rendered negatively charged whereas liposomes composed of negatively charged lipid maintain the same overall net charge in the presence of blood plasma. Acquisition of a negative charge by neutral liposomes seems to be due to adsorption of $alpha_2$-macroglobulin and perhaps other plasma components. However, results presented in this chapter also reveal that pH changes exert a marked effect on the overall charge of liposomes and that the effect is also dependent on the bilayer characteristics of the liposomes being studied. These findings are supplemented by the recent discovery that cations as well as anions associate and even bind to the liposomal bilayers (Kicq et al., 1987; Tatulian, 1987), thus influencing the electrophoretic mobility of liposomes. As ionic dissociation is mainly dependent on pH, one can easily see why pH variation causes such radical changes in net liposome charge. Negative surface charge for particles coated with plasma regardless of the original particle charge has been observed by Wilkins and Myers (1967) using latex microspheres. As with liposomes, negatively charged latex microspheres exhibited a shorter half-life in the circulation than those with a more positive surface charge. A possible explanation for the way in which adsorbed plasma

components can influence particle clearance rates depending on the original particle surface charge has been put forward by Wilkins and Myers (1967) on the basis of a previous finding that surface properties (eg. charge) of the particle could affect the steric arrangement of the adsorbed protein in a manner `recognisable' to the fixed macrophages of the RES. This may also apply to liposomes especially since $alpha_2$-macroglobulin has been recognised as a phagocytosis promoting factor. However, data presented in this chapter suggest that for relatively stable particles like DSPC SUV, clearance from the circulation of both positively and negatively charged vesicles occurs at similar rates and more rapidly than neutral vesicles. The discrepancy could be due to the fact that the bilayer structure of SUV enables molecules to be intercalated as well as adsorbed to the surface in contradistinction to the more inert surface of latex particles.

Conclusions

The influence of charge on the electrophoretic mobility of MLV in vitro and on the clearance from the circulation of SUV after intravenous administration was studied. It was found, contrary to previous supposition, that positively charged lipids (stearylamine and BisHOP were used) incorporated into DSPC SUV were surprisingly cleared from the circulation, in mice, as rapidly as negatively charged ones incorporating phosphatidic acid. Previous findings which indicated that positively-charged stearylamine-containing PC SUV were cleared slowly, at the same rate as neutral PC SUV were confirmed, with the additional discovery that BisHOP-

containing PC SUV were cleared as rapidly as negatively-charged phosphatidic acid PC SUV. These results suggest that positively and negatively charged SUV made by adding charged lipids to neutral phospholipids of a given chain length and transition temperature are cleared at the same rates if the charged lipid is confined in a rigid bilayer (eg. in DSPC SUV). A study of the effect of pH on the electrophoretic mobility of charged MLV in vitro revealed that the net surface charge on liposomes is markedly influenced by changes in pH. This may have important implications for the clinical use of liposomes containing charged lipid, ligands or drugs and in states of acidosis or alkalosis.

Chapter 6

Production of monoclonal antibodies against human hepatocellular carcinoma by immunisation with a cell membrane preparation

Introduction

Hepatocellular carcinoma (HCC) ranks eighth worldwide in terms of cancer mortality. In sub-Saharan Africa and in the Far East, it is one of the major causes of cancer death. The medium survival rate of untreated patients is less than 1 year and even after treatment with surgery or chemotherapy, it is almost 100% fatal. Hepatitis B virus infection is the most prominent factor implicated in the causation of HCC but it will take at least 20 years before an assessment of whether the worldwide vaccination program against hepatitis B reduces the incidence of HCC. Therefore, there is a need to improve the prognosis of patients through early diagnosis and treatment.

The development of hybridoma technology and the production of monoclonal antibodies (Kohler and Milstein, 1975) represent an important new tool in identifying surface markers which are specific for hepatocytes and are associated with transformation and differentiation of liver cells. Monoclonal antibodies with sufficient specificity for human tumours are also potentially important diagnostic and therapeutic agents. Several groups have succeeded in raising monoclonal antibodies that bind to surface determinants expressed on hepatocellular carcinoma cells by immunising

mice with whole cells.

In this chapter, the production of two monoclonal antibodies that bind to epitopes present on HCC cell lines as well as a hepatoma section by the novel approach of immunising mice with HCC membrane preparations is described.

Materials and methods

Production of monoclonal antibodies

Balb/c mice were immunised by three intraperitoneal injections of PLC/PRF/5 membrane preparations emulsified in complete Freund's adjuvant repeated at three-week intervals. 14 days after the last immunisation, mice received an intravenous booster of the PLC/PRF/5 membrane preparation alone and the spleens were removed 4 days later. Splenocytes were fused with myeloma cells (P3-NS1/Ag 4-1) by pelleting the cell mixture at 200g for five minutes and incubating with 1ml polyethylene glycol 1500 (BDH Chemicals Ltd.) 66.4% w/v for seven minutes at $37^{o}C$. The fusion mixture was then gradually diluted by addition of small aliquots of RPMI 1640 (Flow). Finally, the cells ($1x10^{6}$/ml) were dispensed in 2 ml Linbro trays (Flow). Twenty-four hours later, 1ml HAT medium (Flow Labs:- RPMI 1640 containing 10% FCS, 2mM glutamine, 10^{-4}M hypoxanthine, $4x10^{-7}$M aminopterin, $1.6x10^{-5}$M thymidine and antibiotics) was added to each well. Beginning between days 10 to 14, supernatants from wells showing cell growth were tested for antibody activity. Hybridomas secreting specific antibody were isolated by cloning several times by limiting dilution (1 cell/well) in microtitre plates with feeder layers of normal

spleen cells. Antibody isotype was determined by Ouchterlony double diffusion.

Incubation of monoclonal antibodies with cells

Mahlavu cells were maintained in plastic culture flasks (Nunclon) in RPMI 1640 medium containing 10% fetal calf serum, 100 units penicillin/ml and 100 μg streptomycin/ml. The cells were kept at 37°C in a humid atmosphere of 5% CO_2 in air and subcultured by transferring small amounts of cell suspension into fresh flasks with medium. For in vitro experiments, cells were spun down at 1000 rpm for 7 min in a bench centrifuge, resuspended in phosphate-buffered saline supplemented with 5 mg/ml bovine serum albumin and 0.05% (w/v) sodium azide (incubation buffer) and counted in a haemocytometer. Known numbers of cells suspended in 50 ul incubation buffer were placed in triplicate into plastic test tubes and unless otherwise stated, incubated with 50 ul monoclonal antibody at 4°C for one and a half hours. To separate cells from unbound material, incubation mixtures were pipetted onto 1.5 ml 10% Dextran T-40 in incubation buffer and centrifuged at 1200 rpm (400xg) for 25 min. After removal of the Dextran layer, cell pellets were washed once with phosphate-buffered saline at 4°C, centrifuged at 2000 rpm (1000g) for 10 min and counted for ^{125}I radioactivity.

Results and discussion

Production of monoclonal antibodies

Using a membrane preparation derived from cultured

PLC/PRF/5 cells in an immunisation protocol involving three intraperitoneal injections separated by three-week intervals, monoclonal antibodies were raised in Balb/c mice by the method of Kohler and Milstein (1975). Supernatants from hybridoma cell cultures were screened for positivity by a radioimmunoassay procedure developed to detect the binding of monoclonal antibodies to surface determinants on Mahlavu cells. The latter cells were used instead of the PLC/PRF/5 line which was used in the immunising membrane preparation as initial supernatant screenings revealed that monoclonal antibodies secreted by positive hybridomas bound to surface determinants present on all three HCC cell lines ie. PLC/PRF/5, Mahlavu and SK-SF, and because Mahlavu cells were the least fastidious and most rapidly-growing.

Three spleen-myeloma cell fusions were performed and seven antibody-secreting hybridoma clones were initially isolated. However, out of these, four later lost antibody-secreting ability on propagation and repeated cloning by limiting dilution. The three remaining clones were injected intraperitoneally into pristan-primed Balb/c mice to produce antibody-rich ascites after at least three clonings by limiting dilution to ensure purity of the hybridoma lines and to prevent the overgrowth of rapidly-growing non-secreting mutants. Monoclonal antibodies were purified on Sepharose columns and antibody concentrations estimated by nephelometry.

To determine the individual subclasses of the three monoclonals isolated, supernatants from hybridoma cultures were concentrated by PEG osmosis through a semi-permeable membrane and Ouchterlony double diffusion per-

Monoclonal Antibody	SAH tumour	Normal liver	Hepatoma (English,♀)	Hepatoma (Arab,♂)
RF-HCC 1	+ +	+	+ + +	-
RF-HCC 2	+ +	+	+ + +	+

Table 6.1 Immunofluorescence of snap-frozen tissue sections using cloned hybridoma supernatants

Indirect immunofluorescence staining was performed as described on page 91. Scoring of fluorescein staining was expressed on an arbitrary scale ranging from (+): low to (+++): high fluorescence intensity.

formed with rabbit anti-mouse isotype antibodies. This method revealed that the monoclonal antibody designated RF-HCC1 was of the IgG_1 subclass and that designated RF-HCC2 was of the IgM class. No precipitin lines were observed for the third monoclonal probably because antibody concentrations in culture supernatants were present in extremely low concentrations.

On the basis of having identified antibody isotypes, RF-HCC 1 and RF-HCC 2 were further characterised by immunofluorescence staining of snap-frozen tissue sections prepared from HCC cell line xenografts in nude mice, patients' hepatoma sections and normal liver tissue. RF-HCC1 was found to bind strongly to antigenic determinants expressed on one patients' hepatoma section but not at all to a second patient's (Table 6.1). The nationality of the latter patient and consequently, the probable etiology of his hepatoma may be related to the absence of surface antigens which are identified by RF-HCC 1. RF-HCC 1 also bound moderately strongly to PLC/PRF/5 tumour antigens and only very weakly to normal liver tissue. RF-HCC2 also bound strongly to an epitope present on the first patient's tumour and to another antigenic determinant present in the second patient's tumour although this was expressed to a much lesser degree. As with RF-HCC1, RF-HCC2 bound to an epitope present in moderately high density on PLC/PRF/5 tumour xenografts and to a much smaller extent, on normal liver as well.

Interaction of free monoclonal antibodies with Mahlavu cells in vitro

To determine the kinetics of binding of the monoclonal antibodies RF-HCC1 and RF-HCC2 to HCC cells, 10^6 Mahlavu cells were incubated at 4^oC with 0.1 ug free antibody in incubation buffer and cells were separated from unbound antibodies at time intervals by centrifugation through a layer of 10% Dextran T-40.

Results show that binding of monoclonal antibodies to surface epitopes present on Mahlavu cells takes place in a biphasic fashion, with initial rapid binding of each antibody within the first 15 min of incubation, followed by a plateauing-off after this period as the remaining antigenic sites available for binding become depleted (Fig.6.1). RF-HCC1 shows a continued gradual climb in percentage antibody binding values after the initial rapid-binding phase and does not completely reach a plateau by 3h. 14.6% of the total amount added is bound to the cells by this time. RF-HCC2 almost reaches maximal binding values by 1h of incubation time and not much additional antibody is seen to bind after this. By 3h, 25.4% of the amount added had bound to Mahlavu cells. It was found that the commercially available MOPC 21 myeloma IgG_1 (Sigma) monoclonal antibody used a control also bound to a surface antigenic determinant expressed on Mahlavu cells. By 3h, 23.1% of it was shown to have bound to the cells with the kinetic pattern of binding roughly intermediate between that of RF-HCC1 and RF-HCC2.

Tumour cells display distinctively different profiles and structures in cell surface carbohydrates from those of non-transformed progenitor cells. This group of

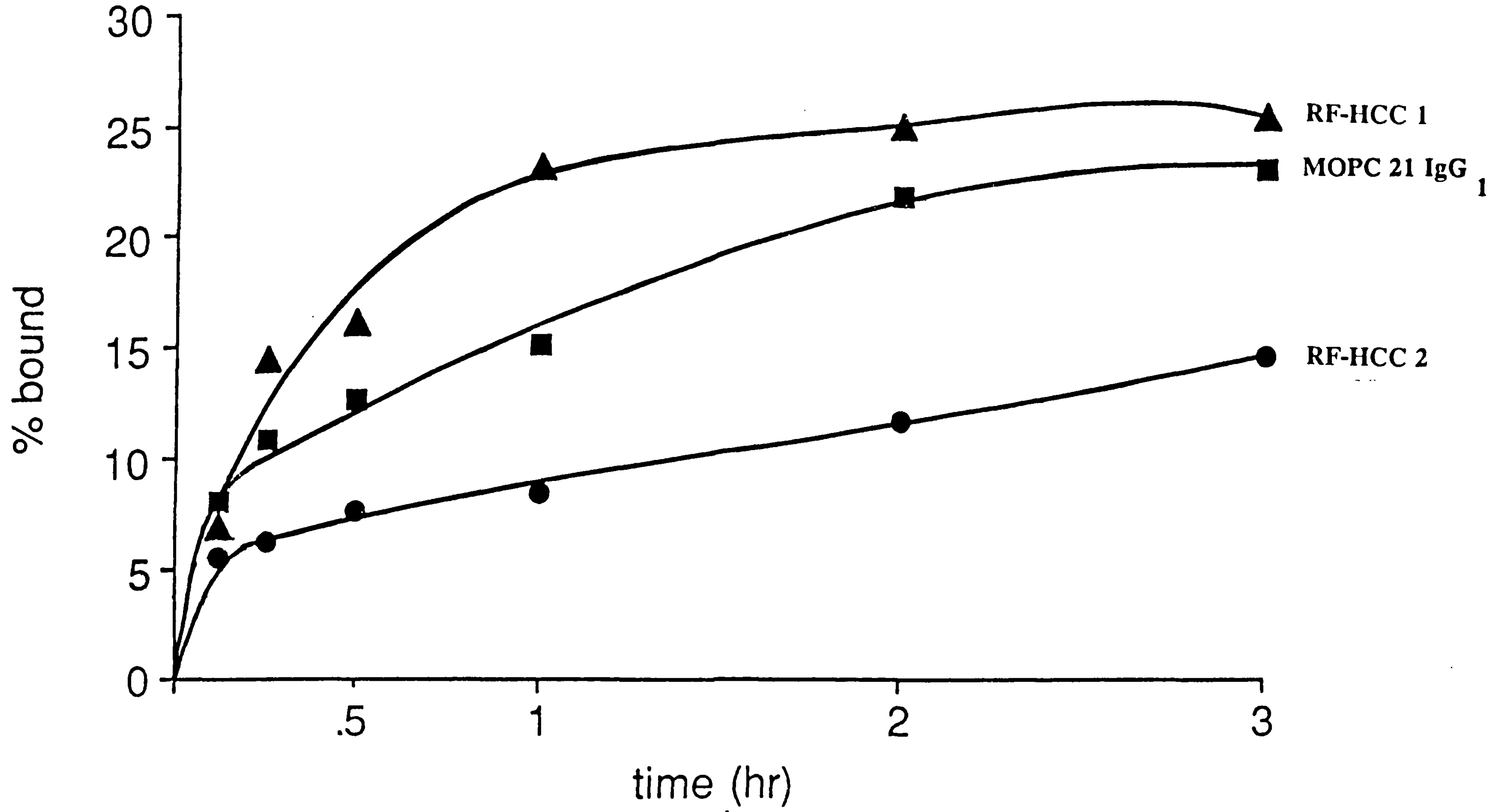


Fig. 6.1 Interaction of free monoclonal antibody with Mahlavu cells in vitro

Mahlavu cells (1.10^6) were incubated at 4°C with free monoclonal antibody (0.1 µg). At time intervals, cells were separated from unbound antibody by centrifugation through Dextran T-40. Points shown are the average of two readings from separate experiments.

carbohydrates may be subdivided into glycolipids and glycopeptides. These surface determinants are recognised by the immune system as tumour associated antigens (TAA). The first TAA described were glycolipids P and P_1 in a blood group O female patient with a gastric tumour (Hakomori, 1981). Anti-P and anti-P_1 antibodies were detected in her serum. On receiving donor blood (containing P and P_1 Ag), she reacted severely, limiting surgery to partial tumour resection. Surprisingly, after surgery, traces of the remaining tumour disappeared and the patient survived for 22 more years. TAA were originally identified by heterologous antibodies but since the breakthrough of hybridoma technology for producing monoclonal antibodies (Kohler and Milstein, 1975) which share a highly specific affinity for a single antigen, the presence of the particular antigen can be detected by attaching a labeling molecule to the monoclonal antibody.

Carlson et al. (1985) developed a panel of monoclonal antibodies by immunisation of Balb/c mice with single cell suspensions of non-trypsin treated human hepatocellular carcinoma cell lines in order to study the antigenic properties of transformed hepatocytes. Three of the antibodies designated P215457, PM4E9917, P232524 recognised separate antigenic determinants on four human hepatoma cell lines and also reacted with epitopes present on chemically-induced rat hepatoma cell lines. In contrast, only 1 of 38 other human malignant and transformed cell lines demonstrated reactivity with the three antibodies. Normal tissues were also found to be unreactive.

Markham et al. (1986) produced a rat monoclonal antibody YPC 2/38.8 after immunising rats with fresh human

colorectal carcinoma. Although the antibody was found to bind to a protein on the cell surface of normal colon and liver tissue, the protein was expressed tenfold more profusely in HCC. After labeling with ^{131}I the antibody was shown to localise HCC xenografts in immunosuppressed mice. 16 patients with HCC were given 1 mg of purified antibody labeled with 1 mCi of ^{131}I by slow i.v. injection. In 8 out of 9 patients with HCC in non-cirrhotic livers, good tumour images were obtained with a gamma camera. In 7 patients who had HCC with underlying cirrhosis, no tumour images were seen. It was concluded that the antibody produced could be useful in localising HCC occurring in livers without underlying cirrhosis.

Monoclonal antibodies directed against HB_sAg have been shown to inhibit growth of HCC xenografts in mice. In addition, these antibodies would be useful in localising HB_sAg-secreting HCC tumours. However, some HCC tumours and cell lines such as Mahlavu and SK-SF do not secrete HBsAg. As such, it would be desirable to produce monoclonal antibodies binding to most HCC tumours.

Shouval et al (1985) raised a monoclonal antibody to HCC, anti-PLC_1, after immunising mice with PLC/PRF/5 cells. It was found to bind to hepatoma associated antigens (HAA) on cell surfaces of 3 hepatoma cell lines but not 11 other tumour cell lines. These HAA were not identified in normal adult liver or foetal liver tissues and a variety of normal tissues. The amount of HAA varied in the 3 HCC cell lines.

Wiedmann et al (1983) raised a monoclonal antibody, K1, against HCC after immunising mice with PLC/PRF/5

cells. It reacted strongly with all 4 hepatomas tested and several other carcinoma tissue sections. It was unreactive with the majority of normal tissues, normal liver and most epithelia. However, it was weakly positive on luminal borders of some ductular secretory epithelia:- gall bladder epithelium, bile duct epithelium, breast acini, vascular endothelium and pancreatic acini and duct epithelium.

In this chapter, the production of monoclonal antibodies binding to three HCC cell lines and to a patient's hepatoma tissue section is described. Although the antibodies may have potential in tumour diagnosis and therapy after full characterisation, the immediate concern regarding their application within the scope of this thesis is their use as ligands for the targeting of liposomes to HCC cells in vitro.

Conclusions

Monoclonal antibodies that bound to 3 human hepatocellular carcinoma cell lines (SAH, SK-SF and Mahlavu) and snap-frozen sections of a patient's tumour were raised by immunising mice with SAH membrane preparations. The monoclonals designated RF-HCC 1 and RF-HCC 2 were of IgG1 and IgM subclasses respectively.

A simple method for the coating of liposomes with proteins

Introduction

Liposomes coated with protein or glycoprotein ligands have been used in many investigations with an aim of developing their potential for clinical applications eg. antibody/lectin-mediated targeting of drug-containing liposomes or the adjuvant effect of liposomes in enhancing the immune response against surface-associated antigens.

Weissmann et al. (1974), observed that heat agglomeration of immunoglobulins increased their membrane perturbing activity and enhanced their association with liposomes. We have investigated the possibility and have determined that the diazotization reagents $NaNO_2$ and HCl/NaCl may be used to produce controllable agglomeration, probably through intermolecular cross-linking, which greatly enhances the adsorption of treated proteins onto the surfaces of both multilamellar and unilamellar liposomes. The method described is proposed as a simple, rapid and efficient way of coating liposomes with proteins for various purposes.

Materials and methods

Preparation of liposomes

All liposomes were prepared from equimolar amounts of 32 µmoles egg PC and Chol as described on pages 58-60.

Coupling of protein to liposomes

2 ml $NaNO_2$ (molar concentration indicated in the legends to the figs.) were added to MLV or SUV composed of 16 μmoles PC:16 μmoles Chol, followed by 0.5 ml of a solution of the protein to be bound. Immediately after shaking, 2 ml equimolar (to $NaNO_2$) HCl/NaCl was added and the mixture left overnight at 4^oC. To determine the rate of protein association with the liposomes, aliquots of the mixture were taken at time intervals, and liposomes separated from unbound radiolabeled protein by centrifugation at 10,000xg for MLV and by elution through a Sepharose CL-4B column in the case of SUV. Radioactivity in the liposomal pellets or eluted fractions was measured to determine the amount of protein bound.

Results and discussion

Effect of treatment with diazotization reagents on the binding of proteins to MLV

Incubation of proteins with equimolar $NaNO_2$ and HCl/NaCl in the presence of MLV liposomes causes a high degree of binding of the protein to the liposomes. Using various concentrations of pig gammaglobulins, it is seen that even with a low molarity of diazotization reagents used (0.1M of both $NaNO_2$ and HCl/NaCl), the amount of binding for any concentration of pig gammaglobulins used is about ten times that achieved when either of the diazotization reagents is present without the other (Fig. 7.1). Thus, using a concentration of pig gammaglobulins of 10 mg/ml, 112.5μg of protein/umole of egg PC are bound when equimolar 0.1M

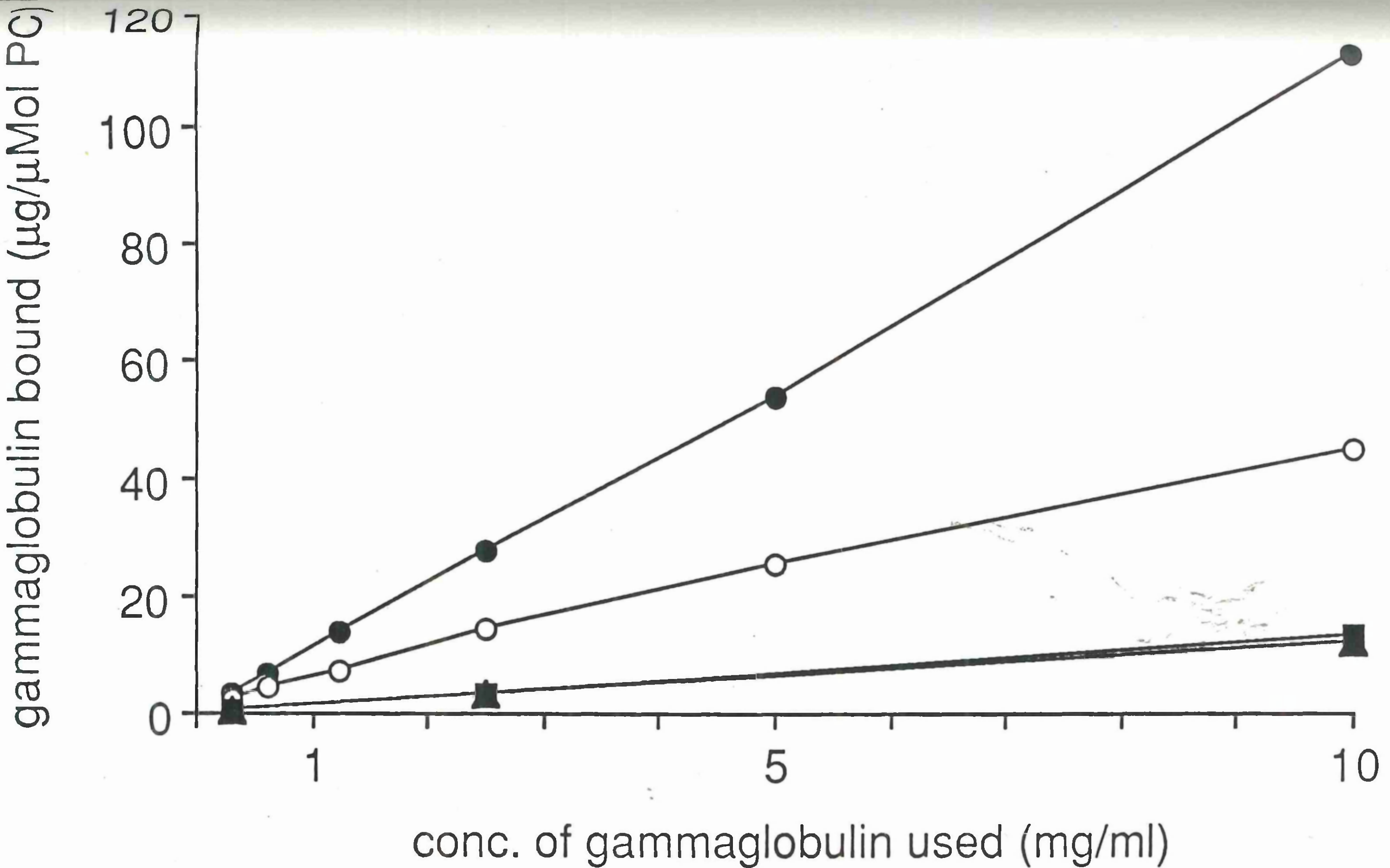


Fig. 7.1 Effect of diazotization reagents on the binding of pig gammaglobulins to MLV

To MLV composed of 16 µmol PC:16 µmol Chol (in 1 ml PBS) were added 2 ml cold $NaNO_2$ ('●' 0.1M,'O' 0.01M) and 0.5 ml pig gammaglobulins followed by 2 ml equimolar (to $NaNO_2$) HCl/NaCl. For controls, 2 ml PBS was used in the place of one or the other diazotization reagent. (■ 0.1M HCl/NaCl alone; ▲ 0.1M $NaNO_2$ alone).

$NaNO_2$ and HCl/NaCl act in concert, as compared to 12.5μg/umole PC bound when each reagent acts alone. This latter small amount of binding represents 2% of the total amount of protein added and is attributed to non-specific adsorption of the pig gammaglobulins to the surface of MLV. Even when an extremely low concentration of diazotization reagents is used (0.01M $NaNO_2$ and HCl/NaCl), a considerable amount of protein, about four times that adhering non-specifically, is bound. When the incubation mixture of diazotization reagents, protein and liposomes is assayed at time intervals by centrifugation of the liposomes to separate them from the reagents in the supernatant, it is seen that the binding reaction is a biphasic one which is more or less complete within four hours (Figs. 7.2). It was observed that the reaction took place almost as rapidly at $4^{o}C$ as at room temperature.

When liposomes coated with pig gammaglobulins by the action of diazotization reagents were further washed three times in PBS adjusted to graded pH values, it was observed that a low pH environment favours the retention of originally bound protein to the surface of MLV, with more than 90% of protein retained when washed in pH 3.5 buffer (Table 7.1). At physiological pH, more than three quarters is retained whilst at a high pH of 10.5, more than 50% becomes dissociated by vigorous vortex resuspension of the liposomes after each centrifugation. Therefore, it is presumed that a large component of the bond between protein and liposomes is electrostatic in nature.

Other proteins that have been bound to MLV using $NaNO_2$ and HCl/NaCl are bovine serum albumin (BSA) and teta-

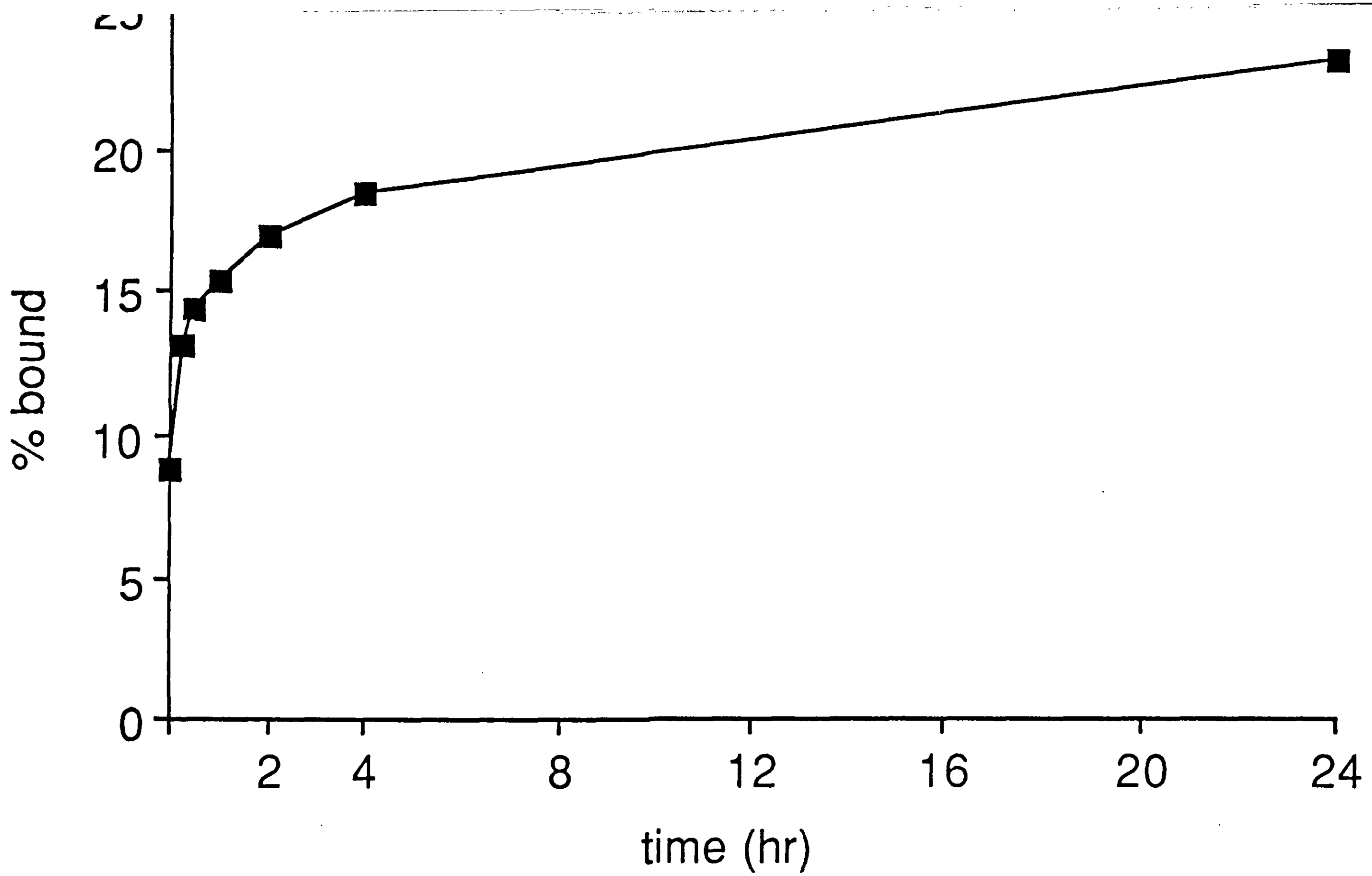


Fig. 7.2 Rate of binding of pig gammaglobulins to MLV in the presence of diazotization reagents

To MLV composed of 16 µmol PC:16 µmol Chol (in 1 ml PBS) were added 2 ml cold 0.1M $NaNO_2$ and 0.5 ml pig gammaglobulins (5 mg/ml) followed by 2 ml 0.1M HCl/NaCl. MLV associated with protein were separated from unbound protein and reagents by centrifugation at time intervals.

pH of washing buffer	% pig gammaglobulin retained on MLV
3.5	91.4
5.0	81.5
7.4	78.2
9.0	75.4
10.5	48.5

Table 7.1 Effect of pH on protein retention on MLV

MLV with pig gammaglobulins surface-linked by $NaNO_2$ and HCl/NaCl treatment were washed by centrifugation at 10,000xg three times in PBS adjusted to various pH values. The percentage of the original amount of protein bound remaining in the pellet after washing is expressed.

nus toxoid (results not shown). The percentage binding values and rates of reaction for these proteins are similar to those obtained for pig gammaglobulins.

Effect of diazotization reagents on the binding of proteins to SUV

The diazotization reagents were used to investigate their ability to bind the proteins BSA and pig gammaglobulins to the surface of SUV in a similar manner to their effect on protein binding to MLV. It was observed that untreated BSA had a low affinity for SUV, with 2.6% non-specifically adsorbed (Table 7.2). However, in the presence of equimolar $NaNO_2$ and HCl/NaCl, binding values rose to greater than tenfold (30.2%) for 1.0M and about eight times (20.3%) for 0.5M diazotization reagent concentration used. 1M $NaNO_2$ acting alone has no effect on increasing non-specific binding values but 1M HCl/NaCl roughly doubles this value.

Pre-treatment of BSA by incubation with equimolar $NaNO_2$ and HCl/NaCl in the absence of liposomes, followed by a subsequent overnight incubation in the presence of SUV led to higher binding values being observed. Thus, even when a relatively low molarity of 0.25M diazotization reagents is used, a binding value of 43.2% is obtained. This appears to be higher than when a four times higher concentration (1.0M) of diazotization reagents is incubated with protein in the presence of SUV. This is probably due to the small amount of ^{125}I liberated from the labeled BSA by high concentrations of HCl.

The rate of reaction of BSA bound to SUV by treat-

BSA treatment	% binding to SUV
Untreated	2.6 + 0.2 (2)
1M HCl/NaCl	5.3
1M $NaNO_2$	2.6
1M ($NaNO_2$ + HCl/NaCl)	30.2
0.5M ($NaNO_2$ + HCl/NaCl)	20.3
*1M ($NaNO_2$ + HCl/NaCl)	42.8
*0.5M ($NaNO_2$ + HCl/NaCl)	48.3
*0.25M($NaNO_2$ + HCl/NaCl)	43.2

Table 7.2 Effect of treatment of BSA with diazotization reagents on binding to SUV

To SUV composed of 16 umol PC:16 umol Chol (in 1 ml PBS) were added 2 ml cold $NaNO_2$ and 0.5 ml BSA (5 mg/ml) followed by 2 ml equimolar HCL/NaCl. SUV associated with protein were separated from unbound protein and reagents after overnight incubation at 4°C by elution through a Sepharose CL-4B column. (*) indicates that BSA was pre-incubated overnight with diazotization reagents before being added to SUV for an additional overnight incubation period.

Pig gammaglobulin treatment	% binding to SUV
Untreated	17.7
1M $NaNO_2$	17.7
1M HCl/NaCl	45.4
0.5M HCl/NaCl	42.2
0.25M HCl/NaCl	40.7
1M ($NaNO_2$ + HCl/NaCl)	59.6
0.5M ($NaNO_2$ + HCl/NaCl)	57.4
0.25M ($NaNO_2$ + HCl/NaCl)	53.8

Table 7.3 Effect of treatment of pig gammaglobulins with diazotization reagents on binding to SUV

To SUV composed of 16 umol PC:16 umol Chol (in 1 ml PBS) were added 2 ml cold $NaNO_2$ and 0.5 ml pig gammaglobulins (5 mg/ml) followed by 2 ml equimolar HCL/NaCl. SUV associated with protein were separated from unbound protein and reagents after overnight incubation at 4°C by elution through a Sepharose CL-4B column.

ment with diazotization reagents was similar to that seen when pig gammaglobulins were linked to MLV (Fig.7.3). The reaction is also biphasic in character with most of the binding having taken place within 4h at room temperature.

Pig gammaglobulins have an intrinsically high affinity for the surface of SUV with 17.7% untreated protein passively adsorbed on overnight incubation. 1M $NaNO_2$ alone has no effect in enhancing non-specific adsorption whereas HCl/NaCl acting alone has a relatively greater effect in enhancing binding values of pig gammaglobulins to SUV compared to its effect on BSA (Table 7.3). However, as is the case with BSA, both diazotization reagents acting together, synergistically boost binding values (to 59.6% for 1.0M $NaNO_2$ and HCl/NaCl). This represents about a three-fold increase over the non-specific adsorption value for pig gammaglobulins in contrast to the greater than ten-fold increase observed for BSA. Therefore, the diazotization reagents have a relatively greater effect in enhancing binding values of proteins with a low intrinsic affinity (eg. BSA) than for those with a higher affinity (eg. pig gammaglobulins) for SUV.

Storage at 4°C for 2wks does not have any appreciable effect on pig gammaglobulin linked to SUV by $NaNO_2$ and HCl/NaCl treatment with more than 90% of the original protein bound being retained, compared to 68.3% of passively adsorbed gammaglobulins still remaining associated with SUV (Fig. 7.4).

Proteins have been bound directly to the surface of liposomes by a variety of methods (Gregoriadis, 1984; Wolff and Gregoriadis,1984). Several investigators have used

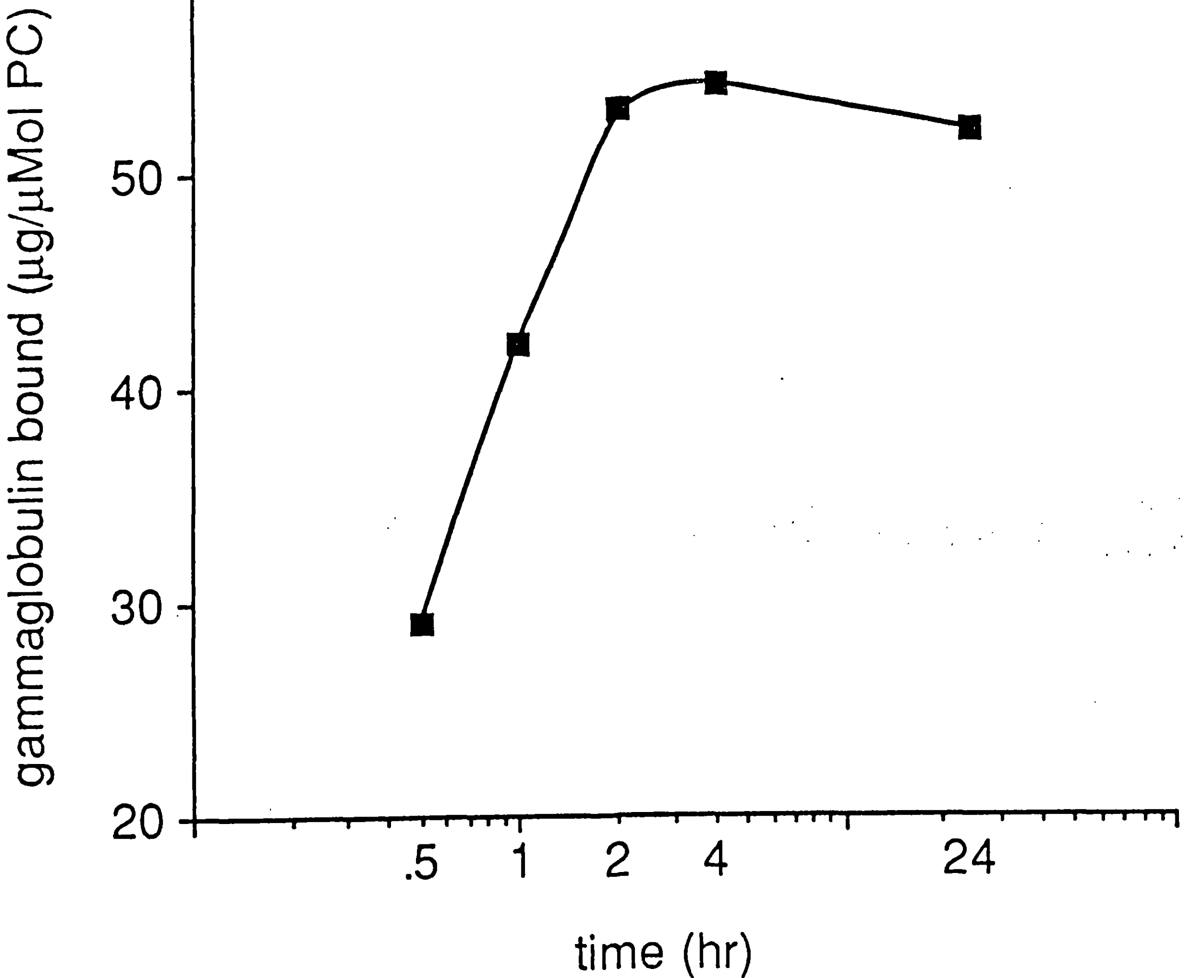
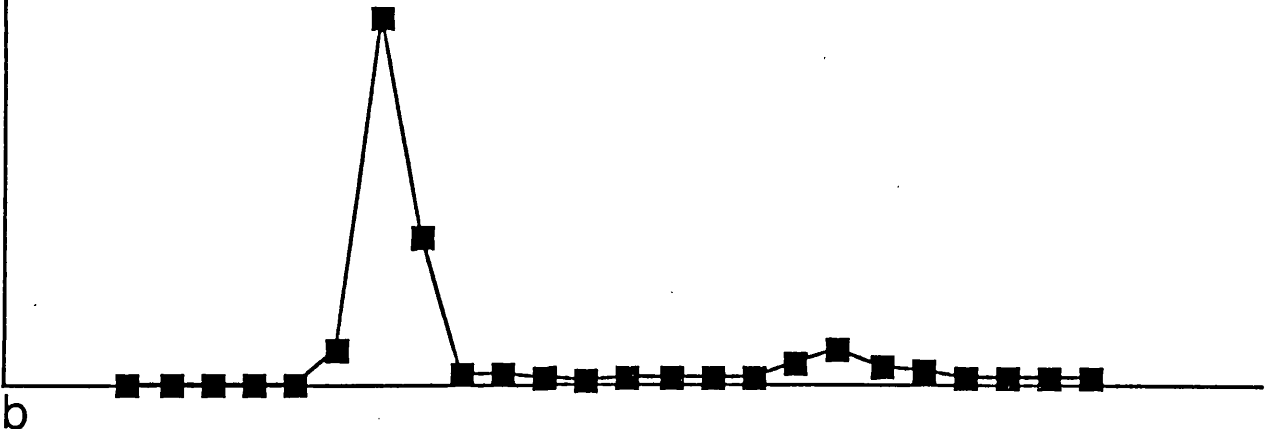


Fig. 7.3 Rate of binding of BSA to SUV in the presence of diazotization reagents

To SUV composed of 16 umol PC:16 umol Chol (in 1 ml PBS) were added 2 ml cold 0.1M $NaNO_2$ and 0.5 ml BSA (5 mg/ml) followed by 2 ml 0.1M HCL/NaCl. SUV associated with protein were separated from unbound protein and reagents by elution at time intervals through a Sepharose CL-4B column.



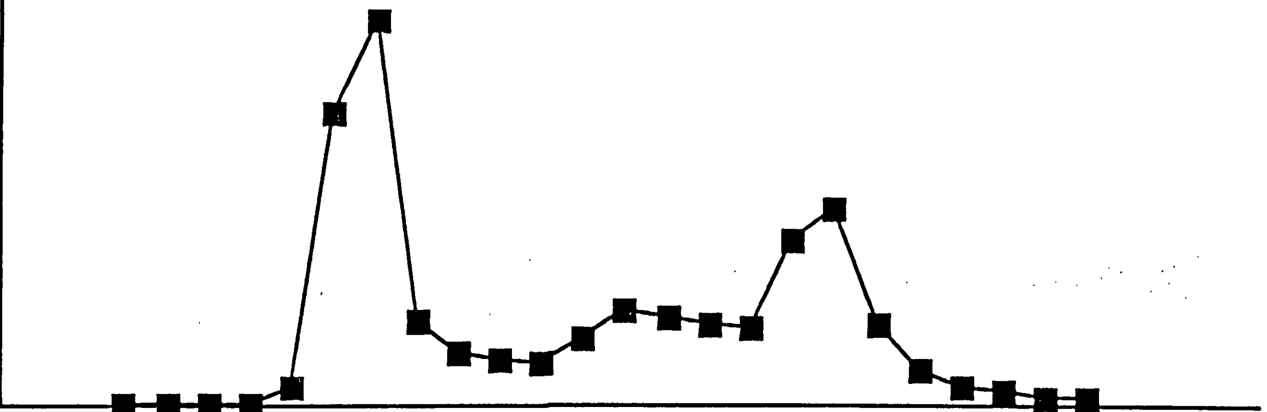


Fig. 7.4 Effect of storage for 2 weeks at 4°C on the retention of pig gammaglobulins on the surface of SUV

SUV coated with pig gammaglobulins by a) treatment with diazotization reagents and b) passive adsorption were stored for 2 weeks at 4°C. Liposomes were then eluted through a Sepharose CL-4B column to determine the amount of radiolabeled protein that had dissociated.

crosslinking reagents such as glutaraldehyde, carbodiimide or suberimidate. A second approach is based of Schiff's base formation between proteins and periodate oxidised vesicles made with glycolipids. More recently, proteins have been bound to liposomes using the heterobifunctional reagent N-succinimidyl-3-(2-pyridyldithio)-proprionate (SPDP) (Barbet et al., 1981; Martin et al., 1981) or by including the sulfhydryl-reactive phospholipid deritive N-(4-(p-maleimido-phenyl)butyryl)phosphatidylethanolamine (MPB-PE) in the membrane surface (Hashimoto et al., 1984). While large amounts of protein (Fab' fragments) have been bound to large unilamellar liposomes using the SPDP or MPB-PE methods both suffer from the limitation that proteins not bearing free sulfhydryl groups must be thiolated before they can be coupled to SPDP or MPB-PE modified liposomes. Although apparently not as efficient as the SPDP or MPB-PE techniques, a method reported by Snyder and Vannier (1984) using diazotization reagents was as efficient as those employing crosslinking reagents without the apparent inherent disadvantage of producing homopolymers.

In the course of recent work with SUV coated with antibodies through the SPDP reaction, it was observed (Senior et al., 1986) that high `coupling' values were obtained in control experiments in which SPDP-modified or non-modified liposomes were incubated with intact IgG. It was suggested that that coupling values obtained by the use of coupling methods entailing lengthy incubation periods may be due to some extent to passive adsorption of the immunoglobulin onto liposomes. Moreover, chemical modification of the protein to be coupled may enhance their intrinsic affin-

ity for the surfaces of liposomes as was aptly demonstrated in this chapter in the case of diazotization reagents. Therefore, one must bear in mind the possibility of this occurrence before proclaiming that all protein molecules associated with liposomes by a covalent coupling procedure are actually covalently bound, as the strength of hydrophobic and other interactions enhanced by chemical modification may be strong enough to enable the protein to be associated with liposomes for prolonged periods of time.

Conclusions

A simple method for the coating of liposomes with proteins was developed. Many proteins (eg. BSA, pig gamma-globulins and tetanus toxoid) could be made to adhere rapidly and efficiently to both MLV and SUV after treatment with $NaNO_2$ and HCl/NaCl. The reaction is complete within four hours and almost all of the protein is retained on the liposomes after storage for 2 weeks at $4^{o}C$.

Antibody-mediated targeting of liposomes to human hepatocellular carcinoma cells - in vitro studies

Introduction

Various studies have demonstrated the feasibility of targeting liposomes via covalently-coupled antibodies to cells in vitro (Leserman et al., 1984; Wolff and Gregoriadis, 1984). A caveat was sounded by Senior et al. (1986) who discovered that extensive immunoglobulin adsorption (34-89%) onto the surface of small unilamellar vesicles (SUV) may lead to misinterpretation of covalent linkage values and that adsorbed antibodies may provide an alternative to preparing liposomes for use in in vitro studies. In this chapter, the adsorption of monoclonal antibodies raised against hepatocellular carcinoma cell lines, onto the surface of SUV and their subsequent use in targeting to these cells in vitro is studied.

Materials and methods

Preparation of antibody-coated SUV liposomes

SUV were prepared from 32 umoles equimolar egg PC and Chol as described on page 60. After ultracentrifugation to remove multilamellar structures and titanium fragments, the supernatant was incubated with 0.5 ml PBS containing a total of 1.0 mg radiolabeled monoclonal antibody and incubated overnight at $4^{o}C$. The incubation mixture was passed

through a Sepharose CL-4B column and SUV obtained at the end of the void volume were collected.

Incubation of liposomes with cells

Mahlavu cells were maintained in plastic culture flasks (Nunclon) in RPMI 1640 medium containing 10% fetal calf serum, 100 units penicillin/ml and 100 μg streptomycin/ml. The cells were kept at 37°C in a humid atmosphere of 5% CO_2 in air and subcultured by transferring small amounts of cell suspension into fresh flasks with medium. For in vitro experiments, cells were spun down at 1000 rpm for 7 min in a bench centrifuge, resuspended in phosphate-buffered saline supplemented with 5 mg/ml bovine serum albumin and 0.05% (w/v) sodium azide (incubation buffer) and counted in a haemocytometer. Known numbers of cells suspended in 50 ul incubation buffer were placed in triplicate into plastic test tubes and unless otherwise stated, incubated with 50 ul IgG_1-coated liposomes at 4°C for one and a half hours. To separate cells from unbound material, incubation mixtures were pipetted onto 1.5 ml 10% Dextran T-40 in incubation buffer and centrifuged at 1200 rpm (400g) for 25 min. After removal of the Dextran layer, cell pellets were washed once with phosphate-buffered saline at 4°C, centrifuged at 2000 rpm (1000g) for 10 min and counted for ^{125}I radioactivity. To measure carboxyfluorescein associated with the cells, these were dissolved in 0.5 ml 5% Triton X-100 in phosphate-buffered saline, if necessary with the help of a bath sonicator (Kerry). After the addition of 2.5 ml PBS, cellular debris were spun down at 3000 rpm (2500g) for 14 min, and CF in the supernatant was meas-

ured.

Results and discussion

In accordance with results obtained by Senior et al. (1986) who observed 52.0% adsorption of mouse immunoglobulins onto PC SUV after passive incubation for 24 hours, it was found that mouse monoclonal antibodies raised against hepatocellular carcinoma also had a high intrinsic affinity for the surface of SUV. RF-HCC1 of IgG_1 subclass displayed a non-specific adsorption value of 35.1% on overnight incubation at $4^{o}C$ with SUV (Fig. 8.1). However, another mouse monoclonal IgG_1 derived from the MOPC 21 myeloma cell line exhibited a `binding' value of only 12.2% despite being from the same subclass . This suggests that the F_c portions may not be the only moieties of the immunoglobulins intercalated between the molecules of the bimolecular leaflet because if this were the case, all antibodies of identical isotype would exhibit the same adsorption values. Thus, it is assumed that there is considerable interaction between the F_{ab} regions, either one arm or both, with the surface of SUV. Monoclonal antibodies of similar isotype but possessing different epitopic specificities would then interact differentially with the liposomal bilayer, depending upon the molecular configuration of their paratopes.

This supposition is further supported by the finding that RF-HCC2, of IgM subclass, gave an adsorption value of 51.6%. This is relatively high compared to the values obtained with IgG_1 antibodies and may be attributed to the fact that IgM is a pentameric molecule with a crab-like configuration, the area of possible interaction between

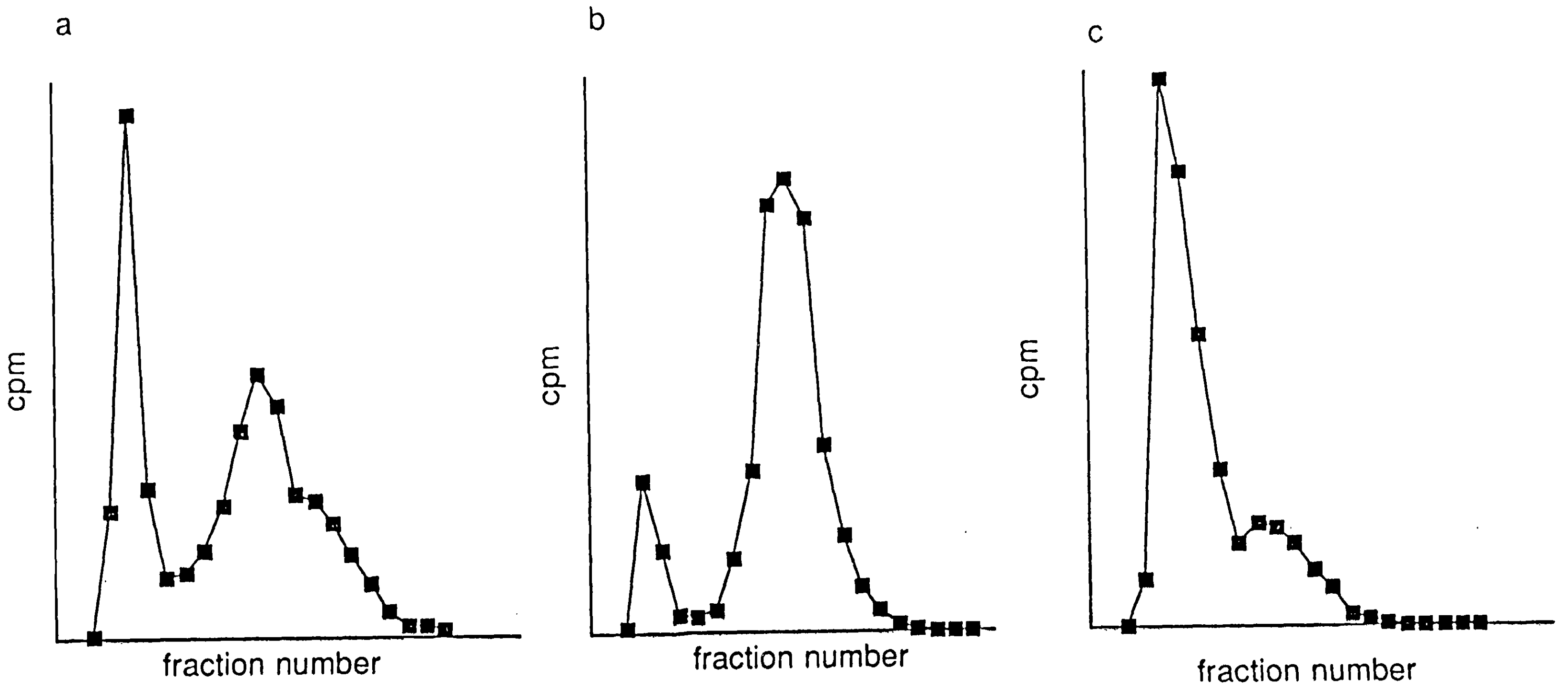


Fig. 8.1 Elution profiles of ^{125}I-labelled monoclonal antibody adsorbed to SUV by passive incubation. a) RF-HCC1; 35.1% bound. b) MOPC 21 IgG_1; 12.2% bound. c) RF-HCC2; 51.6% bound.

the F_{ab} portions and the external liposomal surface being correspondingly greater.

In spite of the fact that some antibody molecules may have their antigen-binding sites interacting non-specifically with the surface of SUV and therefore being unavailable for specific binding to their target sites, it is presumable that those antibodies intercalated in the bimolecular leaflets via their F_c portions or with one F_{ab} arm in the bilayer and the other protruding out into the surrounding medium will still have available paratopes at their disposal for binding to their specific epitopes. These would be sufficient for targeting of the liposomes to which they are attached to the cells expressing the specific antigens on their surfaces.

<u>Interaction of liposome-adsorbed monoclonal antibodies with Mahlavu cells</u>

To investigate the feasibility of using surface epitope-binding monoclonal antibodies passively adsorbed to SUV as cell-specific ligands to target liposomes to cells, CF-containing SUV coated with ^{125}I-labelled RF-HCC1 (IgG_1 isotype) and MOPC 21 IgG_1 were incubated with 10^6 Mahlavu cells at 4^oC (to prevent non-specific endocytosis of the liposomes). Liposomes associated with the HCC cells were separated from free, unbound liposomes by centrifugation through a layer of Dextran T-40 at time intervals.

Results (Fig. 8.2) show that the kinetics of binding of both monoclonal antibodies and CF-containing SUV took place in a biphasic fashion akin to results obtained with incubation of Mahlavu cells with monoclonal antibody

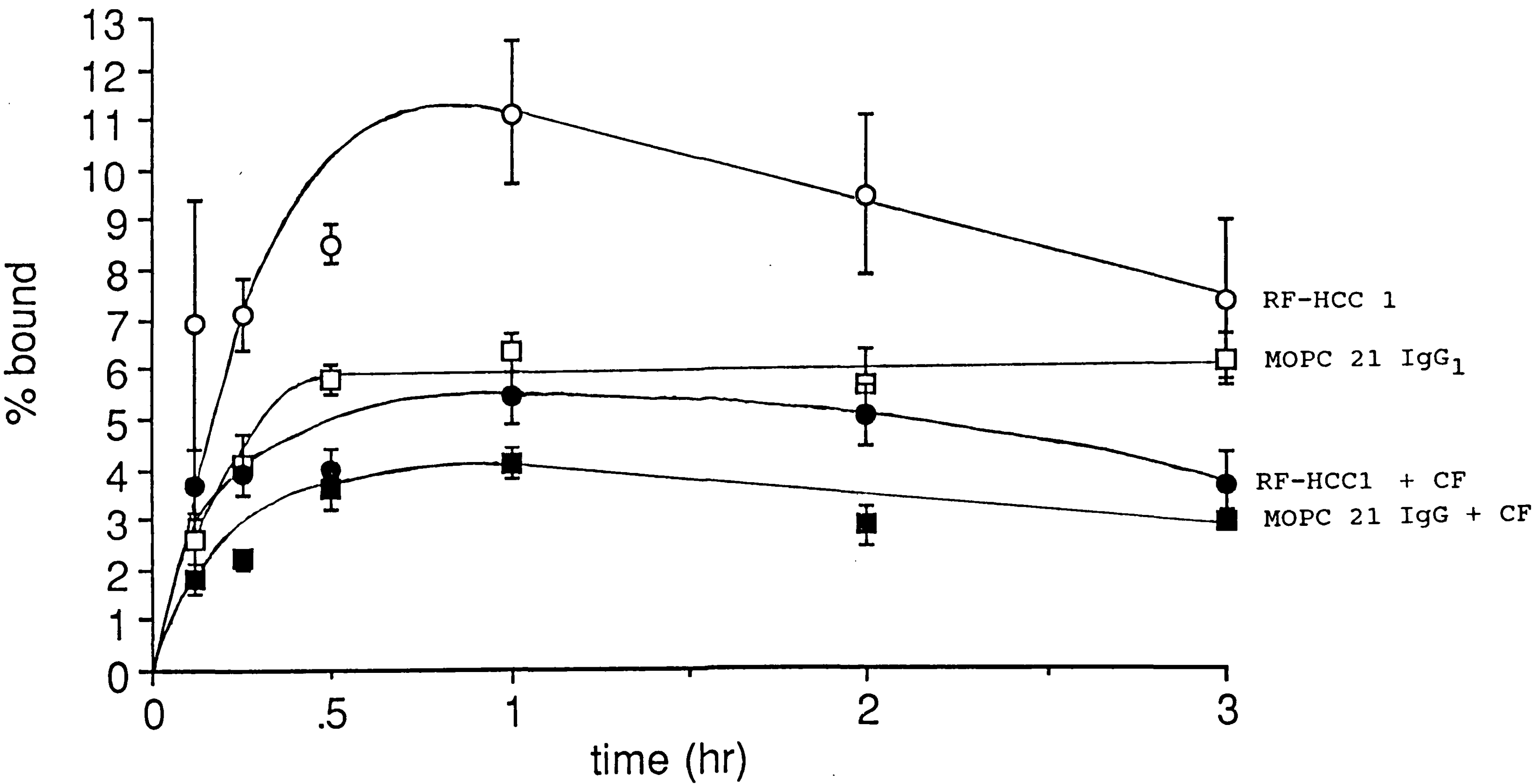


Fig. 8.2 Interaction of liposome-associated monoclonal antibody with Mahlavu cells

Mahlavu cells (1.10^{6}) were incubated at $4^{o}C$ for one and a half hours with CF-containing SUV coated with passively adsorbed RF-HCC1 and MOPC 21 IgG_1. At time intervals, cells were separated from unbound antibody by centrifugation through Dextran T-40. The amounts of antibody and CF associated with the cells was determined by measuring ^{125}I radioactivity and fluorescence respectively.

alone (Chap.6, Fig.6.1). However, an important distinction between the binding kinetics of liposome associated antibody and free antibody is that in the case of the former, maximal binding values are seen at 1h for both RF-HCC1 ($11.1\pm1.5\%$) and MOPC 21 IgG_1 ($6.3\pm0.4\%$) after which, in contrast to free monoclonal antibodies, binding values of radiolabeled RF-HCC1 adsorbed onto SUV are seen to decline after 1h to reach $7.3\pm1.8\%$ at 3h. The percentage of liposomal MOPC 21 IgG_1 bound to Mahlavu cells remains fairly constant as evidenced by the flatness of the plateau in fig. 8.2, unlike the gradual rise seen with free antibody. The difference in binding behaviour may be due to increased sloughing off of the surface determinant to which RF-HCC1 binds when it is associated with liposomes compared to when it is present in the free form although the reason why this should occur is not clear. The presence of antibodies on `fluid' PC SUV may cause capping of the epitope on the Mahlavu cell surface thus facilitating its exocytosis.

As an indication of the percentage of liposomes bound to Mahlavu cells at various time intervals, CF entrapped in the aqueous cavity of SUV was used as a marker. Results show that as with the monoclonal antibodies, maximal binding values were also observed at 1h ($5.4\pm0.5\%$) for liposomes coated with RF-HCC1 and $4.1\pm0.2\%$ for SUV coated with MOPC 21 IgG_1. Binding values are also seen to decline after this period in tandem with the antibodies. There were discrepancies seen between the binding values of ^{125}I-labelled monoclonal antibodies and liposomal binding as assayed by CF fluorescence. A possible explanation for this phenomenon could be that after liposomes have come into

close proximity to the Mahlavu cell surface mediated by the binding of their adsorbed antibodies to cell surface antigens, the PC SUV become `leaky' either through membrane-membrane interaction or capping of antibodies at one pole of the liposomes, thus liberating CF into the surrounding incubation buffer. Evidence for cell-surface induced leakage of liposomal contents has been reported. (Renswoude and Hoekstra, 1981). Another reason could be that after the adsorbed monoclonal antibodies have bound to their antigens, they become detached from the liposomal surface. However, in view of the fact that the patterns of binding of both antibody and CF-containing SUV are very similar, the former postulation would hold more weight. Regardless of whether antibodies become detached from liposomes or not after binding to cells, results indicate that these antibodies are capable of targeting liposomes to Mahlavu cells.

The quantitative uptake of intravenously injected liposomes by the reticuloendothelial system has prompted authors interested in delivering drugs to alternative tissues to develop means of endowing liposomes with target selectivity. This was originally achieved through liposome-bound cytophilic ligands which, in recent years, have included antibodies, glycoproteins, glycolipids and amino sugars. However, because of their great target versatility, antibodies have been the ligand of choice in most of the related work. The advent of hybridoma technology has added further impetus in liposome targeting, which could, under certain conditions, have some advantages over the use of antibodies coupled to drugs directly. For instance, liposomes can incorporate large quantities of a wide range of

drugs, keep them in isolation from the biological milieu and bring them into contact with cells, potentially by a single Ig molecule per vesicle. Also, with liposomes bearing antibodies against more than one type of antigenic determinant, there may be a firmer and more specific binding to cells expressing the determinants.

In the course of recent work with SUV coated with antibodies through the SPDP reaction, it was observed that high `coupling' values were obtained in control experiments in which SPDP-modified or non-modified liposomes were incubated with intact IgG. It was suggested that that coupling values obtained by the use of coupling methods entailing lengthy incubation periods may be due to some extent to passive adsorption of the immunoglobulin onto liposomes. Passively adsorbed antibodies are retained by liposomes upon prolonged exposure to blood plasma and are capable of binding to respective antigens. This simple and mild procedure of liposome coating with antibodies may be preferable to techniques in which modification of immunoglobulins with coupling reagents promotes rapid clearance of the injected liposome-immunoglobulin complex from the circulation. In this chapter, it has been shown that liposomes coated with passively-adsorbed antibodies are able to deliver a considerable amount of their encapsulated load to target cells.

Conclusions

CF-containing SUV coated with passively-adsorbed monoclonal antibody (RF-HCC1 and MOPC 21 IgG_1) were used in in vitro studies for targeting to Mahlavu cells. Significantly greater amounts of the marker entrapped in antibody-

coated SUV were associated with the target cells after incubation for one and a half hours at $4^{o}C$ as compared to plain SUV. These results demonstrate that passively-adsorbed antibodies represent a rapid, convenient and effective method of ligand-mediated targeting of SUV to cells in vitro. The avoidance of harsh conditions (eg. high acidity) which may adversely affect the ligand or encapsulated substance is an added advantage.

General discussion and conclusions

Intense research on the field of liposomes as drug carriers, since the proposal of the concept by Gregoriadis and Ryman in 1972, has led to the expansion of most conceivable aspects of applied liposomology into their present state of development and understanding. Among the more promising avenues for eventual clinical application are the exploitation of liposomes as highly manipulable carriers and adjuvants of immunogens for their use as vaccines, and as antibody-mediated carriers of cytotoxic drugs for targeted chemotherapy.

The initial problems encountered with liposome encapsulation of substances like low entrapment values, poor reproducibility, subjecting the material to potentially damaging conditions, short period of stability and difficulty in scaling-up for industrial use have largely been overcome by imaginative procedures designed to circumvent these problems and improve on existing methodologies. Among the more noteworthy of recent developments is the dehydration-rehydration vesicle (DRV) method which has been exploited in the research done for this thesis to encapsulate a wide variety of substances, namely, reconstituted influenza virus envelopes (RIVE), micellar particles of three strains of influenza haemagglutinin molecules, polio peptides, tetanus toxoid and interleukin-2. In this respect, it has proven to be eminently suitable, yielding high entrapment values with good reproducibility, even for the relatively large (80-120nm) particles of RIVE. The bilayer composition of DRV has also proven to be amenable to manipulation with phospholip-

ids of varying acyl chain lengths and transition temperatures, packing molecules of cholesterol and charged lipids like BisHOP to enhance the adjuvanticity of this particular species of liposome.

Using the versatile dehydration-rehydration method to encapsulate a range of viral and bacterial substances, it was adequately demonstrated in this thesis that liposomes can function as potential carriers and adjuvants in a new generation of non-toxic vaccines. The entrapment of RIVE in DRV (chap. 2) was shown to markedly enhance both the primary and secondary immune responses to RIVE in all IgG subclasses.

Controversy over whether surface-linked or encapsulated antigens are the more effective in eliciting an immune response was attempted to be resolved using a membrane-intercalated hydrophobic chain anchor, to link molecules of two polio virus subunit peptides W1 and W2 to the bilayer membranes of DRV. As expected, free peptide generated only a weak primary response and no detectable secondary response whereas both covalently surface-linked W1 and W2 elicited high primary responses, more markedly so than those of encapsulated peptides. Although primary responses to liposome-entrapped peptides were weaker than those against surface-linked peptides, for liposome-encapsulated W1, a strong secondary response was observed. These findings show that the association of weakly immunogenic peptides with liposomes results in an enhancement of their antigenicity, implying that liposomes can act both as carriers and vehicles of hapten molecules. Moreover, the nature of association of the peptides plays an important part in determining

whether the primary or secondary response is stimulated and to what extent. This presents a further factor which may be manipulated to tailor the immune response to an antigen and it is foreseeable that both covalently surface-linking and entrapping an antigen at the same time would augment the immune response greatly and rapidly, whilst at the same time priming the recipient for a good secondary response.

To further capitalise on the inherent manipulability of liposomes, subsequent chapters dealt with the incorporation of a biological response modifier, charged lipid constituents and mode of administration in an effort to see if the basic adjuvanticity of the liposome-antigen association could be enhanced. Following reports (Anderson et al., 1987; Weinberg and Merigan, 1988) that free interleukin-2 administered together with various antigens could function as an adjuvant for both humoral and cell-mediated immunity, recombinant IL-2 which is presently available with high purity and in large quantities commercially was incorporated into liposomes. Results were interesting and showed that high doses of IL-2 could enhance the primary IgG_1 response to tetanus toxoid co-entrapped with the IL-2 but that the effect was a transient one, rather like an exaggeration of the primary response to the free antigen. Surprisingly, low doses of IL-2 separately entrapped in liposomes suppressed the primary response to liposomal toxoid. In the secondary response, high doses of liposomal IL-2 were especially effective in boosting IgG_{2a} and IgG_{2b} responses whilst tenfold lower doses, especially in separate liposomes, depressed antibody levels in most IgG subclasses. The mechanism by which liposomal IL-2 may enhance or decrease the

immune response against a co-administered antigen is not evident at present but clearly the empirical effects observed augur well for their future use with liposomes in modulating the immune response to achieve a desired goal.

A novel synthetic lipid, BisHOP, which carries a positive charge was tested by incorporating it into liposomes to see if it could increase liposomal adjuvanticity. Results showed that formulating DRV liposomes to include a 20% molar ratio of BisHOP to phospholipid caused a marked and substantial enhancement of the primary IgG response to liposomal tetanus toxoid. An important concomitant finding was that DSPC DRV exhibited more adjuvanticity than PC DRV during the primary response but that the adjuvanticity of PC DRV was most effectively expressed after a booster injection, catching up with and surpassing DSPC DRV during the secondary response for most antibody subclasses tested. The adjuvant effect of BisHOP, similar to that of IL-2 was most evident in the IgG_{2a} and IgG_{2b} subclasses for the secondary response. It is not known for certain why IL-2 and BisHOP both do not further potentiate the IgG_1 secondary response but it may be that the adjuvanticity afforded by the phospholipids in the liposomal structure are sufficient to cause near maximal increases in IgG_1 response and that further attempts to raise this ceiling are of no avail. It would thus appear that liposomes, especially incorporating IL-2 and BisHOP, selectively boost IgG_{2a} and IgG_{2b} responses, the former of which has been shown to be the major isotype involved in host cytoprotection against viral infection. It seems that liposomes share this property with other new-generation adjuvants such as Syntex Adjuvant Formulation 1

(Allison and Byars, 1986).

The properties of the synthetic lipid, BisHOP were studied in another setting different from but related to that of liposomes administered into the tissues to elicit an enhanced antibody response. This was the no less important situation of intravenous administration of SUV for various purposes such as targeted or slow-release drug therapy. An enlightening discovery was that positive as well as negatively-charged constituents incorporated into the bilayer of DSPC SUV caused the latter to be initially cleared from the circulation at similar rates, and more rapidly than control neutral SUV. This implies that charged liposomes, irrespective of being positive or negative are cleared at the same rates and more rapidly than neutral ones, the effect being much more evident with neutral DSPC SUV which exhibit long circulatory half-lives and whose `solid' bilayers hinder removal of the charged lipid constituents. Measurement of microelectrophoretic mobility values of charged MLV in vitro did not reveal direct correlations between the behaviour of charged liposomes in the latter milieu and that of charged SUV in the in vivo circulation because of the markedly different characteristics of the two types of liposome. However, a strong influence of pH on the electrophoretic movement of MLV composed of charged constituents and phospholipids of widely differing T_c was noted.

The possibility of using liposomes as carriers of cytotoxic drugs for targeted delivery to cells was investigated by first raising monoclonal antibodies to surface antigens expressed on hepatocellular carcinoma cell lines. A novel approach employing membrane preparations of PLC/PRF/5

cells grown in tissue culture as the immunising formulation instead of whole cells was found to be more successful in our hands for this purpose. Two monoclonal antibodies designated RF-HCC1 and RF-HCC2 of IgG_1 and IgM isotypes respectively were characterised with respect to their binding patterns to snap-frozen sections of PLC/PRF/5 tumour xenografts grown in nude mice, patients' hepatomas and normal liver tissue as detected by immunofluorescence and radioimmunoassay. It was found that both monoclonals bound to surface determinants expressed on three HCC cell lines and one patient's tumour but very little or not at all to normal liver tissue.

A simple and rapid method was devised to coat large and small liposomes with proteins. Treating antibodies as well as other proteins with the diazotization reagents $NaNO_2$ and HCl/NaCl was found to make them adhere with high avidity and reproducibility to the surfaces of MLV and SUV. Advantages of the method include its rapidity, simplicity, controllability by varying the concentration of reagents used and ability to retain almost all adsorbed protein on storage for two weeks at 4^oC. Low molarities of the reagents used also appeared to cause little denaturation of antigen-binding ability of monoclonal antibodies. Foreseeable applications of the method are the coating of liposomes with antibodies for targeting to macrophages and other antigen-presenting cells subcutaneously as well as intravenously, aggregation of hapten molecules to each other, to carriers or to liposomes for enhancement of their immunogenicity and possibly for antibody-mediated targeting of liposomes.

The final chapter follows up on an earlier paper

which describes the high adsorption rates demonstrated by antibodies to the surfaces of SUV and the proposal that this phenomenon be taken advantage of to target liposomes to cells bearing the surface antigens to which the antibodies specifically bind. In agreement with previous findings, monoclonal antibodies of IgG_1 isotype displayed considerable adsorption to the surfaces of SUV. In addition, IgM was found to have even more marked adsorptive behaviour to SUV possibly due to their pentameric radial configuration which presents a larger area for intermolecular forces to act. Using the monoclonal antibodies RF-HCC1 and MOPC 21 IgG_1 adsorbed to CF-containing SUV, it was shown that these ligands were capable of directing the liposomes to Mahlavu cells which express surface determinants bound by the antibodies.

The aspects of liposomal applications, in the immunological approach to disease prevention and therapy, investigated in this thesis have added more impetus to the proposals to eventually introduce liposomes into the clinical and commercial arena. It must be emphasised that research in optimising useful empirical effects and the elucidation of their mechanisms of action is still in its gestation period. This, however, does not inhibit the more useful effects from being capitalised upon for the benefit of patients and has indeed culminated in the formation of several liposome-based biotechnology companies and the appearance on the market of a liposomal vaccine. One may confidently expect that the promise of using liposomes in the immunoprophylaxis and immunotherapy of disease will eventually be delivered.

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