IMMUNOLIPOCMS IN HAEMATOLOGY

by

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A thesis submitted for the degree of
Doctor of Medicine in the University of Edinburgh

August 1990
TO MY FAMILY.

Quisquam legat, quodlibet inveniet.

Anon.
The aim of this thesis was to use antibody-directed liposomes to enhance the targeting of 1. fluorescent compounds for the detection of cell surface antigens and 2. cytotoxic compounds for delivery to specific cell populations. This involved the selection and production of a suitable species of liposome-protein conjugate from among those described in the literature. The choice was a small unilamellar liposome conjugated either to antibody or protein A via a covalent linkage mediated by the heterobifunctional reagent SPDP.

Anti-immunoglobulin antibody was a more universal ligand than either specific antibody or protein A. The conjugate was made fluorescent by the encapsulation of carboxyfluorescein (CF) in the liposome and the maximally fluorescent concentration was determined at 20 mM CF. The probe was stable on storage over many months and had low non-specific staining characteristics. Applied to lymphocytes it gave a signal enhancement an order of magnitude greater than the conventional fluorescent-antibody method as measured by flow cytometry. This permitted the demonstration of the interleukin-2 receptor on resting polyclonal T-lymphocytes hitherto undetectable by conventional fluorescence. The
threshold of detectability was set at less than 1000 antigen copies per cell.

A range of clinically significant antigens on red cells were visually detectable using the fluorescent liposomes. These included heterozygously expressed Kell, Duffy and Kidd antigens which had not previously been demonstrated by fluorescence due to the fluorescence quenching effect of haemoglobin. The technique was an order of magnitude more sensitive than the fluorescent antibody method and also proved more sensitive than the indirect antiglobulin test in the detection of heterozygous Rh(D) and Fy^a cells. The probe was used to predict foeto-maternal blood group antagonism in a pregnancy at risk for haemolytic disease of the newborn using cells obtained from a chorionic villous sample in the first trimester.

Liposomes containing methotrexate were targeted to T cells among peripheral blood lymphocytes using anti-CD3 antibody. Evidence demonstrating the intracellular delivery of methotrexate is given. The cytotoxic effect was dose-dependent with respect to the concentration of targeting antibody and liposome concentration. There was 80% and 90% growth inhibition after 24 and 48 hours respectively. The kinetics of the response made the technique impractical for the purposes of T cell depletion or purging of harvested bone marrow. However the specificity of the toxicity to targeted cells
validated the approach for use with more rapidly cytotoxic agents.

I hereby confirm that the work contained in this thesis is my own and that the thesis has been composed by myself.

18 APR 1992

Date Athertoh Gray
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ACKNOWLEDGEMENTS

My thanks to Bob Hider for introducing me to the subject of liposomes and to Ernie Huehna for his guidance and encouragement throughout the gestation. Thanks also to David Linch for his constructive criticism and enthusiasm. The work would have taken much longer and been less fun without the practical help and resourcefulness of Janet Morgan. Financial support was granted by the Medical Research Council. A final thanks to all at 98 Cheney Mews and the Human Tumour Immunology Group who gave so freely of their advice, antibodies, friendship, biscuits and blood.
CHAPTER 1

GENERAL INTRODUCTION
1.1 INTRODUCTION

The power and scope of medical treatment is constantly expanding but equally important is the capacity to focus these advances more selectively on the organs, tissues and individual cells involved in the disease process. Selectivity of treatment rests on the dual ability to distinguish more accurately and sensitively between normal and abnormal cells and on the ability to deliver treatment specifically to one or the other. Thus diagnostic and therapeutic refinements are linked in the unitary concept of improved methods of targeting—targeting of detection agents and targeting of therapeutic agents. Liposomes are carrier structures which can be loaded with controllable quantities of analytical and pharmaceutical compounds and which can be directed to specific cells by the attachment of various ligands. The purpose of this thesis is to examine particular contributions which can be made by liposomes in improving both aspects of targeting.

1.2 IMMUNOFLUORESCENT STAINING

This is a commonly used labelling method for the detection of intracellular and cell surface antigens in research and diagnostic laboratories. The most popular fluorophore is still one of the first to be described, namely fluorescein isothiocyanate or FITC (Riggs et al
Antibody is conjugated to this derivative and incubated with the specimen to label cellular antigens. On exposure to blue light FITC-labelled cells emit a yellowish green light which is readily visible and measurable in a fluorimeter. Fluorescein has a high quantum efficiency (the ratio between the number of quanta of light emitted to the number absorbed) and, although it had been in use with fluorescent microscopes long before the advent of flow cytometers, its excitation maximum (493 nm) happens to be very close to the 488 nm argon ion laser wavelength used in almost all flow cytometers.

However the amount of FITC which can be loaded on to each antibody molecule is limited on average to a maximum of four. Higher amounts of fluorophore begin to affect adversely the antigen affinity of the antibody. In addition the quantum efficiency decreases as the degree of coupling increases such that, for example, the fluorescence efficiency of an antibody with 10 fluorescein residues per molecule is approximately 50% less than that with 5 residues (Goldman, 1968). The law of diminishing returns then applies. Finally, raising the amount of fluorescein per antibody molecule increases the overall negative charge of the conjugate. This compromises the specificity of the antibody-antigen reaction by producing more non-specific binding to cells.

A significant enhancement of the fluorescent signal
can be obtained by using indirect staining in which the fluorescent label is attached to a second antibody which binds to molecules of specific antibody already bound to a cellular antigen. It is possible for as many as five molecules of the second antibody to bind to a single cell-bound antibody molecule thereby increasing the number of dye molecules at each antigenic site from 4 to about 20 i.e. an order of magnitude.

Another direction taken in the search for brighter fluorescent labels is towards new fluorophores. Derivatives of the compound rhodamine were developed whose red fluorescence was easily distinguished from the green fluorescence of fluorescein. It then became possible to examine material stained with two different antibodies but in practice a conjugate such as tetramethylrhodamine isothiocyanate (TRITC)-antibody produces an order of magnitude less fluorescence than an equivalent amount of FITC-antibody at the customary excitation wavelength of 488 nm.

A family of fluorophores with performance characteristics that rival fluorescein are the phycobiliproteins which are found in certain algae and have molecular weights circa 240,000. Each molecule contains many bilin chromophores and each of these has a similar quantum yield to fluorescein. An antibody molecule directly conjugated to phycoerythrin may have as many as 30 associated chromophores which compares
favourably with FITC-labelled antibody in the indirect staining procedure. Phycoerythrin-labelled antibodies have become the conjugates of choice for the second label in two-colour immunofluorescence studies and are also preferred by some in single colour work particularly for the detection of small quantities of large molecules on cells. Nonetheless the more recent fluorescent labels have not dislodged FITC-antibodies as the most convenient and economical conjugate for use in the widely-available argon laser flow cytometers.

Attempts to increase the signal from fluorescein-labelled antibodies have used linked carrier structures such as polymers and macromolecules e.g. polyethylamine and dextran as well as synthetic microspheres to which many hundreds molecules of fluorophore had been conjugated in a previous step. These manoeuvres have either inconveniently increased the level of non-specific staining or resulted in disappointing levels of fluorescence due to concentration quenching. This last effect is a form of nonradiative energy transfer wherein the energy of a fluorescent molecule in the excited state is not emitted as a photon of light but transferred to an acceptor molecule of the same species in the low energy or non-excited state. Hence the amount of light which is emitted falls off as the number (i.e. concentration) of acceptor molecules increases.
Entrapment of fluorescein in liposomes has previously been used to study the interaction of the liposomal and cell membranes, to test the homing capability of targeted liposomes and to demonstrate intracellular delivery of liposomal contents. However, this approach has not been fully explored as a method of fluorescent labelling of cells per se. Liposomes have the facility for encapsulating large amounts of fluorescein in a way which can be controlled to prevent concentration quenching. The label is sequestered in a lipid membrane which might reduce interference with the exterior targeting ligand and reduce unwanted interactions with the cell membrane. For these reasons targeted fluorescent liposomes seem a promising means of improving the sensitivity of the FITC-antibody technique.

1.3 DRUG-TARGETING

The ability of liposomes to sequester many hundreds or even thousands of molecules of cytotoxic drugs renders them attractive as carrier agents. The advent of new coupling reagents has made it possible to conjugate liposomes with antibody under mild conditions, producing stable covalent linkages and unimpaired antibody affinity. These features make targeted liposomes potentially more powerful than drug-antibody conjugates which have a more restricted loading capacity. The concept of using liposomes to deliver cytotoxic drugs to
the cell interior is old but major obstacles to in vivo administration have restricted the clinical application of liposomes. These will be reviewed in the next section but are in essence due to instability in plasma, uptake by the reticuloendothelial system and access to the target cells. Removal of these obstacles could allow the potential carrier capacity and targeting ability of liposomes to be realised. A possible role for liposomes was perceived in the ex vivo treatment of bone marrow for transplantation.

Allogeneic bone marrow transplantation has become a recognised treatment in the management of certain malignant haematological diseases, stem cell disorders and genetic diseases. The procedure is complicated by graft versus host disease (GVHD) which is caused by the activity of donor T lymphocytes. Acute GVHD occurs in approximately 40-60% of recipients of HLA-identical transplants and >60% of transplants from HLA-mismatched donors. The case fatality rate of individuals with absent or mild acute GVHD is 10% versus >50% in individuals with moderate to severe disease.

It has been shown that T cell depletion of donor marrow prior to reinfusion reduces the incidence and severity of GVHD. The method by which this is achieved does not seem to affect the clinical outcome provided the viability of the marrow progenitor cells is not
prejudiced and the depletion is greater than a two to four log kill.

Existing methods of T cell depletion include 1. physical methods of removal 2. anti-T cell immunotoxins such as ricin-anti-T-cell monoclonal antibody conjugate and 3. complement-mediated lysis. All methods are labour-intensive and each has its particular restrictions. Complement-mediated T cell lysis which is probably the commonest in use in the U.K. requires prior screening of batches of xenogeneic complement to establish the effectiveness of the lytic activity and the lack of toxicity to progenitor cells in bone marrow culture assays. Harvested bone marrow may neutralise the desired effect through its anticomplement activity and residual activated complement may cause severe reactions in the recipient. On occasion the lysate induces cell clumping leading to difficulties with the reinfusion of donor cells. Some of these problems may be overcome by the use of autologous complement but clearly an ideal method of T cell depletion is not yet available.

Most patients with acute leukaemia do not have HLA-matched siblings but may benefit from the reinfusion of autologous marrow following the administration of potentially curative but marrow-ablative dosages of chemotherapy. Theoretically however the autologous bone marrow may be contaminated with leukaemic cells, even if
the leukaemia is absent morphologically. In an animal model of autologous marrow transplantation it has been demonstrated that such minimal residual disease could cause relapse (Sharkis et al 1980) and that this could be prevented by ex vivo incubation or purging of the marrow using a cytotoxic agent (4-hydroperoxycyclophosphamide or 4-HC).

No randomised clinical trials have documented the need for ex vivo purging and its precise role has yet to be established. Nevertheless there is some evidence for improved disease-free survival in patients with acute myeloblastic leukaemia who received marrow that was adequately purged with 4-HC (Rowley et al 1989). One of the problems with incubating marrow with drug solutions is the untargeted nature of the effects. Thus the haemopoietic stem cells may also be adversely affected and haematological reconstitution delayed. Any selectivity of effect rests solely on the differential sensitivity of normal versus leukaemic progenitor cells. Careful adjustment of the dose of drug is necessary and even then the response may be difficult to predict in individual cases. A means of targeting the cytotoxic agents to residual tumour cells is desirable to improve the margin of safety.

Targeted cytotoxic liposomes may therefore avoid some of the difficulties involved in the complement-mediated lysis of T cells and pharmacological purging of bone
marrow. The technique would certainly remove all of the previous obstacles to the in vivo use of liposomes at a stroke. The cells can be manipulated in a plasma-free medium, there is no reticuloendothelial system uptake to consider and access to cells in suspension is anatomically unrestricted.

The second aim of the thesis is therefore to determine whether targeted cytotoxic liposomes offer any advantages over existing methods of ex vivo manipulation of bone marrow by studying their effects on lymphocyte proliferation. An essential prerequisite to these studies is an informed understanding of the development of liposome targeting.

1.4 LIPOSOMES

1.4.1 Introduction

In 1964 Bangham observed the formation of microscopic rounded particles on addition of water to dried phospholipids on a glass slide (Bangham 1964; Bangham 1965). He guessed that these particles were membranous vesicles enclosing a small amount of the aqueous medium and later suggested that naturally occurring versions played a role in prebiotic evolution (Bangham 1968). The phenomenon follows from one of the most important properties of certain common phospholipids which is to associate into double layers or bilayers in the presence of water. In each layer the molecules are
closely packed together and identically aligned so that the polar headgroups are directed towards the surrounding aqueous medium or the internal aqueous space and the hydrophobic fatty chains compose the interior of the bilayer. The lipid may form as broad sheets of bilayer but these have a tendency to round up and form sealed vesicles or bodies, hence Bangham's term 'liposome'.

Bangham appreciated the resemblance to simple artificial cells and foresaw their role as model biological membranes. Others rapidly recognised their potential as carriers of drugs and the proposal that liposomes could be directed to specific cell types by molecules on their surface attracted considerable scientific and medical interest in the early 1970's. With the advent of monoclonal antibodies it seemed only a question of time before the concept of targeting cytotoxic liposomes to cancer cells in vivo was turned into a reality. The idea quickly caught the public imagination through newspaper and magazine reports which developed a heady militaristic thesaurus of terms like 'magic bullets', 'guided missiles', 'Trojan horses', 'stealth liposomes' etc. Such imagery persists to this day and has contributed to this single application becoming the shibboleth of medical respectability for liposomal research and development.

By the early 1980's however, there was a greater appreciation of the limitations of liposomes as a drug-delivery and targeting system and of the enormous
difficulties in in vivo administration. The early unrealistic representations produced a degree of disappointment even cynicism but a sober scientific interest in liposomes has survived. Publications and meetings have increased and new journals and commercial companies have appeared. There has been no lack of ingenuity in trying to solve some of the difficulties associated with in vivo use, while along the way alternative applications and refinements, though perhaps less newsworthy, have continued to emerge, to fascinate, even to cure.

1.4.2 Physical properties

Lipids

Liposomes can be prepared from a variety of amphiphilic lipids of which phospholipids are the most commonly used. As the name implies these lipids combine a polar and a hydrophobic region within the same molecule. When the shape of the molecule is cylindrical as for example in phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylglycerol (PG), a bilayer arrangement is adopted on dispersion in water. Phospholipids in a bilayer undergo a temperature-dependent phase transition from the gel state at lower temperatures to the liquid-crystalline state at higher temperatures. The transition is an endothermic process and occurs in a narrow temperature range around a characteristic temperature, $T_c$. In the gel state the
fatty acyl chains are tightly packed whereas above the $T_c$ they are more mobile and the thickness of the bilayer is decreased. The $T_c$ is determined by the nature of the acyl chains and of the polar headgroup of the phospholipid. Thus lower transition temperatures are associated with shorter acyl chains (Table 1.1) and a higher degree of unsaturation; the $T_c$ of a phosphatidyl-ethanolamine (PE) species is on average 15-20°C higher than that of the corresponding PC species.

1.4.3 Liposome structure

Three major types of liposomes have been described: multilamellar vesicles (MLV), small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV). MLV are the species which form spontaneously on hydration of dried phospholipids. Their size is heterogeneous and their structure resembles an onion skin of alternating, concentric aqueous and lipid layers.

SUV are formed from MLV by sonication and are single layered. They are the smallest species with a high surface-to-volume ratio and hence have the lowest capture volume of aqueous space to weight of lipid. The minimum size of SUV is determined by the maximum possible
### Table 1.1

**Transition Temperature and Carbon Chain Length**

<table>
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<tr>
<th>Phospholipid</th>
<th>Chain length</th>
<th>( T_c )</th>
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<tbody>
<tr>
<td>DLPC</td>
<td>C12</td>
<td>-1.8°C</td>
</tr>
<tr>
<td>DMPC</td>
<td>C14</td>
<td>23.9</td>
</tr>
<tr>
<td>DPPC</td>
<td>C16</td>
<td>41.4</td>
</tr>
<tr>
<td>DSPC</td>
<td>C18</td>
<td>54.9</td>
</tr>
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The effect of fatty acyl chain length on the transition temperature \( (T_c) \) of various commonly used phospholipids.

DLPC: dilaurylphosphotidylcholine;
DMPC: dimirystoylphospho-tidylcholine;
DPPC: dipalmitoylphosphotidylcholine;
DSPC: distearoylphosphotidylcholine.
curvature of the bilayer compatible with the integrity of the vesicle and is approximately $200 \, \text{Å}^2$.

The third type of liposome, LUV, has a large aqueous compartment and a single (unilamellar) or only a few (oligolamellar) lipid layers. Representative values for size, capture volume and encapsulation efficiency are given in Table 1.2.

1.5 TARGETING IN VITRO

Liposomes as carriers of detection agents or drugs lack target-cell specificity and various attempts to coat the liposomes with 'recognition molecules' have been reported. Following the first report of targeting using antibody (Gregoriadis and Neerunjun, 1975) most investigators now use this method. Alternative approaches have included the incorporation of a glycolipid in the liposome membrane which is preferentially taken up by cells bearing the appropriate carbohydrate receptor and the attachment of hormone molecules for homing to the corresponding receptor-bearing cells.

Antibody molecules used in much of the early work were not covalently attached to liposomes. Attachment was by hydrophobic linkages produced by co-sonication of liposomal lipid and antibody. To provide greater stability, various methods to couple antibody covalently to the liposome were developed. There are two
strategies. The first is to couple antibody directly or indirectly to preformed liposomes using coupling reagents. The second is to derivatise antibody with lipophilic molecules and allow these amphipathic antibodies to partition into the liposomes. Both methods have their adherents but with the advent of newer coupling reagents the first method is probably the most preferred. These reagents are active under mild conditions thus avoiding the potentially deleterious effects on liposome integrity associated with their predecessors.

With the availability of synthetic lipids and large quantities of monoclonal antibodies, liposomes can now be produced which have a high degree of in vitro stability and target specificity. Whether such selective binding can be preserved in vivo and whether it leads to internalisation of the liposome contents will be discussed in the next section.

1.6 TARGETING IN VIVO

A major effort has been devoted to the study of intravenously administered liposomes with the object of discovering methods of targeting them to selected cells, particularly malignant deposits, in the body. Successful targeting however requires overcoming a number of imposing difficulties including instability in plasma, extravasation, evasion of the reticuloendothelial system access to and uptake by the tumour cells. These steps
<table>
<thead>
<tr>
<th>Type</th>
<th>Size (nm)</th>
<th>Encapsulated volume (ul/mg lipid)</th>
<th>Encapsulation efficiency (%)</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLV</td>
<td>400-3500</td>
<td>4.0</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>SUV</td>
<td>20-50</td>
<td>0.5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>LUV</td>
<td>200-1000</td>
<td>11.0</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

Representative values for size, encapsulated volume and encapsulation efficiency of the three major types of liposome. MLV: multilamellar vesicles; SUV: small unilamellar vesicles; LUV: large unilamellar vesicles.

(ref. Weinstein, 1984; Poznansky and Juliano, 1984)
will be examined in turn by following the liposome on its voyage from the plasma to the target cell.

1.6.1 Plasma

Plasma is hostile to the liposomal membrane which is permeabilised by circulating lipoproteins as well as a non-lipoprotein component of plasma (Scherpof et al 1984). The major effect has been ascribed to the interaction of the liposome with high density lipoproteins (HDL) during which there is transfer of liposomal phospholipid to the lipoprotein and release of entrapped solute. The nature and mechanism of the non-lipoprotein constituent of plasma which enhances the influence of HDL is not precisely known although it has been shown to be a plasma protein (Scherpof et al 1984).

Both liposome structure and lipid composition affect vesicle stability in plasma. The most important factors are:

(i) the transition temperature of the lipid. Lipid molecules in bilayers in the 'solid' phase are more tightly packed than in the 'fluid' phase. Thus the average area occupied by a molecule of DPPC is 48Å² in diameter in the former phase compared to 58Å² in the latter (Papahadjopoulos et al 1974). Liposomes whose lipids are in the solid state at the temperature of plasma are less permeable than those in the fluid state.

(ii) the presence of cholesterol. Cholesterol also condenses the area per phospholipid molecule in a fluid
bilayer, reducing permeability and preventing loss of lipid to HDL.

(iii) liposomes other than minimal-size SUV. SUV with a diameter approaching 200Å have a strongly curved bilayer in which the strength of the lateral forces tending to divide the phospholipid molecules closely approximates the strength of the hydrophobic bonds holding them together. In that situation the structure becomes more susceptible to solubilisation by lipoproteins. This is demonstrated by the rapid formation of phospholipid/HDL complexes in a suspension of SUV in plasma during a 24 hour incubation at 37°C compared with the complete lack of solubilising activity in a suspension of large multilamellar vesicles under the same conditions (Scherpof et al 1979).

1.6.2 The extravascular space

For liposomes to reach cells in the extravascular space they must have the ability escape from the circulation. Thirty minutes after I.V. injection only 15% of PC/cholesterol liposomes remain in the blood; the remainder are found in the liver, spleen and, to a lesser extent, in the bone marrow (Allen et al 1987). In these organs the endothelial cells of the sinusoidal capillaries are separated by distances of several thousand Angstroms and the basement membrane is either absent or discontinuous. Thus large and small liposomes have no difficulty in leaving the vascular compartment
whereupon the majority are retained in these organs by the fixed phagocytes of the reticuloendothelial system (RES).

Parenchymal cells of the liver have also been reported to take up SUV as intact structures and to acquire liposomal lipid either by direct exchange or indirectly from HDL or Kupffer cells (reviewed by Poate et al 1984). LUV are taken up by hepatocytes to a minor extent (Nicolau et al 1987). This can be augmented by inclusion in the membrane of glycolipids such as lactosyl ceramide which has a terminal galactose residue capable of interacting with a galactose-specific lectin present on hepatocytes.

Elsewhere in the body the endothelial cell lining and basement membrane are continuous with the exception of the renal glomeruli where the endothelium is interrupted by fenestrae varying from 300 to 800Å° but the basement membrane is complete. Continuous and fenestrated capillaries therefore represent a major anatomical barrier to liposome extravasation. Materials smaller than 90Å° diameter may cross the capillary wall via the so called 'small pore' pathway between adjacent endothelial cells, whereas material between 100Å° and 700Å° may be transported in endothelial vesicles (transcytosis). SUV with a minimum size limit of approximately 200Å° would be excluded from the former but evidence is lacking that they are accommodated to a significant degree by the latter.
This has been put to the test in a range of tissues including mouse lung, cat muscle, dog colon and cat submandibular gland (reviewed by Poste et al 1984). Extravasation of neither SUV nor MLV was detectable with the exception of mouse lung where intravenously injected MLV were observed in pulmonary macrophages recovered from alveoli by pulmonary lavage. This finding, however, could be abolished by prior monocyte depletion of the blood indicating that the extravasation of liposomes was mediated by phagocytosis and subsequent migration of monocytes into the alveoli. Extravasation of liposomes into tissues other than those supplied by sinusoidal capillaries is therefore negligible.

1.6.3 The reticuloendothelial system

Numerous studies have shown that the majority of liposomes injected intravenously are retained in the liver and spleen and to a lesser extent the bone marrow (reviewed by Gregoriadis 1976). Tissue fractionation studies have confirmed that liposome uptake is primarily associated with the RE component of these organs (Tanaka 1975). The reason for the efficient uptake of injected liposomes by the RES is not well understood but is thought to be related to their association with and opsonisation by the plasma proteins (Hoekstra and Scherpof 1979).

The challenge of evading RES uptake has prompted strategies to make liposomes less attractive to Kupffer
cells and splenic macrophages. The rate of clearance from the blood is directly related to size with small liposomes being more slowly removed from the circulation than large ones (Allen and Everest 1983).

The rate of clearance is also dependent on the surface charge with negatively charged liposomes being removed more rapidly than those of neutral or positive charge. However, the nature of the negatively charged lipid may be very important since decreased liver and spleen uptake of liposomes containing negatively charged, carbohydrate-bearing lipids (gangliosides) has been reported (Allen and Choun 1987; Gabizon and Papahadjopoulos 1988). The presence of a small molar fraction of neutral monoasialylganglioside produced a slight decrease in the blood/RES ratio 2 hours after injection whereas the same fraction of negatively charged monosialylganglioside increased the ratio 6-fold. The results indicate the importance of sialic acid rather than neutral carbohydrate in the observed effect (Allen and Choun 1987). When combined with an optimal selection of other lipids 60-fold increases have been achieved in the fraction of the recovered dose present in blood 24 hours after injection (Gabizon and Papahadjopoulos 1988).

Cell surface carbohydrates and electrical charge have a widely appreciated role in cellular interaction and recognition phenomena and it may be that liposomes bearing sialic acid residues are not able to approach so
closely to the negatively charged surface of the fixed macrophages.

Liposome size, lipid composition and charge therefore exert a marked effect on the rate of liposome clearance from the blood with half lives varying from 30 minutes in the case of PC/cholesterol liposomes to 15 hours in the case of distearoyl phosphotidylcholine/cholesterol/monosialoganglioside liposomes (Gabizon and Papahadjopoulos 1988). However, despite the alteration in kinetics the basic pattern of tissue distribution remains unchanged with RES uptake predominating (Poste et al. 1984; Gabizon and Papahadjopoulos 1988).

One proposal for reducing the dominant effect of RES uptake is to pre-condition the RES with 'empty' liposomes so as to block the ability of the RES to phagocytose a subsequent administration of 'active' liposomes (reviewed by Poste et al. 1984). Whilst it is relatively easy to induce a state of reversible RES blockade in this way, it is likely that several injections of cytotoxic liposomes would be needed as treatment for a hypothetical tumour and each might require premedication with empty liposomes. As with alterations in liposome structure and composition, it has not been shown that such a manoeuvre actually increases liposome uptake elsewhere in the body.

There are also serious reservations over the degree of RES blockade which would be induced and the potentially deleterious effects on immune function. The predominant uptake by the liver, spleen and bone marrow of liposome
encapsulated cytotoxic drugs targeted to cells outside the RES, may be not only diversionary, but also harmful. Mild histological changes were reported in the livers and severe atrophy was found in the red and white pulp of the spleens of mice given liposome-encapsulated adriamycin though neither was more severe than free adriamycin in equivalent doses (Gabizon et al 1983). The possibility exists that macrophages which engulf these liposomes may be inhibited or destroyed and the functioning of the RES may be seriously impaired. Adjacent parenchymal cells in these organs may also be affected by local concentration of cytotoxic agents. After systemic administration of liposome-encapsulated bleomycin the metastatic spread of a number of malignant tumours in animals has been reported to actually increase (Poste et al 1984).

1.6.4 Tumour access

There have been several reports of animal studies showing that entrapment of a drug in liposomes increased its effect on tumour growth with modest increases in therapeutic indices (Gabizon et al 1982; Olson et al 1982; Gabizon et al 1983; Gabizon et al 1985). Assuming our injected liposome has withstood the permeabilising environment of the plasma, and evaded the phagocytes of the RES, how then would it gain access to the tumour to achieve such an effect?

The mechanism and precise location of liposome accumulation in implanted tumours is presently unclear.
The permeability of tumour vasculature may, like that of new or injured vessels, be increased compared with normal tissues (Underwood and Carr 1972) but this can be highly unpredictable. Enhanced tumour uptake is seen with liposomes having prolonged circulation time (Gabizon et al 1988), and it has been suggested that accumulation of liposomes in tumours is due to endocytic uptake and transcytosis of the liposomes by capillary endothelial cells.

Alternatively endocytosed liposomes may cause endothelial cell damage leading to tumour regression either by impairment of the blood supply or by promotion of passage of liposomes to the tumour due to capillary leakage. These processes are purely speculative and no morphological studies to clarify these issues have been reported (Gabizon et al 1988).

Administration of adriamycin (ADM) encapsulated in liposomes has been advocated following animal studies and in vitro work which showed a maintained or improved therapeutic activity (Gabizon et al 1982, 1983, 1985; Olson et al 1982). Tumour cell killing was enhanced by a factor of 100 in a murine metastatic tumour model (Gabizon et al 1985). Preliminary results have been reported of a phase I clinical trial involving patients with hepatic metastases from primary gastrointestinal adenocarcinomata (Sella et al 1987). No alopecia, ulceration, nausea, vomiting or phlebitis was noted in any of the six patients. No thrombocytopenia and only
one episode of leucopenia occurred. Two partial responses were observed.

The enhanced tumouricidal effect of liposomal ADM is difficult to explain. The use of phagocytic cell lines as tumour models may explain some results (Mayhew et al 1983). Other studies have used lymphoma tumours with metastases only in the liver and spleen. By virtue of the discontinuous endothelial cell lining and basement membrane as well as the phagocytic macrophages, high concentrations of ADM are achieved in the tissues surrounding the metastases. When a lymphoma cell-line was used to produce an intramuscular tumour liposomal ADM was much less effective than free ADM on local growth (Gabizon et al 1985). ADM transfer from macrophages to tumour cells has been reported (Martin et al 1984) and this may account for the observed responses.

Part of the therapeutic enhancement caused by liposomal encapsulation may be due to the sustained release action of liposomes. In the case of cytosine arabinoside it has been demonstrated that a single dose of liposomal drug was effective in prolonging the lives of mice having L1210 leukaemia whereas a single-dose treatment with free drug was totally ineffective (Mayhew et al 1978). However when the free drug was given as a sustained infusion the effect in some circumstances was just as effective as the liposomal preparation.

Injected free ADM is rapidly cleared from the plasma in a triphasic manner with a half-life of less than 12
minutes in the first phase (Martindale 1982). The plasma clearance of liposomal ADM is two- to three-fold higher than free drug up to 24 hours after injection (Olaon et al 1982). Thus any claim for a tumour-localising effect or increased therapeutic index using markers or drugs encapsulated in liposomes must be compared with optimal administration schedules of the free agent. In clinical practice this is reflected in the recent trend towards sustained infusions of chemotherapeutic agents. Suffice it to say that none of these mechanisms can be described as true targeting relying as they do on an ill-defined and non-specific property of the endothelial cells, local macrophages or the liposomes themselves.

A serious side-effect of ADM is irreversible cardiotoxicity and this has led to a recommended maximum total dose of 550 mg/m² (Association of British Pharmaceutical Industry 1985). Liposomal ADM has been reported to reduce the cardiac concentration of anthracycline 15-fold (Gabizon et al 1982) and the incidence of cardiac histopathological lesions (Olaon et al 1982). This may prove to be clinically useful but the mechanism by which it is achieved remains obscure.

A contributory factor may be the low RE cell content of cardiac muscle as this may reduce the local concentration of ADM-liposomes. In addition the endothelial cell lining and basement membrane in the capillaries of the myocardium is continuous which
prevents contact between ADM-liposomes and parenchymal cells compared with free ADM. Finally cardiac uptake of liposomal ADM may be minimised by partitioning of the drug in the lipid bilayer. The high degree of ADM incorporation in liposomes cannot be accounted for by their entrapped water volume which implies some interaction or intercalation of the drug in the lipid phase (Gabizon et al 1982). The availability of drug for partitioning into the myocardial cell membrane may therefore be limited.

Extrapolating the use of liposomal ADM from a selected group of tumour models to the broad church of neoplasia cannot be foreseen as yet but even if the only advantage is a reduction in cardiotoxicity the effort may still be considered worthwhile.

1.6.5 Uptake of liposomes by tumour cells

Let us assume that liposomes, one way or another, succeed in coming into contact with tumour cells; the next question to consider is how they would deliver their contents to the cell cytoplasm. Some tumour cell lines used as models are actively phagocytic (Mayhew et al 1983; Fruhling et al 1980) which no doubt helped in localisation of liposomes in these cases but this is not universally true of 'wild' tumours. In order to facilitate targeting to non-phagocytic cells, the attachment of antibody to the liposome bilayer has been
proposed (Gregoriadis and Neerunjn 1975; Heath et al 1980).

Antibodies used for this purpose have been those raised with a specific cell line or tumour model in mind (Urdal and Hakomori 1980; Bragman et al 1983). In practice though it is not practical to raise monoclonal antibodies which are specific to each tumour. In addition the phenotypic diversity of neoplastic cells in a tumour means that only a proportion of cells can be targeted at one time. More commonly utilised have been antibodies raised to class I (H-2K\textsuperscript{k}) and class II determinants coded for by the murine major histocompatibility complex (Machy et al 1982; Heath et al 1983; Huang et al 1983; Machy and Leserman 1983). They have proved to be highly specific in \textit{in vitro} studies but their \textit{in vivo} use is precluded by the presence of these antigens on many other types of host cell. In general, tumour specific antigens have proved an elusive target.

An important finding from the work on cell antigens is that binding of a liposome antibody conjugate to the corresponding cell surface antigen, however selective, does not ensure effective transfer of liposomal drug. Thus while anti-H-2Kk liposomes bound well to mitogen-stimulated lymphocytes of both T and B cell origin from mouse spleen, methotrexate transfer occurred only in T cells. B cells were sensitive to liposomes containing methotrexate only when conjugated with anti-I\textsubscript{a} antibody (Machy et al 1982). The conclusion is that the
effectiveness of a surface antigen for liposome targeting does not rest solely in the sufficiency of its own expression but equally in its functional relationship with the cell.

The mechanism by which antibody conjugated liposomes deliver their contents to cells has been extensively investigated and is thought to occur by receptor-mediated endocytosis (Huang et al 1983). This is similar to the formation of endocytic vesicles observed in endothelial cells (see section 1.6.2) but is triggered by binding to the specific antigen or receptor on the cell surface. The adjacent plasma membrane invaginates and the whole complex is internalized as a cytoplasmic vacuole. The receptor and its ligand separate after which the receptor may be recycled to the cell surface or degraded. The vacuole fuses with lysosomes and the ligand is exposed to their content of acidic enzymes.

The evidence that antibody conjugated liposomes may be handled in this way comes from several sources. Endocytic inhibitors such as cytochalasin B and a combination of 2-deoxyglucose and sodium azide strongly inhibited uptake of antibody conjugated liposomes (Huang et al 1983). Cells were more sensitive to drug delivered from small than from large liposomes despite greater binding of methotrexate in the larger liposomes (Machy and Leaerman 1983). The average diameter of the two liposome species was 2000Å and 800Å whereas that of endocytic vesicles is about 800Å. Thus the non-
proportionality of drug binding and drug effect may be explained by a failure to engulf the larger liposomes which supports endocytosis as the major mechanism of liposome internalisation.

Why some receptors mediate this type of endocytosis and other do not and why the same receptor expressed on different cells can have opposite effects indicates a fascinating, but as yet unknown, relationship between these structures and the cytoskeletal elements necessary for internalisation. The relevance is that the phenomenon places further stringent limitations on the properties of a hypothetical tumour specific antigen.

The pathway of liposome uptake by cells has practical implications for liposome targeting. Liposomal contents are thought to be released in lysosomes which are an acidic compartment containing many proteolytic and other enzymes. The cytotoxic agent may therefore degraded and rendered ineffective before it reaches the cytoplasm or cell nucleus. This is thought to be the case with cytosine arabinoside (ara C) which when encapsulated in liposomes conjugated with anti-H-2 antibody was no more cytotoxic than untargeted liposomes (Huang et al 1983). On the other hand, methotrexate exhibited target cell-specific cytotoxicity when entrapped in anti-H-2 liposomes and this could be partially reversed by chloroquine, a lysosomotropic amine which raises intralysosomal pH.
The most likely explanation is that cytosine arabinoside, which is known to be labile to lysosomal hydrolases (Papahadjopoulos 1978) is degraded in the lysosomal system before it can reach its site of action in the nucleus. Methotrexate being a weakly acidic molecule becomes protonated at the acid pH of liposomes and as an uncharged species diffuses across the lysosomal membrane to its cytoplasmic site of action. Therefore, in addition to all other details about liposome composition, size, antibody specificity and receptor characteristics, it is essential to consider also the chemical properties of the compounds which are to be encapsulated.

An ingenious solution is to encapsulate the drug in liposomes which are pH sensitive. Certain lipids such as phosphatidylethanolamine, oleic acid and palmitic acid cause liposomes containing them to lose their integrity at low pH and fuse with adjacent membranes. Endosomes, which are the pre-lysosomal compartment into which liposomes are delivered by receptor-mediated endocytosis, have an internal pH of 5.0 to 6.5 which is sufficient to induce this phenomenon. Thus the cytotoxic effect of ara-C which is otherwise negligible, when encapsulated in such liposomes, can be restored (Connor and Huang 1986). This would predict that agents which raise the internal pH of endosomes and lysosomes would once more nullify the cytotoxic effect of ara-C encapsulated in pH sensitive liposomes. Pretreatment of target cells with chloroquine
and $\text{NH}_4\text{Cl}$ has precisely this effect (Connor and Huang 1986).

1.7 PASSIVE TARGETING

The anatomical barrier of the continuous capillary system and the waiting phagosomes seem to set at naught efforts to target liposomes to any other type of cell. This had led many workers to make a virtue out of a necessity and concentrate on disorders of the RES which might be amenable to liposomal delivery of biologically active compounds. Passive targeting, as this has been called, exploits a function which is native to the targeted cell and not a homing property of the liposome. Nevertheless, promising applications have been reported and are described next.

1.7.1 Liposomes and antimicrobial agents

Some agents used for the treatment of bacterial, viral, fungal and parasitic diseases are very toxic and the infections themselves may be refractory to conventional doses of treatment. Liposomal encapsulation by altering the tissue distribution and pharmacokinetics of these drugs might have a favourable effect on the infection. Though perhaps less glamorous than cancer therapy the use of liposomal drugs in infectious diseases is a closer clinical reality.
Amphotericin B (AMB) is an effective but toxic antifungal whose activity is related to its ability to react with membrane sterols, creating channels through which cellular contents leak. It binds preferentially to ergosterol, a fungal membrane sterol but it also binds to cholesterol abundant in mammalian cell membranes and this may account for its notable toxicity. Being highly lipophilic, however, makes AMB a good choice for liposomal incorporation.

Adverse effects of AMB include fever, chills, electrolyte disturbances and impaired renal function. Several clinical trials of liposomal AMB have reported the absence of severe side-effects in patients who had previously reacted badly to free AMB (Sculler et al 1988; Lopez-Berestein et al 1985; Lopez-Berestein et al 1987). The half-life of liposomal AMB is prolonged; serum AMB concentrations have been reported to be ten times higher than those obtained after infusions of free AMB (Scullier et al 1988). Cumulative doses of up to 3g of liposomal AMB have been administered without the toxicity seen with much lower doses of the free drug.

In a study of 19 patients with systemic fungal infections and haematological malignancies, 18 had previous antifungal therapy (Lopez-Beresein et al 1985 and 1987). Nine were cured, six had partial responses and four showed no response. Nine patients remained neutropenic throughout; treatment with liposomal AMB was well tolerated by all. Responses to the liposomal
preparation appear to be at least as good if not better than conventional AMB and an improved therapeutic index is achieved by the reduction in toxicity.

Why liposomal incorporation improves efficacy and reduces toxicity is uncertain. Liver, spleen and bone marrow are the main sites of systemic fungal infections and passive targeting of liposomes into the RES of these organs may result in local concentrations of the antibiotic. Combining AMB with the lipid of a liposome may reduce its ability to interact with cell membranes of the host but interfere less with its interaction with the fungal cell membrane. Lipid composition does appear to affect the activity of liposomal AMB since liposomes containing phospholipids alone were more effective in a murine candidiasis model than those containing ergosterol or cholesterol (Lopez-Bereatein et al 1983).

Nevertheless, treatment failures are recorded in which context it is interesting to note the results of an animal study using AMB incorporated in liposomes conjugated to an antibody specific to Candida albicans (Hospenthal et al 1989). The survival rates of mice with disseminated candidiasis were higher in those treated with the targeted compared with the untargeted AMB liposome. No in vivo toxicity was observed with either preparation. Since fungal infections are spread via the blood stream, the added benefit of antibody conjugation may be an example of successful intravascular targeting.
Liposomal AMB is the first formulation of liposomes to reach large scale clinical use and may be expected to be applied to the treatment of other fungal infections such as aspergillosis, cryptococcosis and histoplasmosis in the near future.

Leishmaniasis is another infection successfully treated in experimental animals with liposomal drugs. Liposomally delivered therapy seems to be a major advance over previous treatment regimes for this intracellular parasite. The antimonial drugs used in treating leishmaniasis are also quite toxic particularly to the kidney, liver and heart. Meglumine antimoniate given to hamsters with visceral leishmaniasis was 276 times more effective when incorporated in liposomes compared with free antimonial as measured by the amount of drug required to cause 50% suppression of hepatic parasites (Alving et al 1980). In addition to antimonials, other antileishmanial agents such as amphotericin B and 8-aminoquinolones show an enhanced therapeutic index when encapsulated in liposomes (Alving et al 1980). Leishmania protozoa of an infected host colonize the macrophages of the RES and would seem to be a natural choice for passively targeted liposomes. In addition, electron microscopy has shown that the organisms multiply within the same endocytotic vacuole into which liposomes are ingested (Alving 1983). Liposomal delivery to a specific subcellular organelle is the most exquisite example of targeting yet described.
Liposome encapsulation of a range of antibiotics has been reported to have increased the effectiveness of treatment of other facultative intracellular organisms including brucella, salmonella, mycobacteria, staphylococcal, Escherichia coli, legionella and listeria species (reviewed by Coune 1988). Treatment of certain viral infections which are characterised by viral replication in Kupffer cells and other macrophages may also be improved by liposomal delivery as is the case with experimental Rift Valley fever and ribavirin. As well as delivering antimicrobial agents, liposomes have been used to incorporate macrophage activators for the treatment of herpes, hepatitis and fungal infections. The rationale of immune modulation is similar to that discussed in the activation of macrophages for antitumour activities (see below).

1.7.2 Liposomes and biological response modifiers

In the treatment of neoplastic disease the phenomenon of passive targeting of liposomes to the RES has been exploited indirectly. Activated macrophages may contribute to the host's tumour-defence by their ability to recognise and destroy neoplastic cells selectively. This ability may become attenuated in the presence of an excessive tumour burden but could be regained by successful activation of tumouricidal macrophages.

Muramyl dipeptide (MDP) is a synthetic analogue of structures found in bacterial cell walls and stimulates a
variety of macrophage functions \textit{in vitro} but is less effective \textit{in vivo} probably due to its extremely rapid renal clearance. Encapsulated in liposomes MDP renders macrophages tumouricidal \textit{in vitro} far more effectively than free MDP and can also activate cells which have become refractory to the free agent (Kirsh and Poste 1987). Macrophage activation factor (MAF), a T-cell-derived lymphokine, has also been encapsulated in liposomes with similar results. This has been confirmed in mouse experiments with model lung and lymph node metastases (Fidler et al 1982).

Liposomal delivery of biological response modifiers to macrophages, albeit passive, may provide a strategy for adjunctive therapy in malignant disease but no clinical studies have yet been published.

1.7.3 Diagnostic imaging

Encapsulated contrast media have been applied in some areas of diagnostic imaging including scintigraphy, computed axial tomography (CT) and magnetic resonance (MR) (reviewed by Seltzer 1989).

Radionuclides such as Tc-99m and In-111 have been encapsulated in liposomes and used to generate images of liver metastases, myocardial infarcts, abscesses, lymph nodes, tumours and synovial tissue in arthritic joints (Williams et al 1986). In the case of liver metastases the principle relies on reduced uptake of liposomes by the pathological area. Passive targeting to the Kupffer
cells which are lacking in the tumour deposits results in a photon-deficient area. The opposite is true in the case of an inflammatory process where the mononuclear and polymorphonuclear phagocytes produce a local increase in radioactivity compared with normal tissue.

In CT scanning the encapsulation of iodinated contrast media have been shown to produce a selective enhancement of normal liver and spleen tissue. In MR scanning contrast agents also play an important role and both water soluble and hydrophobic compounds have been successful as image enhancers. In a blinded analysis of MR images (Unger et al 1989) liposomal gadolinium diethylenetriaminepentacetic acid (Gd-DTPA) was compared with free Gd-DTPA in the imaging of hepatic metastases in rats and there was a significant improvement in metastasis detection.

In all of these areas the clinical role of liposomally delivered contrast material has still to be determined. Their advantages over standard imaging agents, how well they will be tolerated and their precise realm of clinical application have not been established.

1.7.4 Haemosomes

If the intravascular space itself can be considered a target then the interesting studies with liposomally encapsulated haemoglobin can justifiably be included here. A red cell-free blood surrogate would be useful for emergency blood replacement and for patients with
multiple alloantibodies for whom compatible blood cannot be found. Blood substitutes have been mainly of two types a) polymers and other derivatives of haemoglobin and b) perfluorinated hydrocarbons. Problems with the former have included haemoglobin stability, increased oxygen affinity, excessive colloid osmotic pressure and rapid renal clearance. Perfluorochemicals have remarkable oxygen-carrying capacity at high oxygen tensions but their oxygen-carrying capacity is only a few percent of that of whole blood at alveolar oxygen tensions. The proposal to encapsulate haemoglobin in liposomes offers certain advantages over haemoglobin solutions such as the avoidance of chemical procedures to decrease the oxygen affinity and the rapid filtration by the kidneys. The liposomal membrane also prevents the oxidative conversion of haemoglobin to methaemoglobin (Gaber et al 1983).

'Haemosomes', as liposome-encapsulated haemoglobin suspensions have been called, show some remarkable similarities to red cells and an important difference. They undergo reversible oxygenation with a Hill constant of 2.8 compared with the normal value for haemoglobin in red cells of 2.9. No significant loss of function occurs on encapsulation nor is any difference detected in the rate of oxygen binding and release compared with red cells. The haemoglobin shows no evidence of leakage from liposomes in physiological conditions. The main difference is that the in vivo half life of haemosomes is
at best 20 hours while that of transfused red cells is 120 days (Farmer and Gaber 1987) for which RES uptake is once again responsible. Whether this leads to serious side-effects from RES blockade and whether the continuing efforts to produce liposomes with reduced RES uptake will be successful remains to be seen. It has been pointed out though that for the purpose of emergency blood replacement, only a few hours of efficacy are required (Farmer and Gaber 1987).

1.7.5 Liposomes and gene transfer

DNA transfection into eukaryotic cells is an important tool for the investigation of gene function and expression. It has also led to proposals for genetic treatment of inherited disorders using the pure, cloned genes provided in quantity by recombinant DNA technology. Several techniques have been developed for the introduction of foreign genetic material into somatic cells such as microinjection, electroporation, calcium phosphate precipitation, DEAE-dextran and modified viruses.

The first use of liposomes for gene transfer was reported by Mukherjee et al in 1978. Liposome technology has evolved substantially since then but the efficiency of this procedure has not generally exceeded that of the other methods. Recently, however, Itani et al (1987) have reported gene transfer as much as four orders of magnitude more efficient than the calcium
phosphate method. Liposomes may provide a useful alternative for cells which are refractory to gene transfer by conventional methods.

Successful liposome-mediated gene transfer requires efficient encapsulation of undegraded genetic material, selective binding to the target cell, delivery to the cytoplasm with avoidance of lysosomal degradation, migration to the nucleus, incorporation in the genome, stable and effective expression of the genetic message.

Encapsulation efficiencies of 30 to 50% have been achieved using large unilamellar liposomes but exposure of DNA to sonication and organic solvents will limit the applicability of the procedure in some circumstances (reviewed by Mannino and Gould-Fogerite 1988). The calcium-EDTA-chelation technique avoids exposure to detergents, sonication and solvents in liposome synthesis but the encapsulation efficiency is lower (1-10%). A recently described modification, however, allowed almost 40% of nucleic acid to be encapsulated (Mannino and Gould-Fogerite 1988).

Not surprisingly liposome composition has an important influence on the process of gene transfer. Increased infectivity was associated with the inclusion of negatively charged PS in PC vesicles (Frahley et al 1981) probably by promotion of fusion with the cell membrane and avoidance of lysosomal enzymes.

Fusion of liposomes with cells, in addition to directing their contents away from the lysosomal
compartment, results in the incorporation of the lipid bilayer in the cell membrane. However this can have an independent effect on the efficiency of nuclear uptake of exogenous DNA. Nicolau and Sene have shown with PC liposomes that the nuclear uptake was 40% of the whole cellular uptake of $^{32}$P-DNA, but only 28% with PC/PS liposomes. Inhibition of endocytosis with cytochalasin B confirmed that 85% of the uptake of PC liposomes was by this route but was only 36% for that of PC/PS liposomes. These data suggest that following endocytosis liposomal phospholipid can have an important protective function during the subsequent migration of DNA to the nucleus.

Cell cycle kinetics also has a major influence on the degree of nuclear incorporation. Cells in $G_1$ incorporated 37% of exogenous DNA in nuclear DNA when it was presented in PC liposomes whereas cells in mitosis, when the nuclear membrane has broken down, incorporated twice that amount (Nicolau and Sene 1982). Synchronisation of cells in either phase had only a small effect on the efficacy of PC/PS liposomes (40 to 46%).

Liposomes targeted with antibodies and other ligands bind efficiently to target cells and delivery of vesicle contents is via receptor mediated endocytosis. The effect of exposure to the lysosomal compartment can be diminished by pretreatment of cells with lysosomotropic agents like chloroquine (Frahley et al 1981). A similar effect was seen using pH-sensitive liposomes (Wang and Huang 1987).
A favoured strategy for increasing the effectiveness of liposome-cell interaction is to reconstitute membrane proteins from enveloped viruses into the liposomal bilayer to form proteoliposomes. Envelope proteins from for example Sendai, Semliki forest and influenza viruses mediate both attachment to target cells and fusion of tightly juxtaposed membrane bilayers resulting in cytoplasmic delivery of vesicle contents without exposure to lysosomal enzymes.

Viral proteins may also have a function in protecting exogenous DNA on its way through the cytoplasm and in mediating its incorporation in the nucleus. Nuclear protein itself has been co-introduced with DNA in proteoliposomes and led to a five-fold increase in expression of DNA compared with non-nuclear protein (Kaneda et al 1989).

The greatest potential for liposomes in gene transfer is that none of the conventional methods can be used to transfect intact adult animals. In vivo gene transfer using liposomes has been reported by several groups (Nicolau et al 1987; Wang et al 1987). Gene expression has usually been transient although in one case (Mannino and Gould-Fogerite 1988), proteins encoded by sequences of transfected bovine papilloma virus were detected at sacrifice after eight months.

The previously described problems of RES uptake remain. Kupffer cells and splenic macrophages are not ideal cells for the incorporation and expression of
transfected genes. Even the achievement of increased hepatocyte uptake can not guarantee sustained expression since all these cells are end-cells and gene expression is likely to be quenched however stable the incorporation.

In this respect, the most attractive somatic cell target in the body is the bone marrow stem cell. Stem cells have the potential to differentiate and thereby repopulate haemopoietic tissue. Bone marrow is readily accessible for in vitro manipulation and reinfusion and this might be a future strategy for the treatment of severe genetic disorders affecting haemopoietic cells e.g. adenosine deaminase deficiency, purine nucleoside phosphorylase deficiency, thalassaemia and sickle cell disease. The clear superiority of liposomes over other methods of gene transfer would need to be demonstrated.

1.8. FUTURE OF LIPOSOMES

Many promising results have been described and potential applications proposed in the quarter century since liposomes were first described, but liposomal adriamycin and amphotericin are the only preparations which have reached the stage of preliminary clinical trials. It is legitimate then to ask why liposomes have not had more clinical impact.

In a sense, liposomes have been the victim of their own variety and versatility. This has arisen from the wide choice of phospholipids and phospholipid mixtures,
the numerous methods of production and the variable size and structure of the end-product. The result has been a complete lack of standardisation which makes it difficult to compare studies and leads to uncertainty about the interpretation of results.

Another uncertainty which has slowed progress towards clinical usefulness is the precise mode of action of liposomally presented drugs. In the case of the neoplastic agents it is still not clear whether the effects are mediated by a slow release mechanism and, if so, whether this might be operating by systemic release in the circulation or by local release from macrophages. Such uncertainty makes rational scheduling and the interpretation of beneficial or adverse effects conjectural.

Although liposomes may reduce the toxic effects of drugs like ADM, amphotericin and the antimonials, there is concern that the altered tissue distribution may produce side-effects of its own. The accumulation of liposomes in the liver and spleen may lead to RES blockade which may be undesirable in patients who are already heavily immunosuppressed. Liposomes containing cytotoxic agents may produce even more prolonged injury to phagocytic cells. The opposite effect could be produced by administration of liposomally incorporated biological response modifiers. Activated macrophages may not limit their response to neoplastic or infected cells but produce unwanted inflammatory or autoimmune
phenomena. These possibilities have to be tested in long-term studies in appropriate animal models before large clinical trials can be approved.

A final problem concerns scaling-up production of liposomes from the requirements of laboratory experiments to those for commercial diagnostic agents and for the treatment of large numbers of patients. Methods of preparation such as sonication and rotary evaporation of solvents present serious engineering and technical problems when applied on an industrial scale. Batch to batch variations in the physical characteristics of liposomes can be foreseen which may seriously alter efficacy and toxicity. Recovery levels, impurities and sterility are common problems with column chromatography which is frequently used to separate constituents in liposome production.

Investment in the technology necessary to ensure good quality control will only be attracted when questions about effectiveness, mode of action, toxicity and likely demand have been settled.

In summary, targeting of diagnostic or therapeutic agents using liposomes has an uncertain future but will probably continue to be researched. Liposomes have many of the properties desirable in laboratory agents and may be safer to use than radioactive reagents. They may also have a role in improving the specificity and sensitivity of selected solid-phase assays and immunophenotyping techniques but they will be competing
with an increasing array of alternative technologies. Their early promise as highly specific carriers of drugs to treat cancer has not been realised but revised options such as targeting of anti-infectious agents to the RES and attenuation of the toxic side-effects of drugs are on the threshold of clinical exploitation. This will be limited at first, but may pioneer further applications as in vivo experience and confidence accumulates.

The series of experiments to be described in this thesis address two areas of immunofluorescent phenotyping where signal enhancement is required. An optimally fluorescent species of liposome is developed and its performance is compared with a conventional fluorescent technique in the detection of leucocyte antigens. The probe is used to look for the interleukin-2 receptor on peripheral blood lymphocytes where it is not normally detected by conventional staining.

Red cell antigens are difficult to demonstrate by fluorescence and are conventionally detected by agglutination methods. In situations where the cell number or percentage in a mixture of cells is low these methods may be impractical and a sensitive fluorescent method more suitable. The sensitivity of fluorescent liposomes is compared with that of the fluorescent antibody technique and the indirect antiglobulin test in red cell phenotyping. The probe is used to look for the existence of foeto-maternal blood group antagonism in two pregnancies at risk of haemolytic disease of the newborn
using cells obtained from chorionic villous samples in the first trimester.

Finally liposomes containing methotrexate are targeted to T cells among peripheral blood lymphocytes using anti-CD3 antibody. Evidence for the intracellular delivery of methotrexate is given and the applicability of this approach to T cell depletion and purging of bone marrow is discussed.
CHAPTER 2

MATERIALS AND METHODS
2.1 MATERIALS

2.1.1 Lipids

Synthetic dipalmitoyl L-\(\alpha\)-phosphatidylcholine: Sigma
Synthetic dipalmitoyl L-\(\alpha\)-phosphatidylethanolamine: Calbiochem
Cholesterol: British Drug House (BDH)

2.1.1 Reagents

All reagents were Analar or tissue culture grade where possible.

Anhydrous sodium sulphate: BDH
Sodium hydroxide: BDH
Sodium chloride: BDH
Sodium iodide: BDH
Sodium acetate: BDH
Iodine (resublimed): BDH
Hepea (N-2-hydroxy ethylpiperazine-N-2-ethane sulphonic acid): BDH
Ammonium thiocyanate: BDH
Ferric chloride hexahydrate: BDH
Dithiothreitol: Sigma
Sodium azide: Sigma
Triton-X-100: Sigma
N-hydroxysuccinimidyl 3-(2-pyridyldithio) propionate: Pharmacia.

Protein A: Pharmacia
Sephadex G50: Pharmacia
Sephrose CL-4B and CL-6B: Pharmacia
Sephadex G 25M PD 10 columns: Pharmacia
5(6)-carboxyfluorescein: Eastman Kodak
Phytohaemagglutinin (PHA): Wellcome Pharmaceuticals

2.1.3 Solvents and solutions
Methanol: May and Baker
Chloroform: "
Ethanol: "
Acetone: "
Formaldehyde: "
Triethylenediamine: Sigma
Scintillator 299: Packard

2.1.4 Radioactive materials
\[ ^{125} \text{I} \] sodium iodide (1mCi/ml): Amersham International
\[ ^{3} \text{H} \] deoxyuridine (1 mCi/ml): " "

2.1.5 Media
RPMI 1640 with L-glutamine and 20mM Hepes: Gibco
Foetal calf serum: Gibco (inactivated by heating at 56°C for one hour).
Ficoll-Hypaque: Pharmacia

2.1.6 Chromatography plates
Linear K silica gel 5 x 20cm (250um thickness): Whatman
2.1.7 Column chromatography

Sephadex G50 and LH20 granules were swollen, washed and degassed in distilled water before pouring into 1 x 20cm glass columns plugged with glass wool. The gel was equilibrated with the relevant buffer and, where appropriate, loaded with liposomes and protein to saturate non-specific binding sites. Separate columns were used for coupling of protein A and IgG.

2.1.8 Buffers

Phosphate buffered saline (pH 7.4): Oxoid Ltd.

One tablet was dissolved in 100ml distilled water and the pH adjusted by dropwise addition of hydrochloric acid or sodium hydroxide as appropriate.

Hepes buffer (pH 7.4 or 8.0)

10mM Hepes and 145mM sodium chloride (NaCl).

2.38g Hepes and 8.468g NaCl were dissolved and made up to 1 litre with double distilled water. The pH was adjusted with 5M sodium hydroxide.

Acetate buffer (pH 5.0)

Solution A: (0.1M)

6.8g sodium acetate and 2.98g NaCl were dissolved and made up to 500ml with distilled water.

Solution B: (0.1M)

2.87ml glacial acetic acid and 2.93g NaCl were made up
to 500ml with distilled water.

63 parts of solution A were added to 37 parts of solution B and the final pH adjusted to 5.0 with acetic acid or sodium hydroxide.

Glycine buffer (pH 2.5, 0.2M)
25ml 0.2M glycine and 15ml 0.2M HCl were made up to 100ml with distilled water.

Tris buffer (pH 8.8)
50ml 0.1 M Tris (hydroxymethyl)aminomethane and 8.5ml 0.1 M HCl were made up to 100ml with distilled water.

2.2 METHODS

2.2.1 Purification of carboxyfluorescein
Carboxyfluorescein (CF) obtained from the manufacturer contained hydrophobic impurities capable of binding to phospholipids and disturbing the liposomal bilayer. Purification was carried out according to the method of Ralston et al 1981. Briefly, CF (5g) was mixed with activated charcoal and boiled in hot absolute ethanol (100ml). The solution was filtered (Whatman No. 1 filter paper) to remove the charcoal and recrystallised by cooling. The precipitate was washed in cold water in a Buchner funnel and dissolved in 10 M KOH. This solution (the potassium salt of CF) was passed down a
Sephadex LH 20 (Pharmacia) column equilibrated with distilled water. The impurities were retarded as a broad brown band behind the clear orange of the purified salt. This was lyophilised and stored in the dark as the crystalline material.

2.2.2 Synthesis of DPPE-DTP

Dipalmitoyl L-a-phosphatidylethanolamine 3-(2-pyridyldithio) propionate (DPPE-DTP) was prepared in the laboratory. Ten umol of dipalmitoyl L-a-phosphatidyl ethanolamine (DPPE) was dissolved in 700 ul chloroform : methanol (9:1) and reacted for 2 hr at room temperature with SPDP (12 umol in 300 ul methanol) in the presence of 20 umol triethylamine. The organic phase was washed once with 2 ml PBS and twice with water before drying with nitrogen and lyophilisation for 2 hr. The product was stored as a 5 mM solution in chloroform : methanol.

The purity of the derived product (DPPE-DTP) was assessed by thin layer chromatography on a 5 x 20 cm Whatman linear K plate. The solvent system was comprised of chloroform/methanol/distilled water in the ratio
Thin layer chromatography of lipids. Lanes 1 and 2 were spotted with DPPE following reaction with SPDP to form DPPE-DTP. Lanes 3 and 4 were spotted with native DPPE. See text for Rf values.
14:5:0.7 and the lipid was stained by exposure of the plate to iodine vapour. DPPE yielded a single spot with a Rf value of 0.36 (Fig. 2.1). DPPE-DTP also gave a single spot but with a Rf value of 0.41 indicating complete conversion of the reactant to the slightly more hydrophobic derivative.

2.2.3 Phospholipid assay

The phospholipid concentration in solutions of DPPE-DTP and suspensions of liposomes was assayed by the colorimetric method of Stewart (1980). This is based on the formation of a coloured complex between ammonium ferrothiocyanate and phospholipid which is in the ratio 1:1 in the case of DPPC and 1:2 with DPPE. The method is rapid and sensitive and requires the generation of a standard absorption curve using serial dilutions of lipid. The concentration of phospholipid in the test samples is adjusted to fall within the straight portion of the curve.

The assay was carried out as follows.

1) A solution of ammonium ferrothiocyanate was prepared by the addition of 27.03g of ferric chloride hexahydrate and 30.4g of ammonium thiocyanate to deionised distilled water and made up to 1 litre.

2) Stock solutions of DPPC and DPPE at a concentration of
0.1 mg/ml in chloroform were prepared and volumes from 0.1 to 1ml were transferred to glass test tubes in duplicate. Each tube was made up to 2 ml with chloroform.

3) Test samples were treated identically using volumes selected according to the expected phospholipid content, usually between 0.05 and 0.1 ml.

4) 2 ml of ammonium ferrothiocyanate solution was added to each tube which was covered with parafilm and mixed vigorously for a minute. The two phases were separated by centrifugation for 10 minutes at 750g and the lower organic phase was removed with a glass Pasteur pipette. The aqueous solution was clarified with a pinch of anhydrous sodium sulphate before measuring the absorbance at 488nm.

5) A standard curve was constructed from the mean absorbance and the measured phospholipid concentration. The concentration of the samples was seen to fall on the straight part of the curve and the final result was obtained by correction for the dilution factor. Since the phospholipid content of the liposomes used in these studies was 99% DPPC, the standard curve of this lipid was used in the assay of liposomal phospholipid.

2.2.4 Liposome synthesis

Small unilamellar vesicles or liposomes (SUV) were
composed of dipalmitoyl phosphatidylcholine (DPPC), DPPE-DTP and cholesterol in a molar ratio of 66:1:33. Suspensions of liposomes were prepared by probe ultrasonication in volumes of 3 ml and utilising 40 umol of total lipid. The lipids (total 40 umol) were dissolved in chloroform : methanol and applied to the walls of a round-bottomed flask by rotary evaporation. Traces of solvent were removed by lyophilisation for a minimum of 2 hr. The solute to be encapsulated was dissolved in 3ml of buffer (0.01 M Hepes, 0.145 M sodium chloride, pH 7.4) and added to the lipid. The mixture was brought to 45°C for a few minutes and shaken vigorously for 20 min.

Once all the lipid was hydrated, the suspension was transferred to a water-cooled vessel at 41°C in the cabinet of a probe sonicator (Soniprep 150, MSE). Sonication was carried out at power setting mark 8 for a total of 45 min. This was delivered by a process timer in 80 cycles of 30 sec on, 10 sec off in order to avoid cavitation and frothing. An atmosphere of nitrogen was maintained above the liposomes (Fig 2.2) to minimise oxidation of the phospholipid.

The resulting suspension was centrifuged at 1000g for 20 min to remove titanium particles from the tip of
Probe sonicator (Soniprep 150, MSE) in operation. Inside the cabinet the liposomal suspension is held in a water-cooled glass vessel surrounding the probe. A process timer (on top of the cabinet) controls the duration of sonication.

A close-up view shows the inflow and outflow tubing of the water-jacket and the narrower tube delivering a constant stream of O₂-free nitrogen.
the probe and applied to a short column of Sephadex G50 (equilibrated in Hepes buffer pH 8.0) in order to separate the encapsulated from the unencapsulated solute. The liposome peak appeared in the void volume and since all the solutes used in this study were coloured it was easy to determine its position by simple visual inspection. Liposomes were stored under N₂ at 4°C until ready for coupling to protein.

2.2.5 Purification of sheep anti-mouse IgG immunoglobulin

Sheep serum containing antibodies to injected mouse immunoglobulin was a gift from Prof. P. Beverley, Human Tumour Immunology Group, ICRF, UCMSM, London. This contained proteins cross-reacting with human Ig as well as other unwanted ovine proteins. A pure preparation of polyclonal anti-mouse IgG was therefore required in order to avoid the coupling to liposomes of protein which was irrelevant or capable of non-specific binding to human cells.

Whole sheep serum was centrifuged at 700g for 20 min to remove aggregates and debris and dialysed overnight against PBS at pH 7.4. Fifty ml of dialysed serum was circulated overnight through a HIG (human Ig coupled to Sepharose, Serotec) column equilibrated with PBS. The eluate was circulated on a MIG (mouse Ig coupled to Sepharose, Sigma) column in a similar manner. The column
was washed with PBS and the eluate monitored with a U.V. flow cell until no further protein was detected. The eluate was discarded.

The bound sheep anti-mouse Ig immunoglobulin (S&M-Ig) was eluted with glycine buffer at pH 2.5. The protein-containing fractions were identified by the flow cell, pooled and titrated to neutral pH with Tris buffer at pH 8.8. The S&M-Ig was dialysed for 24 hr with several changes of PBS. The concentration of protein was determined by the absorbance at 280 nm according to the formula:

\[
\text{Protein conc. (mg/ml)} = \frac{\text{Absorbance}}{1.4}
\]

The concentration was adjusted to 5 mg/ml in an Amicon apparatus under N₂ pressure and the protein was stored in aliquots at -20°C.

2.2.6 Iodination of protein

S&M-Ig and protein A were iodinated in order to track the proteins during derivatisation reactions and column chromatography and to measure the efficiency of the coupling reaction with liposomes. In the case of protein A, radioactivity was also used to calculate the degree of DTP substitution (see 2.2.8). Iodination was performed using the chloroamide 1,3,4,6-tetrachloro-
3a,6a-diphenylglycoluril which was first described by Fraker and Speck (1978) and is marketed as Iodo-Gen (Pierce). This mediates the rapid iodination of protein with aqueous I\(^-\) while remaining in the solid phase by virtue of its insolubility in water.

A small amount (20 \(\mu\)g) of Iodo-Gen was dissolved in 200 \(\mu\)l of methylene chloride and the solution was transferred to the reaction vessel (microfuge tube). The organic solvent was evaporated under a gentle stream of nitrogen and the tube was rinsed with water to remove loose flakes of reagent. The sample of protein to be labelled (200 \(\mu\)g in 100 \(\mu\)l PBS) was added followed by 500 \(\mu\)Ci of Na\(^{125}\)I (10\(\mu\)l). The reaction was allowed to proceed for 15 min during which time the vessel was gently agitated. The reaction was stopped by removal of the sample from the reaction vessel. A drop of 'cold' Na I was added (final concentration 0.25 M) to ensure safe handling. Unreacted Na\(^{125}\)I was removed by passing the sample through a short column of Sephadex G25 equilibrated with PBS.

2.2.7 DTP substitution of immunoproteins

Protein A (PA) powder was dissolved in water and a trace of \(^{125}\)I-labelled PA was added. Reactive dithiopyridyl (DTP) groups were introduced using SPDP. This was prepared as a 40\(\text{mM}\) solution in methanol and sufficient was added to produce a 10 molar excess. The
mixture was incubated for 30 min at room temperature and transferred to Hepes buffer (pH 7.4) by gel filtration through an equilibrated PD 10 column. This removed unwanted reaction products and unreacted SPDP. The radioactivity in each fraction of eluate was measured in a gamma counter and the peak fractions were pooled.

The substituted protein A (PA-DTP) was activated by conversion of the DTP groups to free thiol groups using the reducing agent dithiothreitol (DTT). DTT was freshly prepared as a 500 mM solution in Hepes buffer and sufficient was added to the PA-DTP to give a final concentration of 50 mM. The mixture was incubated for 20 min at room temperature and filtered through the PD 10 column now re-equilibrated with Hepes buffer (pH 8.0). The radioactivity of the fractions was counted as before and the three most radioactive were mixed immediately with a suspension of liposomes.

DTP-substitution of purified S&M-Ig was carried out in a similar manner with the exception that SPDP was added in 15 molar excess and after derivatisation the Ig-DTP was transferred to acetate buffer (pH 5.0) in order to protect the native disulphide bonds during DTT reduction.

2.2.8 Determination of DTP to protein ratio

The optimal DTP substitution of antibody for maximal binding to liposomes and minimal denaturation has been
shown elsewhere to be approximately 5 moles of DTP per mole of protein (Barbet et al, 1981). This was determined by the method of Carlson et al (1978) and was routinely performed on each batch of DTP-substituted protein as a quality control measure.

Twenty-five microlitres of Ig-DTP was diluted to 500ul with Hepes buffer in a quartz cuvette and the absorbance was measured at 280nm (for calculation of IgG concentration) and at 343nm which is the absorbance peak of free pyridine-2-thione (P2T). The instrument was zeroed on a buffer blank. Ten ul of 0.5M DTT in buffer was added to release the P2T and the increment in optical density at 343nm was recorded. The formula for calculating the P2T concentration was:

\[ c = \frac{A_2 - A_1}{E} \]

where \( c \) = molar concentration, \( A_2 \) = absorbance at 343nm after DTT, \( A_1 \) = absorbance at 343nm before DTT and \( E \) = molar extinction coefficient of P2T (8.08 x 10^{-3} M^{-1} cm^{-1}) at 343nm.

The concentration of IgG was measured by the absorbance method at 280nm but a correction factor was required to allow for the presence of the DTP groups. Thus:

\[ A_{280} \text{ due to IgG} = A_{280} - (B \times 5.1 \times 10^3) \]

where \( B \) is the molar concentration of P2T as calculated above and 5.1 x 10^3 is the molar extinction coefficient
of P2T at 280nm.

The concentration of IgG in mg/ml was calculated by dividing the corrected absorbance by 1.4 (see above) and converting to the molar concentration by dividing by the molecular weight (150,000).

The number of DTP residues per IgG molecule was calculated by dividing the molar concentration of P2T by the molar concentration of IgG.

The concentration of PA could not be measured by absorbance and was calculated by measuring the radioactivity in an aliquot as a fraction of the activity in the native starting solution whose concentration was known. Protein derivations used in these studies averaged 3-9 DTP residues for protein A and 9-13 for IgG.

2.2.9 Coupling of immunoproteins and liposomes

Thiolated PA or IgG was added to a suspension of liposomes and incubated at room temperature for 24 hours. Unattached protein was removed by passage through a small column of Sepharose CL-6B equilibrated in Hepes buffer (pH 7.4). Two peaks of radioactivity were obtained enabling the efficiency of the coupling reaction between protein and liposomes to be monitored and calculated.

2.2.10 Antibodies

UCHT1 is a monoclonal anti-CD3 antibody (murine)
The original antibody is subclass IgG1 but IgG2b and IgG2a switch-variants of the same antibody have also been produced (Beverley and Cellard 1981, Smith et al 1986). Saturating concentrations (>2.5 μg/ml) of culture supernatant or ascitic fluid were used except where specifically mentioned.

As well as its affinity for CD3, UCHT1 is notable for inducing a mitogenic response in T lymphocytes in certain individuals (responders). Therefore when present in cell suspensions, the antibody was able to be used both to target liposomes conjugated with PA or S&M-IgG and to induce T-cell proliferation in cytotoxicity experiments.

Fluorescent goat anti-mouse immunoglobulin (G&M-FITC) was obtained from Nordic Laboratories.

2.2.11 Preparation of lymphocytes

Peripheral blood from healthy volunteers was taken into preservative free heparin (2 i.u./ml). Samples were diluted to approximately twice the original volume with cell culture medium (RPMI 1640, Gibco) and layered on to an approximately equal volume of Ficoll-Hypaque (Pharmacia). The tubes were centrifuged at 1000g for 20 minutes at room temperature and low density mononuclear cells were collected from the interface using a Pasteur pipette. The cells were washed twice (350g, 7.5 minutes, room temperature) and resuspended in RPMI 1640 with 10% FCS.
PHA-lymphoblasts were obtained by incubation of peripheral blood mononuclear cells (1 x 10^6/ml) for 72 h in cell culture medium containing 10% fetal calf serum and purified phytohaemagglutinin (PHA; 4ug/ml).

2.2.12 E-rosetting method

Peripheral blood mononuclear cells (PBMC) were enriched for T lymphocytes (E positive) by rosette formation of CD2 positive T lymphocytes with AET-treated sheep red blood cells (SRBC) (Kaplan and Clark, 1974). Briefly, suspensions of PBMC at 10^7/ml in RPMI 1640 were mixed with an equal volume of 4% AET treated SRBC and 20% (final volume) FCS. The cells were pelleted (400g for 5 min) and kept on ice for 45-60 min. The pellet was gently resuspended, layered on to Ficoll-Hypaque and centrifuged (100g for 15 min at room temperature).

E negative cells were collected from the interface and washed twice in RPMI 1640 with 10% FCS. E positive cells were separated from the rosetted SRBC by hypotonic lysis with double distilled water for approximately 30 sec, washed and resuspended as for the E negative fraction.

2.2.13 Immunofluorescent phenotyping

Aliquots of 2 x 10^5 peripheral blood lymphocytes (PBL) were reacted with monoclonal antibodies in microtitre plates as previously described by Linch et al
Fifty microlitres of antibody (first layer) were followed by 50mcl of liposomes or 50mcl of FITC-S&K (second layer). Both first and second layer incubations were for 30 minutes at 4°C with 3 washes in ice-cold medium after each incubation.

2.2.14 Flow cytometry

Cells were analysed on a FACS IV (Becton Dickinson) or an Epics Profile (Coulter Electronics) flow cytometer using a 488nm light source from an argon laser (300mW). For each analysis an average of 10,000 cells was counted. The fluorescent level at which cells were determined to be positive was established by visual inspection of the histogram of negative (control) cells such that less than 5% appeared in the positive zone. The fluorescence intensity per cell (as the channel-index number) was recorded using a linear amplifier and the mean cell fluorescence (MCF) was calculated as the sum of the products of the number of cells per channel and the channel-index number divided by the total number of cells recorded:

\[
\text{MCF} = \frac{\text{cells per channel} \times \text{channel-index number}}{\text{total number of cells}}
\]

Where specified, certain photographs display the relative fluorescence intensity on a log scale.

Reference is made throughout the text to the use of UV microscopy. Whereas the mercury vapour lamp of the instrument was capable of emitting light at UV wavelengths, it was blue light (wavelength circa 498nm) which was used to excite fluorescein. A more accurate term might be fluorescent microscopy.
CHAPTER 3

PRELIMINARY EXPERIMENTS IN LIPOSOme PRODUCTION AND PROTEIN CONJUGATION
3.1 INTRODUCTION

For the purpose of encapsulating fluorescent dyes and cytotoxic drugs the properties required of liposomes are the same: (i) low permeability of encapsulated solutes (ii) retention of targeting ability over reasonable lengths of time i.e. a good shelf life and (iii) minimal non-specific interaction with cells. This chapter aims to select the most appropriate type of liposome for conjugation to antibody or protein A and to test the physical properties of the product.

Liposomes come in a bewildering variety of sizes, structures and compositions and it would be impossible rigorously to test each species before making the most appropriate choice. A random selection can nevertheless be avoided by the following considerations.

The lipids conferring the most stability are those which are fully saturated (hydrogenated). The presence of double bonds in the carbon chains of the phospholipid molecule prevents tight packing in the liposomal bilayer which increases the permeability of encapsulated solutes. Another disadvantage is that double bonds are susceptible to oxidation which again interferes with the packing of the molecules and hence to an increase in permeability. Careful storage of lipids and liposomes under an inert atmosphere is necessary.

Lipids which are in the 'solid' state at 37°C are less permeable than those in the 'fluid' state. This
suggests the selection of lipids with a $T_c$ above $37^\circ C$ and this is largely dictated by the fatty acyl chain length. In general an increase in the number of carbon atoms results in an increase in $T_c$. The $T_c$ and carbon chain lengths of various commonly used phospholipids are given in Table 1.1.

Phospholipids can be obtained by chemical synthesis or in the native form extracted from sources such as egg lecithin. The latter are usually mixed-chain phospholipids with unequal chain-lengths in each molecule as well as being only partially saturated. Synthetic lipids are preferrable since they are of more uniform chain-length, have a more clearly defined $T_c$ and can be obtained in the fully saturated state. Neutral as opposed to charged phospholipids are also preferrable to minimise non-specific interaction with cells.

Cholesterol which is a major component of almost all biological membranes has an important architectural role in reducing the surface area occupied by phospholipid molecules arranged in bilayers and this is accompanied by a decrease in permeability.

With regard to liposome structure the greatest stability is associated with SUV. MLV and LUV while possessing larger internal aqueous compartments compared to SUV are more permeable and unstable (see Table 1.2).

The method of coupling of the targeting protein to the liposomal membrane is also an important
consideration. Ideally a strong covalent bond is preferred to weaker forms of association such as hydrophobic interaction but the method must also avoid the risk of homopolymerisation or denaturation of the protein. Likewise reaction conditions must be mild such that the integrity of the liposome is not compromised.

All of these requirements were taken into account in selecting the most suitable methodology and species of targetable liposome from the wide variety described in the literature. The most suitable appeared to be SUV composed of DPPC and cholesterol targeted using immunoprotein (Protein A or IgG) covalently coupled to the liposomes using SPDP. The characteristics of this species of liposome were assessed in a series of preliminary experiments.

3.2 RESULTS
3.2.1 Encapsulation of solute

Liposomes were produced as described in Chapter 2 but omitting DPPE-DTP since protein was not coupled for this purpose. The concentration of encapsulated CF was 100mM. The suspension was applied to a short (5 cm) column of Sephadex G25 without prior removal of unencapsulated CF and the fractions (0.5 ml) were analysed for both lipid and fluorescence.

As shown in Fig. 3.1 inorganic phosphate appeared in the eluate in the fractions corresponding to 6 to 10ml
(the void volume of the column) indicating the presence of liposomes. CF appeared in two peaks corresponding to the void and elution volumes (Fig. 3.2). Due to the high initial concentration of CF (100 mM) the encapsulated CF is strongly quenched and fluorescence is only detectable in the elution volume (12 to 20 ml). Quenching can be relieved by treatment with detergent and after addition of Triton-x there was a marked increase in fluorescence of the fractions from the void volume (liposome peak). No such increase was observed in the fractions from the elution volume.

3.2.2 Coupling of protein

Attachment of immunoprotein was carried out as described in Chapter 2 utilising DTP-substituted DPPE as the anchor lipid and protein derivatised with the same reagent and activated by mild reduction. After 24 hr the mixture was applied to a 20 cm column of Sepharose 4B. Representative profiles of the radioactivity counts in each fraction (0.4 ml) for IgG (UCHT1) and Protein A are given in Fig. 3.3. As measured by radioactive counting approximately 40% of available IgG and 20% of PA became coupled to liposomes. The final concentration of liposomes (total phospholipid) was assayed at 2.5 mM (see Chapter 2.2.3), that of PA 1.3 uM and that of antibody 0.4 uM.
Column chromatography of liposomes. An aliquot of liposomes was applied to a 5cm column of Sephadex G25 and the phospholipid content of the eluate was assayed. Liposomes appeared as a narrow peak corresponding to the void volume of the column.
Column chromatography of liposomes as in Fig 3.1. The liposomes contained 100mM carboxyfluorescein and fluorimetry was performed on intact liposomes (—□—) and liposomes after the addition of Triton X-100 (—◆—).
Coupling of liposomes to Protein A and IgG. Small unilamellar liposomes (SUV) were covalently coupled to Protein A (---) and IgG (---) using the heterobifunctional cross-linking agent SPDP. The reaction was followed using trace amounts of $^{125}$I-labelled protein. Uncoupled protein (second peak) was removed by column chromatography (Sepharose 4B). Protein coupled to liposomes appeared in the void volume of the column (first peak).
A control incubation was performed using derivatised IgG activated with DTT and maintained under the same coupling conditions i.e. 24 h at room temperature but in the absence of liposomes. The radioactivity profile (Fig. 3.4 upper trace) showed most of the IgG in the elution volume with only a trace (<0.5%) of protein in the void volume. A similar elution profile was obtained after incubation of liposomes and unactivated protein under the same conditions (Fig. 3.4 lower trace).

3.2.3 Targeting of liposomes

The ability to attach to a specific cell population was assessed using protein A-bearing liposomes and peripheral blood lymphocytes (PBL) labelled with UCHT1, a monoclonal anti-T cell receptor (anti-CD3) antibody (see Chapter 2.2.10). PBL are 65 to 75% T cells, with B cells and non-B, non-T lymphocytes comprising the majority of the remainder. Because B cells do not express CD3 and hence do not bind UCHT1, they are not detected by the fluorescent second layer antibody technique (indirect immunofluorescence) and do not attach liposomes. The non-T cell population acts as a useful internal control for any non-specific attachment of liposomes.

Flow cytometric analysis of PBL labelled with UCHT1 antibody and SUV-PA containing 40 mM CF at 4°C is shown in Table 3.1. The results are given as the percentage
Homopolymerisation of protein and passive uptake by liposomes. IgG derivatised with SPDP and activated by DTT was incubated for 24 hr at room temperature. A trace amount of $^{125}$I labelled IgG was included. Virtually all IgG was recovered in the elution volume (——). SUV incubated under the same conditions with unactivated protein failed to couple IgG (---).
of fluorescence positive cells and the relative fluorescence intensity (peak channel number). The negative control in each case was cells incubated with the fluorescent layer alone i.e. without UCHT1, and the positive control was cells incubated with UCHT1 and the conventional second layer antibody labelled with FITC (G&M-FITC).

Protein A bearing liposomes attached to approximately equal numbers of PBL (65-70%) as G&M-FITC when UCHT1 (IgG2a & IgG2b) was present. When UCHT1 was omitted the cells became negative and the small number of positives was again approximately equal in both cases. Protein A liposomes therefore bound to cells labelled with antibody. The 30% or so of PBL which were not fluorescent were the B cells and non-B, non-T lymphocytes which, though ontologically closely related to the T cells, did not bear the targeting antibody.

Protein A binds poorly to IgG1 subclass antibodies at physiological pH (Ey et al 1976). This characteristic was exploited to exclude the possibility that T-cell binding was not a non-specific interaction between cell-bound antibody and liposomes. The staining performance of the IgG1, IgG2b and IgG2a switch variants of the antibody were compared and although the IgG1 subclass of UCHT1 was present on the cell surface as indicated by its ability to bind G&M-FITC (Table 3.1), it was unable to bind protein A liposomes. However the
Peripheral blood lymphocytes were incubated with the IgG1, IgG2\textsubscript{a} and IgG2\textsubscript{b} switch variants of the anti-CD3 antibody (UCHT1) and stained with either FITC-conjugated goat anti-mouse antibody (G&M-FITC) or protein A bearing liposomes containing 40mM carboxyfluorescein (SUV-PA). Negative controls were cells incubated with the second layer alone. Fluorescence was measured by flow cytometry in a FACS IV (Becton Dickinson) and the results expressed as the percentage of fluorescence-positive cells and the relative fluorescence intensity. The ability of these antibodies to bind G&M-FITC and SUV-PA was compared.

The percentage of positives and their relative fluorescent intensity in the control cells has been subtracted from the values given for the positive cells.

### Table 3.1

<table>
<thead>
<tr>
<th></th>
<th>% of positive cells</th>
<th>Relative Fluorescence Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>cells + G&amp;M-FITC</td>
<td>7</td>
<td>27</td>
</tr>
<tr>
<td>cells + SUV-PA</td>
<td>8</td>
<td>29</td>
</tr>
<tr>
<td>cells + UCHT1+(IgG1)+G&amp;M-FITC</td>
<td>67</td>
<td>96</td>
</tr>
<tr>
<td>cells + UCHT1(IgG1)+SUV-PA</td>
<td>10</td>
<td>43</td>
</tr>
<tr>
<td>cells + UCHT1(IgG2\textsubscript{b})+G&amp;M-FITC</td>
<td>65</td>
<td>95</td>
</tr>
<tr>
<td>cells + UCHT1(IgG2\textsubscript{b})+SUV-PA</td>
<td>65</td>
<td>91</td>
</tr>
<tr>
<td>cells + UCHT1(IgG2\textsubscript{a})+G&amp;M-FITC</td>
<td>64</td>
<td>97</td>
</tr>
<tr>
<td>cells + UCHT1(IgG2\textsubscript{a})+SUV-PA</td>
<td>65</td>
<td>95</td>
</tr>
</tbody>
</table>
IgG2β and IgG2α variants were able to bind liposomes and G&M-FITC equally well and to bind the latter as effectively as the IgG1. These data indicate that the interaction of protein A liposomes with T cells is solely by means of protein A-antibody bridging.

3.2.4 Retention of targeting ability

This was assessed by comparison of the staining performance of freshly made SUV-PA with that of the same batch of liposomes after 3 months storage at 4°C in the dark. FITC-conjugated anti-mouse antibody was used as a reference standard and the same batch stored at -20°C was used for the phenotyping 3 months later. As shown in Table 3.3 the percentage of positive cells with each fluorescent label was the same on each occasion although different between occasions (different volunteer donor). The relative fluorescence intensity of the liposome compared to the FITC-labelled cells was unchanged (106.5% versus 105.8%) over this interval of time.

3.2.5 Liposomal stability

Retention of CF by a suspension of Protein A-coupled liposomes was tested after 4 weeks at 4°C by passage of an aliquot through a column of Sephadex G50 thereby separating free CF from that remaining in the liposomes. A small amount of Triton-X was added to each fraction of eluate to release encapsulated CF and the amount of
Table 3.2

**Effect of Storage on Liposome Targeting**

<table>
<thead>
<tr>
<th></th>
<th>Fresh</th>
<th>4 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>RFI</td>
</tr>
<tr>
<td>FITC</td>
<td>65</td>
<td>155</td>
</tr>
<tr>
<td>SUV-PA</td>
<td>64</td>
<td>164</td>
</tr>
</tbody>
</table>

**Ratio of RFI (%)**

<table>
<thead>
<tr>
<th>Fresh</th>
<th>105.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 months</td>
<td>106.5</td>
</tr>
</tbody>
</table>

Peripheral blood lymphocytes were labelled with UCHT1 and either freshly made liposomes (SUV-PA) or FITC conjugated anti-mouse antibody then analysed by flow cytometry. The staining performance was compared to the results obtained after storage of the liposomes for four months. The figures represent the percentage positive cells and the relative fluorescence intensity (RFI).
fluorescence in the encapsulated peak was expressed as a percentage of the total fluorescence present.

Retention of CF was also tested after incubation in conditions known to promote liposome permeability, namely the presence of serum and a higher temperature. An aliquot of SUV-PA was incubated at 37°C for an hour in tissue culture medium containing 10% foetal calf serum after which it was processed as above. There was 96% retention of liposomal CF after a month of storage at 4°C; after an hour’s incubation the retention was 99.4%.

3.2.6 Electron microscopy

SUV were negatively stained with 1% uranyl acetate and applied to carbon coated grids. Photomicrographs (Fig. 3.5) were taken at a magnification of 100,000 and were themselves twice actual size. The approximate size of the liposomes was 50 nm.

3.3 DISCUSSION

Column chromatography with gels of appropriate fractionation range is useful for demonstrating both the encapsulation of a solute in the aqueous phase and the attachment of protein to the lipid phase of liposomes. In each case the free material (CF and immunoprotein) appears in the elution volume of the column and co-elutes with liposomes in the void volume only in the bound form.
Electron photomicrographs of SUV examined with a Jeol JEM 1200 EX instrument and the technical assistance of Dr. G. McPhail, UCMSM.
True encapsulation as opposed to simple partitioning of CF into the bilayer is ensured by purification of the salt to remove hydrophobic contaminants prior to liposomas synthesis. The marked effect of detergent also implies release of encapsulated CF with unquenching of fluorescence rather than a lipid partitioning phenomenon.

The attachment of protein is a covalent as opposed to a hydrophobic interaction since DTP-substituted protein does not associate with liposomas unless reduced with DTT (Fig. 3.4). This results in free thiol groups capable of forming di sulphide bonds with the DTP-substituted lipid in the liposomas. The theoretical possibility of free thiol groups reacting together to produce polymers could result in protein aggregates sufficiently large to co-elute with the liposomas thereby mimicking covalent attachment. However homopolymerisation is excluded by the control incubation (Fig. 3.4) of activated protein without liposomas whereupon significant amounts of protein did not appear in the void volume.

Ultrastructurally the liposomas appeared as vesicles containing a central unstained zone representing the aqueous compartment, bounded by a single membrane in which a bilayer arrangement was faintly visible. No structural differences were discernible between protein and non-protein bearing liposomas.
Successful conjugation of immunoprotein to the liposome is no guarantee per se of immunological targeting. Preservation of protein function may be impaired by chemical modifications at the antigen combining site or by steric interference from the liposome. A simple global test of these effects is indirect immunofluorescent phenotyping comparing the performance of PA-liposomes with that of the standard FITC-antibody method. Since both methods gave the same percentage of fluorescent cells with satisfactory negative controls (Table 3.1), and since true protein A-antibody bridging was demonstrable by the use of different subclasses of IgG (Table 3.1), it can be concluded that protein function is not seriously compromised by conjugation to liposomes.

The choice of lipid and coupling method also favour a liposome species with maximal stability. No significant deterioration in either targeting or fluorescence was detected after at least 3 months storage. This long 'shelf-life' is achieved by the use of saturated lipids of relatively high transition temperature and the inclusion of cholesterol, features which have been shown to reduce the permeability of solutes across the membrane (Papahadjopoulos et al 1971). The coupling method is such that good levels of antibody attachment are achieved without the exposure of the liposome to oxidising agents.
or detergents that characterise other methods of production.

In summary this chapter has explored the methodology of synthesis and evaluated the physical characteristics of a selected liposome species. The techniques were found to be relatively easy to master and reproduce. The size, structure, encapsulation volume, efficiency of protein attachment and targeting function were found to agree closely with those described by others (Barbet et al 1981). The targeting ability and remarkable stability of the product justified its adoption in further applied studies.
CHAPTER 4

IMMUNOPHENOTYPING OF LYMPHOCYTES BY FLUORESCENT LIPOSOMES
4.1 INTRODUCTION

Fluorescein isothiocyanate conjugated anti-mouse immunoglobulin is the most commonly used probe for phenotyping with monoclonal antibodies and for fluorescence activated cell sorting. It is ideal for high density antigens but is less satisfactory for antigens less than a few thousand per cell. Many important antigens such as the interleukin-2 (IL-2) receptor on resting lymphocytes are present at such low numbers for which an improved fluorescent detection system is required. Leucocytes have a relatively high autofluorescence due to the presence of pyridine and flavin nucleotides. This broad band of autofluorescence overlies the emission wavelength of fluorescein and instruments are incapable of distinguishing the contributions from each source. Signals from small quantities of cell-bound FITC-antibody may therefore be subsumed into the proportionately larger autofluorescence component.

Liposomes by virtue of their relatively large internal aqueous space can carry increased numbers of fluorescent molecules but this is only of value if the signal due to non-specific binding is not increased pari passu. In this chapter liposomes are optimised with respect to fluorescent signal and choice of ligand for use as a second layer in indirect immunofluorescent phenotyping of peripheral blood lymphocytes. The results
are compared with the conventional FITC-antibody technique. The IL-2 receptor which is not detectable by the latter method was sought using fluorescent liposomes.

4.2 MATERIALS AND METHODS

4.2.1 Antibodies

UCHT1 (subclasses IgG<sub>1</sub>, IgG<sub>2a</sub>, and IgG<sub>2b</sub>), UCHT2, UCHT4, LFA-2 and unpurified sheep anti-mouse IgG antibody were a kind gift of Prof. P. Beverley as were 7G7 and anti-Tac; a third anti-IL-2 receptor antibody was obtained from Serotec. UCH gamma was obtained from the Department of Haematology, University College London. My9 was obtained from the third International Workshop and Conference on Human Leucocyte Differentiation Antigens (1987). Sheep anti-mouse IgG antibody was produced as described in Chapter 2.2.5. Conjugation with fluorescein isothiocyanate was carried out using the method of Hudson and Hay (1980).

4.2.2 Liposomes and FITC-labelled antibody

A family of unconjugated liposomes was generated which was identical in all but CF concentration and this was varied from 1mM to 200mM. The concentration of CF giving maximally fluorescent SUV was determined by fluorimetry. Protein A (Pharmacia) or sheep anti-mouse IgG antibody was covalently coupled to liposomes as
previously described (Chapter 2). The same batch of sheep anti-mouse IgG antibody was conjugated to FITC to produce a conventional label with the same antigen specificity as the antibody conjugated liposomes. The IgG concentration in the liposome and FITC conjugate was determined from the $^{125}$I-labeled protein counts and by spectrophotometry at 280nm respectively. The fluorescein concentration was obtained by fluorimetry at 495nm.

4.3 RESULTS

4.3.1 Choice of ligand

SUV (40mM CF) coupled to protein A (SUV-PA) or sheep anti-mouse IgG antibody (SUV-S&M) were compared to FITC-labeled sheep anti-mouse antibody (FITC-S&M) in their ability to bind to PBL previously reacted with the three available switch-variants of the anti-CD3 monoclonal antibody UCHT1 (Table 4.1). The SUV-S&M gave the same percentage (approximately 65%) of positive cells as the FITC-S&M regardless of the anti-CD3 antibody subclass.

4.3.2 Concentration of CF and F/P ratio

The concentration of CF which gave maximally fluorescent liposomes was 20mM (Fig. 4.1). Although more CF was contained in the other liposomes the signal was weaker due to the increasing effect of concentration quenching as demonstrated by lysis of the liposomal
Table 4.1

Targeting of Liposomes to UCHTI-coated PBL

<table>
<thead>
<tr>
<th>Fluorescent</th>
<th>First layer antibody</th>
<th>UCHT1</th>
<th>Antibody subclass</th>
</tr>
</thead>
<tbody>
<tr>
<td>second layer</td>
<td>None</td>
<td>UCHTI</td>
<td></td>
</tr>
<tr>
<td>reagent</td>
<td></td>
<td></td>
<td>IgG1</td>
</tr>
<tr>
<td>FITC-S&amp;M</td>
<td>7%</td>
<td></td>
<td>67%</td>
</tr>
<tr>
<td>SUV-S&amp;M</td>
<td>5</td>
<td></td>
<td>66</td>
</tr>
<tr>
<td>SUV-PA</td>
<td>8</td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

Figures are the means of two experiments using two different batches each of SUV-PA and SUV-S&M in PBL from healthy volunteers. The SUV-PA as seen in the previous chapter failed to bind to PBL coated with the IgG1 subclass of antibody at pH 7.4. Staining with the fluorescent layer alone gave comparably negative background levels with each type.
membrane with Triton-X. Quenching was calculated according to the formula:

\[ Q = 100 - \left( \frac{Fluorescence \text{ before Triton-X}}{Fluorescence \text{ after Triton-X}} \right) \times 100\% \]

The molar fluorescein / protein (F/P) ratio for conjugated liposomes containing 20mM CF and for that of the corresponding FITC conjugate was 60 and 4 respectively.

4.3.3 Sensitivity and specificity

The preferred liposome species was therefore considered to be SUV containing 20 mM CF and coupled to sheep anti-mouse antibody. The optimal dilution of the liposome suspension to obtain maximum signal to noise ratio was approximately 1 in 4 although dilutions of the liposomes up to 1 in 128 provided a similar value for the percentage of PBL reacting with UCHT1 (Table 4.2). The fluorescence characteristics of the 1 in 4 dilution and those of a similarly optimised concentration of FITC-S&M were compared using a panel of monoclonal antibodies and the corresponding antigens expressed on resting PBL (Table 4.3). The enhancement of mean cell fluorescence (calculated after subtraction of background) was increased approximately 9-fold in the case of UCHT1 and from 3.1 to 7.8 times with antibodies against a range of
The fluorescence of liposomes containing various concentrations of CF was measured (—□—). CF was released from liposomes by detergent lysis using Triton-x and the percentage of fluorescence quenching (—○—) was calculated according to the formula:

\[ Q = 100 - \frac{\text{Fluorescence before Triton-x}}{\text{Fluorescence after Triton-x}} \times 100 \]
### Table 4.2

**Titration of Liposomes Coupled with Sheep Anti-mouse Antibody**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution of SUV-S&amp;M</th>
<th>% positive cells</th>
<th>Relative fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>cells + Ab + SUV-S&amp;M</td>
<td>1:1</td>
<td>65</td>
<td>249</td>
</tr>
<tr>
<td></td>
<td>1:2(a)</td>
<td>71</td>
<td>241</td>
</tr>
<tr>
<td></td>
<td>1:2(b)</td>
<td>73</td>
<td>244</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>75</td>
<td>217</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>75</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td>1:16</td>
<td>71</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td>1:32</td>
<td>66</td>
<td>164</td>
</tr>
<tr>
<td></td>
<td>1:64</td>
<td>71</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>1:128</td>
<td>62</td>
<td>121</td>
</tr>
<tr>
<td>cells + SUV-S&amp;M</td>
<td>1:1</td>
<td>6</td>
<td>38</td>
</tr>
</tbody>
</table>

PBL were labelled with antibody (UCHT1) and various dilutions of liposomes coupled to sheep anti-mouse antibody (SUV-S&M). The percentage of positive cells and the relative fluorescence intensity measured by flow cytometry were compared. The optimum working dilution was established as 1:4. Cells with a 1:2 dilution were compared before (a) and after (b) fixation with 1% formaldehyde.
other lymphocyte antigens. The percentage of positive cells was the same with each antibody by either technique indicating that the increase in sensitivity was not at the expense of specificity. This was confirmed by comparison of the background staining due to SUV-S&M with that of FITC-S&M. In 4 experiments using 2 different liposome preparations the MCF was 20.1 +/- 4.4 compared to 26.1 +/- 1.9 with FITC-S&M. Similarly in an experiment comparing the autofluorescence of the lymphocytes with the fluorescence of the cells after incubation with SUV-S&M in the absence of UCHT1 (autofluorescence of cells plus non-specific binding of label) it was found that at the optimal dilution (1:4) non-specific binding of SUV-S&M added only minimally to the autofluorescence (Table 4.4, 15.4 v 14.5).

Figure 4.2 is the FACS histograms of PBL reacted with and without UCHT1 using either FITC-S&M (Fig. 4.2 a) or SUV-S&M (Fig. 4.2 b) as the second layer. The SUV-S&M results in a clear superiority in brightness of the positive cells without a parallel increase in background staining.

4.3.4 Detection of interleukin-2 receptor

Optimised liposomes were used to probe for the IL-2 receptor against which a number of monoclonal antibodies have been raised and characterised. The E-rosette positive fraction of PBL was isolated (Chapter 2.2.12)
**Table 4.3**

Enhancement of mean cell fluorescence by SUV-S&M

<table>
<thead>
<tr>
<th>Ab</th>
<th>CD no. and Isotype</th>
<th>% positive cells</th>
<th>Mean cell fluorescence FITC-S&amp;M</th>
<th>Mean cell fluorescence SUV-S&amp;M</th>
<th>Mean cell fluorescence enhancement &amp; S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>8</td>
<td>16</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>UCHT1</td>
<td>CD3 IgG1</td>
<td>77</td>
<td>50</td>
<td>351</td>
<td>8.9 &amp; 0.9</td>
</tr>
<tr>
<td>UCHT4</td>
<td>CD8 IgG1</td>
<td>25</td>
<td>28.5</td>
<td>591</td>
<td>3.1 &amp; 0.8</td>
</tr>
<tr>
<td>Leu 3a</td>
<td>CD4 IgG1</td>
<td>55</td>
<td>59.5</td>
<td>180</td>
<td>7.8 &amp; 0.5</td>
</tr>
<tr>
<td>UCHT2</td>
<td>CD5 IgG1</td>
<td>79</td>
<td>80.5</td>
<td>170</td>
<td>3.3 &amp; 1.4</td>
</tr>
<tr>
<td>LFA-2</td>
<td>CD2 IgG1</td>
<td>84</td>
<td>86</td>
<td>145</td>
<td>3.7 &amp; 0.7</td>
</tr>
</tbody>
</table>

PBL were reacted with a range of monoclonal antibodies and labelled with sheep anti-mouse antibody conjugated to either FITC or liposomes containing 20mM CF. A representative example of the percentage positive cells and MCFs is given. The mean cell fluorescence (MCF) enhancement factor of liposome-labelled over FITC-labelled cells was averaged over three experiments using three separately synthesised batches of liposomes. The enhancement factor was calculated as follows: the MCF of the negative cells was subtracted from that of the positive cells to give an increment for each label and the increments were divided to give the enhancement factor for liposomes.
FACS histograms of PBL labelled with (a) FITC-S&M or (b) SUV-S&M. Relative fluorescence intensity (log scale) and cell number (linear scale) are on the x- and y-axes respectively. The right-hand and left-hand peaks are the cell profiles with (positive) and without (negative) first-layer antibody respectively.
Table 4.4

Comparison of Autofluorescence with Background Staining due to SUV-S&M

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution of SUV-S&amp;M</th>
<th>Mean cell fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>cells + SUV-S&amp;M</td>
<td>1:1</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>16.1</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td><strong>15.4</strong></td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>1:16</td>
<td>15.1</td>
</tr>
<tr>
<td></td>
<td>1:32</td>
<td>14.4</td>
</tr>
<tr>
<td>cells alone</td>
<td>Nil</td>
<td>14.5</td>
</tr>
</tbody>
</table>

PBL were incubated for 30 minutes at 4°C with various dilutions of liposomes and their fluorescence was compared to that of unmodified cells. The optimum working dilution (underlined) which produced the maximum signal to noise ratio was determined by titration (Table 4.2).
and reacted with three different anti-IL-2 receptor antibodies namely 7G7, Serotec and anti-Tac. As negative controls, cells were incubated with irrelevant antibodies of the same IgG subclass as the anti-IL-2 receptor antibodies. The irrelevant antibodies were My9, an anti-myeloid antibody and an antibody to the gamma chain of human foetal haemoglobin.

E-positive cells stained with anti-IL-2 receptor antibodies and FITC-conjugate gave the same percentage of positive cells and mean cell fluorescence as the negative controls (approximately 5 and 20 respectively, Table 4.5a). This compared with 19% of cells with an MCF of 36 when using sheep anti-mouse IgG liposomes. Positive controls were lymphocytes which had been stimulated with PHA in culture for five days. Between 35 and 58% of such cells were positive and the percentage with each antibody was similar using either fluorescent label (Table 4.5b). In contrast, no IL-2 receptor positive cells were detectable by either method in the E-negative fraction (Table 4.5c).

4.4 DISCUSSION

Targeting of liposomes to cells has been achieved in the past by covalent attachment to the liposomal bilayer of antibody itself or protein A (Leserman et al. 1980).
### Table 4.5

Detection of IL-2 receptor on E-rosette positive lymphocytes

<table>
<thead>
<tr>
<th>Antibody (IgG subclass)</th>
<th>FITC</th>
<th>MCF</th>
<th>SUV</th>
<th>MCF</th>
</tr>
</thead>
<tbody>
<tr>
<td>2nd layer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>My9 (2a)</td>
<td>6.5±4.1</td>
<td>22.0±8.3</td>
<td>4.8±3.3</td>
<td>20.1±6.5</td>
</tr>
<tr>
<td>UCH gamma (1)</td>
<td>6.0±5.6</td>
<td>17.3±8.3</td>
<td>3.3±1.2</td>
<td>15.6±1.6</td>
</tr>
<tr>
<td>7G7 (2a)</td>
<td>4.5±2.6</td>
<td>21.3±7.9</td>
<td>19.8±6.1</td>
<td>34.8±14.2</td>
</tr>
<tr>
<td>Serotec (1)</td>
<td>4.2±2.1</td>
<td>17.0±7.7</td>
<td>18.6±5.2</td>
<td>36.0±9.9</td>
</tr>
<tr>
<td>anti-Tac (2a)</td>
<td>5.3±1.9</td>
<td>22.0±8.0</td>
<td>19.0±7.1</td>
<td>36.5±11.2</td>
</tr>
</tbody>
</table>

b)  
2nd layer  |  3.0 |  20.0 |  0.6 |  15  
My9        |  3.0 |  18.0 |  1.0 |  16  
UCH gamma  |  4.0 |  19.0 |  0.7 |  17  
7G7        |  35  |  44   |  39  |  47  
Serotec    |  48  |  55   |  58  |  74  

c)  
2nd layer  |  5.0 |  38   |  6.0 |  44  
My9        |  3.0 |  39   |  5.0 |  40  
UCH gamma  |  6.0 |  42   |  10.0|  48  
7G7        |  5.0 |  43   |  5.0 |  41  
Anti-Tac   |  6.0 |  44   |  10.0|  49  

Legend to Table 4.5

E-rosette positive lymphocytes (a) were labelled with three anti-IL-2 receptor antibodies (7G7, Serotec, anti-Tac) and either FITC or liposome conjugate. The percentage positive cells and mean cell fluorescence (MCF) were measured by flow cytometry. Negative control cells were incubated with irrelevant antibodies (My9 and UCH gamma) or with second layer reagent only. PHA-lymphoblasts (b) and E-rosette negative lymphocytes (c) were labelled and analysed as in (a).

The experiment used a single batch of liposomes and PBL from each of three volunteers. PHA-lymphoblasts and E-rosette negative lymphocytes were derived from one of the volunteers.
Both approaches however have serious limitations in applicability. Direct attachment of antibody has the disadvantage of committing a suspension of liposomes to a single antigen target creating the need for a different liposome preparation for each antigen. The protein A system relies upon the affinity of this Staphylococcal protein for the Fc portion of the immunoglobulin molecule. Cells are incubated with targeting antibody and the excess is washed off prior to addition of the liposomes which are therefore uncommitted or polyspecific. The disadvantage is that protein A does not bind to all subclasses of mouse antibody at pH 7.4. At this pH it has only low affinity for IgG1, a commonly used subclass of mouse monoclonal (Table 4.1). Another potential disadvantage is that protein A may bind to endogenous surface (human) IgG expressed on non-targeted cells e.g. B lymphocytes. Protein G, also derived from bacteria and recently purified, binds to a wider range of immunoglobulin species but still has poor affinity for mouse IgG1 (Bjorck et al 1984). It was reasoned therefore that a better ligand to be coupled to liposomes would be polyclonal antimouse immunoglobulin and it is shown here that sheep anti-mouse antibody can be covalently coupled to liposomes using SPDP without loss of reactivity. This liposome species detects IgG1 antibodies as well as IgG2a and IgG2b.
The amount of CF which can be encapsulated in liposomes is dependent on the volume of the internal aqueous space and the solubility of the CF salt. However high concentrations result in only weakly fluorescent liposomes due to the phenomenon of concentration quenching. Measurements on a family of liposomes encapsulating different concentrations of CF (Fig. 4.1) determined the maximally fluorescent liposome as containing approximately 20mM CF. In a previous study liposomes with only half this concentration of CF were selected (Truneh et al 1987). However these liposomes (which were directly coupled to the targeting monoclonal antibody) were evaluated at concentrations of only 1, 10 and 50 mM CF. Since small variations around the optimal CF concentration have a critical effect on fluorescence, it is essential to study liposomes with small incremental differences in the concentration of the fluorophore.

At 20 mM CF the F/P ratio of liposomes coupled to S&M is 60 whereas FITC-conjugated antibody can carry up to only 4 molecules of fluorescein (F/P ratio = 4) without affecting the affinity of the antibody. In comparative tests with FITC-S&M the mean cell fluorescence (MCF) of PBL incubated with various antibodies was 3.1 to 8.9 fold higher with SUV-S&M. On the FACS histogram (Fig. 4.2), this is illustrated by the clear shift to the right of the fluorescent peak of the liposome-labelled cells. That this enhancement was not
achieved at the expense of increased background staining is shown by comparison of the cellular fluorescence after incubation with either label in the absence of first layer antibody. The MCF is very similar in both and close to the autofluorescence of the cells.

The theoretically possible 15-fold increase in MCF predicted by comparison of the F/P ratios was not observed with any of the antibodies tested. Since antibody-conjugated liposomes are large (approximately 500 Å) when compared to FITC-antibody conjugates, some of the shortfall may be explained by steric hindrance around the antigen epitopes on the cell-bound antibody. It also needs to be explained why some antibodies e.g. UCHT1 gave a consistently higher MCF enhancement factor than others e.g. UCHT4. It may be that the epitopes on the monoclonal antibodies differ in their accessibility to sheep anti-mouse antibody when it is linked to FITC compared to liposomes. At the level of the cell surface antigen it is also conceivable that adjacent membrane structures and the configuration or clustering of cell surface antigens could interfere with the binding of the larger antibody-coupled liposomes.

Optimised liposomes also proved superior to the FITC-conjugate in the detection of the IL-2 receptor on resting peripheral blood T cells. This receptor is not normally detected on resting T cells and reduced expression has been demonstrated in patients with
acquired immunodeficiency syndrome and common variable hypogammaglobulinaemia. IL-2 plays a crucial role in T cell proliferation and cellular immune responses require that T cells change from a resting to an activated state.

To exert its biologic effect, IL-2 must interact with specific membrane receptors. These have been previously demonstrated on resting T cells only by means of sensitive methods such as radiolabelling, using which it has been calculated that there are approximately 1000 such receptors per cell (Smith 1980).

Using fluorescent liposomes, 20% of cells were consistently positive for this antigen and negative with the FITC label. The most likely explanation for the negative result with FITC-conjugate is that the signal is too weak relative to the autofluorescence of the lymphocyte. It has been calculated that the fluorescence emission from unstained murine lymphocytes excited at 488nm and measured through the same filters used to detect FITC-antibody fluorescence, have a mean intensity equivalent to that which would be expected from 10,000 molecules of cell-associated, antibody-bound FITC (Shapiro 1985).

Confirmation that what the liposomes were detecting was the IL-2 receptor and not some non-specific adherence of the anti-IL-2 receptor antibody to the lymphocytes was given in three ways. Firstly incubation of the cells with irrelevant antibodies of the same immunoglobulin subclass
as the anti-IL-2 receptor antibodies gave the same results as the negative controls stained with fluorescent layer alone. Secondly, three different anti-IL-2 receptor antibodies were tested and all three gave closely similar percentages of positive cells (19.8, 18.6 and 19.0) and mean cell fluorescences (34.8, 36.0 and 36.5). Two of these antibodies (7G7 and anti-Tac) are directed to different epitopea on the IL-2 receptor (Rubin et al 1985). Less consistent results might have been expected were non-specific interactions between the antibodies and the cells occurring.

Finally, confirmation was made possible by the nature of the IL-2 receptor itself. Synthesis and expression of the receptor in peripheral blood T-cells can be induced by a variety of mitogens which increase both the number of positive cells and the fluorescence intensity. Thus when 5-day PHA lymphoblasts were tested, approximately twice the number of positive cells and a two-fold increase in mean cell fluorescence was observed. The FITC-conjugate also proved capable of detecting the IL-2 receptor on these cells albeit at lower levels of fluorescence than with the liposomes.

It was concluded that liposomes are capable of detecting the IL-2 receptor on resting T-cells. At an antigen density of 1000 copies per cell, this is below the threshold of detectability of FITC-conjugates. A simple and rapid technique for the detection of low level
expression of this receptor has potential in the study of IL-2 mediated responses and its disorders.

Previous attempts to boost the fluorescent signal with carrier structures have been hampered by either concentration quenching resulting in weak emissions (Shapiro 1985) or a parallel increase in background giving a reduced signal to noise ratio (Kieran et al 1984). Liposomes combine the advantage of a large internal aqueous space with the ability to encapsulate a precisely optimised concentration of fluorophore thus avoiding the possibility of quenching.

The low background signal from these highly fluorescent liposomes may be attributable to several factors. Non-specific attachment is minimised by the use of a neutral, saturated lipid which is in the crystalline-gel phase at temperatures below 37°C. Another potential cause of non-specific binding is interaction of the antibody component with Fc receptors on the cell surface. However coupling of antibody to liposomes using SPDP occurs by way of amino groups many of which are present in the Fc portion of the Ig molecule. These bonds probably result in a tethering of the Fc portion to the liposome membrane such that it is probably no longer fully available for Fc receptor binding unlike the same portion of the molecule in FITC-conjugates which remains capable of such interactions.
In the case of the FITC-conjugate each fluorescein isothiocyanate molecule replaces a positively charged amino group on a lysine residue with a negatively charged carboxyl group. The net increase of two negative charges leads to increased non-specific binding of the antibody protein to the cell surface. In the case of the liposome-conjugate antibody and label are not in direct contact and this difference may contribute to the very low non-specific binding to the cell membrane. As the purified CF exists in a compartment which is completely separated from the cell membrane by the liposomal bilayer the possibility of interaction with surface protein and carbohydrate structures and also partitioning of the fluorophore into the cell membrane is effectively eliminated.

Applications can be envisaged in situations requiring enhancement of an existing fluorescence signal e.g. cell sorting, and in the demonstration of low density antigens at present only detectable by radiolabelling or rosetting techniques e.g. cell phenotyping for leukaemia diagnosis and the detection of early activation antigens. Liposomes are very versatile in terms of how much material, and of what sort, can be encapsulated. Thus the principle could be extended to provide even higher levels of fluorescence by using liposomes with larger internal aqueous volumes e.g. multilamellar or reverse phase vesicles, and fluorophores.
with different emission spectra could be encapsulated for multi-colour fluorescence work. Cells can be examined by flow cytometry at any convenient time after labelling by simple fixation in formaldehyde and sterile sorting can be performed since the probe readily passes through a 0.22μm filter.

It has been demonstrated that the versatility of fluorescent liposomes can be extended further by the attachment of a polyspecific ligand such as an anti-species antibody rather than a target-specific monoclonal antibody or protein A.
CHAPTER 5

RED CELL PHENOTYPING BY FLUORESCENT LIPOSOMES
5.1 INTRODUCTION

This chapter aims to investigate whether the signal enhancement which can be achieved using optimally fluorescent liposomes in leucocyte phenotyping can also be obtained with erythrocytes. Haemoglobin in erythrocytes absorbs significantly at the emission maximum of fluorescein so that only weak signals are obtained with fluorescent antibodies (Cohen et al 1960). This explains in part why immunofluorescent staining has not played a more important role in red cell phenotyping despite the presence of relatively high density antigens and the availability of avid antibodies. Only a few antigens e.g. in the ABO and Rhesus systems have been demonstrated by this method (Gemke et al 1986) and this could be attributed to the high antigen density and antibody avidity in these cases. On the other hand, Kell, Duffy and Kidd antigens cannot be demonstrated by conventional immunofluorescence techniques.

Standard methods of red cell phenotyping are based on visible agglutination which is sensitive enough for most purposes. However when the absolute number of red cells is very small or when the cell of interest constitutes a minor subpopulation in a mixture of cells agglutination may be difficult or impossible to observe. Conversely these circumstances are well suited to fluorescent techniques which can analyse single cells in high dilution. Such a technique could be used in
situations where the standard agglutination methods are impracticable.

5.2 MATERIALS AND METHODS

Three modalities of the indirect antiglobulin technique (IAT) were compared: 1) IAT with anti-human globulin 2) IAT with fluorescein labelled anti-IgG and 3) IAT with fluorescent liposome-labelled anti-IgG.

Small unilamellar liposomes encapsulating purified carboxyfluorescein at a concentration of 20 mM were prepared as previously described. Goat anti-human antibody (Seralab) was covalently attached to the liposomes using SPDP. A FITC conjugate of the same antibody was purchased commercially. Anti-D, -E, -C, -K, -Jk<sup>a</sup> and -Fy<sup>a</sup> sera and corresponding heterozygous donor red cells were prepared by the North London Blood Transfusion Centre, Colindale. Antisera from two different donors were tested for each antigen. Cells were sensitised by incubation of one volume of a 5% suspension in phosphate buffered saline (PBS) with sixteen volumes of neat antisera for 60 minutes at 37°C.

After washing four times in PBS, the cells were incubated for 30 minutes at room temperature with anti-IgG either bound to fluorescent liposomes or conjugated with FITC. Cells were washed a further three times and examined by eye under an ultraviolet microscope and by flow cytometry (Becton Dickinson FACS IV and Coulter Epics).

Cytometric analysis was immediately preceded by vigorous mixing of the cell suspensions to avoid clumping.
The conventional IAT was performed using the same ratio of cells to sera and the same incubation conditions. Cells were washed four times in PBS and mixed with two volumes of a broad spectrum anti-human globulin (Blood Products Laboratory, Diagnostics). After centrifugation at 1000 rpm for 60 seconds the tubes were read with the naked eye and microscopically.

5.3 RESULTS

5.3.1 Flow cytometry

Table 5.1 shows the mean cell fluorescence (MCF) of an average of 10,000 cells for each red cell phenotype and for both types of fluorescent label (SUV and FITC). The results using two different panels of typing antisera are given in a) and b). All tests using liposome-labelled cells were more strongly positive than with FITC-labelled cells and gave an enhancement factor (MCF ratio SUV/FITC) ranging from 3.0 to 54.6 depending on the antigen specificity being typed. Only R1r were easily discriminated from background with FITC conjugate. The non-specific fluorescence of unsensitized control cells did not increase pari passu.

Fig. 5.1 shows an example of the histograms obtained from the flow cytometer using R1r cells and anti-D. In the top trace the middle peak is the fluorescence profile of the negative control cells and
Comparison of the MCF of various heterozygous donor cells sensitised with two panels of antisera (a & b) and labelled with either G&H-SUV or G&H-FITC.

The MCF values are presented without subtraction of background which varied slightly between each cell phenotype and between the SUV and FITC labels. The controls are a mean of all the backgrounds and are included as an indication of the level of signal which was obtained.
Histograms from the flow cytometer (FACS IV) of Rhesus (D) heterozygous (R1r) cells sensitised with anti-D. Top photograph: cells labelled with liposomes compared with negative control cells; bottom photograph: cells labelled with liposomes compared with FITC-labelled cells.
the right-hand peak shows the strong right shift obtained with cells labelled with fluorescent liposomes. The second photograph contrasts the relatively weak fluorescence of FITC labelled cells (middle peak) with the much brighter liposome-coated cells (right-hand peak).

5.3.2 Comparison of red cell phenotyping methods

In a comparison of the IAT, SUV and FITC techniques, the FITC label was the least sensitive (Table 5.2). The titration end-point of the liposome label was at least two doubling dilutions higher in the case of R1r cells and anti-D and three doubling dilutions for Fy^a+b^v cells and anti-Fy^a compared to the IAT. The minimum number of IgG molecules detectable by the routine IAT is between 100 and 150 per cell (Stratton et al 1983). A range of from 5 to 90 IgG molecules are present on normal cells (Merry et al 1984; Jeje et al 1984) and although these will contribute to the background signal of the negative control cells used in flow cytometry, they are insufficient to produce a positive direct antiglobulin test. It can be assumed therefore that the liposome technique is capable of detecting between 90 and 150 IgG molecules per cell.
5.3.3 Ultraviolet microscopy

Sensitised cells of the phenotypes examined in Table 5.1 were all readily visible using the liposome conjugate. In contrast, only \( R_1r \) cells sensitised with anti-D were visible using the FITC conjugate. No fluorescence was observed with antigen-negative control cells incubated with antisera or with antigen-positive cells incubated with fluorescent liposomes alone. Antigen positive cells diluted 1 in 10 in an antigen negative population were labelled with liposomes; the positive and negative populations were easily distinguished (Fig. 5.2a, b, c, d, e, f and g).

5.3.4 Case 1

A chorionic villous sample (CVS) was obtained at 11 weeks gestation from a woman (PS) who was pregnant for the fifth time. Her first child was Rh(D) negative and the second was Rh(D) positive. Standard anti-D prophylaxis was given but a high titre anti-D antibody developed during the third pregnancy. She was delivered of a healthy Rh(D) positive baby at 32 weeks gestation by caesarian section. Her fourth pregnancy ended in a late second trimester still birth. The foetus was Rh(D) positive with a haemoglobin of 8.8g/dl and the cause of death was given as hydrops foetalis secondary to Rhesus(D) immunisation.
Table 5.2

Titration of typing antisera by IAT and two fluorescent labels (FITC and SUV)

<table>
<thead>
<tr>
<th>Cells</th>
<th>Antibody dilution</th>
<th>R_{R}</th>
<th>FITC</th>
<th>SUV</th>
<th>IAT</th>
<th>FITC</th>
<th>SUV</th>
</tr>
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<tr>
<td>Neat</td>
<td>+++</td>
<td>64.3</td>
<td>762.9</td>
<td>++</td>
<td>17.1</td>
<td>112.8</td>
<td></td>
</tr>
<tr>
<td>1/2</td>
<td>+++</td>
<td>65.3</td>
<td>756.0</td>
<td>++</td>
<td>8.3</td>
<td>95.0</td>
<td></td>
</tr>
<tr>
<td>1/4</td>
<td>+++</td>
<td>61.1</td>
<td>579.3</td>
<td>++</td>
<td>8.8</td>
<td>93.5</td>
<td></td>
</tr>
<tr>
<td>1/8</td>
<td>+++</td>
<td>44.0</td>
<td>453.0</td>
<td>++</td>
<td>9.1</td>
<td>89.9</td>
<td></td>
</tr>
<tr>
<td>1/16</td>
<td>+</td>
<td>32.0</td>
<td>337.0</td>
<td>++</td>
<td>8.4</td>
<td>89.9</td>
<td></td>
</tr>
<tr>
<td>1/32</td>
<td>+</td>
<td>19.3</td>
<td>192.0</td>
<td>++</td>
<td>7.9</td>
<td>89.8</td>
<td></td>
</tr>
<tr>
<td>1/64</td>
<td>+</td>
<td>11.7</td>
<td>94.7</td>
<td>+</td>
<td>7.5</td>
<td>88.6</td>
<td></td>
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<tr>
<td>1/128</td>
<td>+</td>
<td>6.5</td>
<td>68.0</td>
<td>+</td>
<td>6.7</td>
<td>83.3</td>
<td></td>
</tr>
<tr>
<td>1/256</td>
<td>+</td>
<td>5.3</td>
<td>59.0</td>
<td>+</td>
<td>5.5</td>
<td>62.4</td>
<td></td>
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<tr>
<td>1/512</td>
<td>+/-</td>
<td>4.6</td>
<td>46.0</td>
<td>-</td>
<td>5.3</td>
<td>43.7</td>
<td></td>
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<tr>
<td>1/1024</td>
<td>-</td>
<td>4.8</td>
<td>41.0</td>
<td>-</td>
<td>5.2</td>
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<tr>
<td>1/2048</td>
<td>-</td>
<td>4.3</td>
<td>38.0</td>
<td>-</td>
<td>5.2</td>
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<td>29.5</td>
<td>-</td>
<td>5.1</td>
<td>19.4</td>
<td></td>
</tr>
</tbody>
</table>

Heterozygous donor cells (Rh D and Fy^a) were sensitised with the corresponding antisera in serial dilution. The titration end-points were determined by IAT and flow cytometry after labelling with G&H-FITC or G&H-SUV.
Photomicrographs (visible light and UV) of sensitised heterozygous R^rd donor red cells labelled with G&H-SUV. Cells suspensions were 1:10 dilutions of antigen-positive cells in antigen-negative control cells.
Photomicrographs (visible light and UV) of sensitised heterozygous $R_1 r$ donor red cells labelled with G&H-FITC. Cell suspensions were 1:10 dilutions of antigen-positive cells in antigen-negative control cells.
Photomicrographs (visible light and UV) of sensitised heterozygous E (CcDEe) donor red cells labelled with G&H-SUV. Cell suspensions were 1:10 dilutions of antigen-positive cells in antigen-negative control cells.
Fig 5.2d

Photomicrographs (visible light and UV) of sensitised heterozygous c(CcDse) donor red cells labelled with G&H-SUV. Cell suspensions were 1:10 dilutions of antigen-positive cells in antigen-negative control cells.
Photomicrographs (visible light and UV) of sensitised heterozygous K (Kk) donor red cells labelled with G&H-SUV. Cell suspensions were 1:10 dilutions of antigen-positive cells in antigen-negative control cells.
Fig 5.2f

Photomicrographs (visible light and UV) of sensitised heterozygous Fy\textsuperscript{a} (Fy\textsuperscript{a}+\textsuperscript{b}+) donor red cells labelled with G\&H-SUV. Cell suspensions were 1:10 dilutions of antigen-positive cells in antigen-negative control cells.
Photomicrographs (visible light and UV) of sensitised heterozygous Jk\textsuperscript{a} (Jk\textsuperscript{a+b+}) donor red cells labelled with G&H-SUV. Cell suspensions were 1:10 dilutions of antigen-positive cells in antigen-negative control cells.
Cells were extracted from the CVS and sensitised with anti-D typing serum and anti-IgG fluorescent liposomes as described above. Positive (R_1^e_r) and negative (rr) control cells were treated in parallel. A drop of cell suspension on a glass slide was examined by UV microscopy. The number of foetal cells in the washings was determined by a modification of the acid elution method of Kleihauer (Dacie and Lewis 1984) and the fluorescent anti-Hb F techniques (Popat et al 1977). The number of Hb F containing cells was 7% and 8% respectively by the Kleihauer and fluorescent anti-Hb F techniques. One hundred percent of liposomally labelled R_1^e_r controls were brightly fluorescent, the rr cells were completely negative. No fluorescent cells were seen in the washings from the CVS and therefore no evidence of an at-risk pregnancy was obtained.

The pregnancy continued but the baby died in utero in the second trimester due to hydrops presumed secondary to Rh(D)-induced haemolysis.

5.3.8 Case 2

A second CVS was obtained from a woman (JP) who was ten weeks into her seventh pregnancy. She had had one healthy child, two spontaneous abortions and three intrauterine deaths from hydrops due to Kell immunisation.

Foetal red cells were extracted in a Petri dish
using a dissecting microscope and cleaned of tissue debris by filtration. The total yield was 100,000 cells of which half were sensitised with anti-Kell typing serum and labelled with liposomes. Approximately 30% of the red cells were fluorescent with satisfactory positive (Kk) and negative (kk) controls. The Kleihauer technique was applied to the remainder of the sample but there were insufficient cells to determine the percentage of Hb F containing cells.

The foetus was considered to be at risk. In view of the poor obstetric history close observation was maintained and the mother received weekly intravenous immunoglobulin therapy. At approximately 19 weeks gestation foetal ultrasound showed no evidence of hydrops but foetal blood sampling was performed and the result was a Hb of 4 g/dl with a haematocrit of 14%. The direct Coombs' test was moderately strongly positive. The indirect Coombs' test using anti-Kell typing sera gave the same strength of agglutination. The sample was insufficient for elution and identification of the antibody. An intravenous transfusion of 115mls of packed cells brought the haematocrit up to 47%.

The pregnancy was supported with six intravascular transfusions until 36 weeks when a healthy 7lb infant was delivered by caesarian section. The haematocrit was 50% and the phenotype Kell negative. A little phototherapy was given but no transfusion was required.
5.4 DISCUSSION

The ability to determine blood group antigens on small numbers or minor subpopulations of red cells can be clinically important e.g. in the investigation of a delayed haemolytic transfusion reaction, the detection and quantitation of transplacental haemorrhage at the time of delivery and the detection of foetal blood group antagonism in chorionic villous biopsy samples. In these situations standard agglutination techniques can be impracticable.

Immunofluorescent techniques are well adapted to small numbers of cells but the standard method using FITC-antibody conjugates is disappointingly insensitive. The reason is that the absorption spectrum of oxyhaemoglobin has two major peaks at 540 and 576 nm which overlap the main emission peak of fluorescein isothiocyanate. The latter peak is maximal at 520 nm, with half maximal values at approximately 510 and 560 nm. The red cell is therefore particularly well-equipped to produce significant quenching of the fluorescent signal through its haemoglobin content.

This has led to attempts to overcome the phenomenon using red cell ghosts. Combined with double indirect immunofluorescence using two different FITC conjugates it has been possible to demonstrate the blood group antigens A, B, H, Rhesus D, C, E, c and e in a solid phase
microfluorescent test (Gemke et al 1986). Homozygous KK red cells sensitised with anti-K were weakly fluorescent; sensitised Kk cells were negative.

Another approach involved fluorescent microspheres coated with anti-IgG but the requirement for leucocyte depletion of the sample and removal of unbound microspheres, each by a different density centrifugation step, added considerably to the complexity of the method and to the minimum number of cells needed (de Man et al 1988). No immunofluorescent method has simply or reliably demonstrated heterozygous expression outside the Rh system.

Liposomes conjugated to anti-IgG and containing a maximally fluorescent concentration of carboxyfluorescein can be employed in an identical way to FITC conjugates and the method takes only 30 minutes longer than the conventional indirect antiglobulin test. The signal enhancement conferred by fluorescent liposomes can be demonstrated over a range of clinically important blood group antigens with different typing sera (Table 5.1 a and b). Unlike the fluorescent microsphere and solid phase microfluorescent techniques cells labelled with liposomes are suitable for analysis by flow cytometry. The technique was more sensitive than the IAT as shown by the titration end-points of two serially diluted antisera (Table 5.2) and it allows accurate quantitative and comparative studies. In addition flow cytometry would
enable enrichment of minor subpopulations of cells by addition of a sorting capacity.

An additional advantage of the enhanced fluorescence is the ability to visualise the result by UV microscopy. Of the heterozygous phenotypes examined, only \( R_1 R \) cells sensitised with \(-D\) were visible using FITC conjugate. Fluorescent liposomes extended the range of visually detectable antigens to include heterozygous \( E, c, K, F_y^a \) and \( J_k^b \) cells. This allowed typing of very small numbers of cells \(<50,000\) and of mixed populations where the concentration of the cell of interest was only 10% (Fig. 5.2).

Practical application of the liposome technique to first trimester foetal blood grouping is illustrated by two cases. In case 1 the apparent failure of the fluorescent liposomes to detect the presence of 7-8% 'foetal' cells sensitised with anti-\( D \) led to the false surmise of an unaffected pregnancy. This may be explained by the fact that Hb-F containing cells are not necessarily of foetal origin. In a study of 11 normal pregnancies there was a significant increase in the number of F cells in the maternal circulation which reached a peak at 18 to 22 weeks gestation (Pop et al 1977). In two cases, however, an earlier peak of 9 to 10% was seen at 12 weeks. Neither the Kleihauer nor the more sensitive fluorescent anti-Hb F test is specific for Hb F in foetal cells.
In a CV sample which is heavily contaminated with maternal blood, tests cannot be relied upon to indicate the presence of foetal cells if the percentage of Hb F containing cells falls below approximately 10%. Gemke et al in their description of a solid-phase microfluorescence technique for phenotyping foetal erythrocytes in the first trimester of pregnancy emphasised the requirement that, in the case of a negative result, the ratio of foetal to maternal RBCs is shown to be above the sensitivity threshold of the fluorescent technique employed. This is reported as 1 in 4000 or 0.025% foetal cells using their method.

However, in view of the rise in maternal F cells during the first trimester it is possible for a CV sample to contain up to 10% F-cells but no foetal RBCs. By their criterion, such a sample would be reported as showing no evidence of blood group antagonism i.e. a false negative result. For negative results where the percentage of F-cells is less than 10% (as in case 1) it would be more accurate to describe the analysis as inconclusive and suggest a second biopsy. Case 1 draws attention to the pitfalls in using sample washings which are heavily contaminated with maternal blood and the benefits of enriching the foetal RBC component by dissection of the biopsy sample.

In case 2 the detection of Kell positive cells at 10 weeks was confirmed by the subsequent development of
immune haemolysis in the foetus at 18 weeks. Final confirmation of the diagnosis of haemolytic disease of the newborn due to Kell sensitisation must await phenotyping post-natally. With this assumption, the result demonstrates the expression of Kell antigen early in foetal life and the feasibility of typing by fluorescent liposomes utilising as few as 50,000 cells. There was insufficient sample for an F cell count but in the case of a positive result this matters less, even if the percentage is below 10%, since Kell positive cells can only be of foetal origin.

Early detection of an at-risk foetus may lead to intervention with ultrasound-guided intrauterine transfusions before the appearance of hydrops as in case 2. The result might be the survival of a higher proportion of these severely alloimmunised pregnancies.

Further applications of the technique can be envisaged in other situations where the absolute number of cells is low or the cell of interest is a minor subpopulation. This includes the investigation of delayed haemolytic transfusion reactions and the quantitation of foeto-maternal haemorrhage. Non-radioisotopic red cell survival studies might be feasible if the technique could be made sufficiently sensitive at extreme dilutions of cells and an antigen disparity between the homologous and autologous blood was available.
Combined with flow cytometry, the liposome technique was more sensitive than the antiglobulin test in two antigen systems (Rhesus and Duffy) representing a relatively high antigen density (9,900 - 14,600 D sites; ref Rochna and Hughes-Jones 1965) and a low one (6,900 Fy\(^a\) sites; ref Masouredia et al 1980). This would lend itself to the investigation of 'Coombs' negative' autoimmune haemolytic anaemia, as a more sensitive antibody screening method and in the cross-matching of blood where donor units appear compatible in vitro but have a shortened survival in vivo. The increasing use of flow cytometry and the availability of a sensitive fluorescent probe may stimulate the future application of this technique in transfusion medicine.
CHAPTER 6

UPTAKE OF ANTIBODY-DIRECTED CYTOTOXIC LIPOSOMES

BY CD3 ON HUMAN T CELLS
6.1 INTRODUCTION

Having established the feasibility of targeting liposomes specifically to the CD3-Ti complex on T-cells (Chapter 3) this chapter explores the question of internalisation of liposome contents and the effectiveness of encapsulated methotrexate (MTX) in inhibiting lymphocyte growth in culture. This will be used as a model for determining the experimental conditions applicable to depleting bone marrow of unwanted cells. Such cells would include T lymphocytes in allogeneic and residual malignant cells in autologous marrow. The rationale behind both T cell depletion and purging is discussed in Chapter I.

The use of antibody-directed cytotoxic liposomes in this context would allow the latter a clinical application unfettered by considerations of plasma stability, RES uptake and target-cell accessibility.

6.2 MATERIALS AND METHODS
6.2.1 Liposomes, antibodies and cells

Protein A liposomes containing 40 mM CF, 200 mM CF or 20 mM MTX were synthesised. PHA-lymphoblasts were obtained by incubation of peripheral blood mononuclear cells (1 x 10^6 /ml) for 72 h in cell culture medium containing 10% fetal calf serum and purified PHA.
(4ug/ml). The targeting antibody was UCHT1 (CD3, IgG2b subclass).

6.2.2 Liposome uptake by peripheral blood lymphocytes

Samples of $2 \times 10^5$ cells were reacted with UCHT1 and fluorescent second layer antibody in microtitre plates as described in Chapter 2.2.13. The effect of temperature on cell fluorescence was studied by placing cells labelled at 4°C with SUV-PA or G&M-FITC in a waterbath at 37°C. At specified intervals, cells were removed and the fluorescence analysed by flow cytometry.

6.2.3 UV microscopy

Cells labelled with fluorescent liposomes or fluorescent second layer antibody were placed on glass slides and sealed under cover slips at 4°C. They were examined immediately under a UV microscope using phase optics and a X60 magnification oil-immersion lens. Modulation of surface fluorescence was studied by allowing the chilled slides to warm on the microscope stage. The readings of a thermometer laid alongside the slides was 25°C after 5 min and 35°C after 20 min. Photographs were taken at 10 min intervals using a standard exposure time of 15 sec. The UV source was occluded between examinations to prevent bleaching. All prints were handled identically.
6.2.4 Treatment of cells with ZZAP

Peripheral blood lymphocytes were labelled at 4°C with antibody and SUV-PA containing 40mM CF. After thorough washing in ice-cold medium, fluorescence was measured by flow cytometry immediately and after 60 min at either 4°C or 37°C. Cells were then treated for 15 min at room temperature with medium containing 0.1M dithiothreitol and 0.1% cysteine-activated papain (NBTS, Edgware) in a modified version of the ZZAP method (Branch and Petz 1982). After further washing at 4°C cells were re-analysed on the FACS.

6.2.5 Incubation of liposome-encapsulated MTX with lymphocytes

a) Effect of liposome concentration

PBL (2 x 10^5 cells /well) were plated in triplicate cultures in 96-well microtitre plates. Fifty ul of mitogenic antibody (UCHT1, final concentration 2.5ug/ml) and 50 ul of various dilutions of a protein A-conjugated MTX-liposome suspension (SUV[MTX]-PA) were added to each well which was topped-up with 100 ul of culture medium containing 10% FCS. Control cells were cultured with antibody and unconjugated MTX-liposomes. Cells were cultured for 72 hr in a fully humidified atmosphere of 5% CO_2 at 37°C. One microcurie per well of ^3H dUr was added
for the last 6 hr of culture. Cells were harvested on to fibreglass filters (Whatman) using an Automaah (Dynatech) and counts were measured on a scintillation counter set for $^3$H.

b) Effect of antibody concentration

PBL were incubated with various dilutions of antibody (50 ul volume) and a fixed dilution (50 ul of 1 in 5 dilution) of protein A-conjugated MTX-liposomes. Conditions and controls were otherwise as in a). The growth index was calculated as follows:

\[
\frac{\text{ radioactivity counts (targeted cells)}}{\text{ radioactivity counts (untargeted cells)}} \times 100\%
\]

c) Effect of incubation time on liposome-mediated inhibition

PBL were incubated with UCHT1 (2.5ug/ml final concentration) and SUV(MTX)-PA (1 in 5 dilution). At specified times the wells were washed to remove liposomes and the cells were re-cultured in medium supplemented with FCS, antibody and IL-2. Conditions were otherwise as in a).

d) Effect of liposome-encapsulated MTX on PHA-lymphoblasts
PHA-lymphoblasts ($2 \times 10^5$ cell per well) were plated in triplicate cultures. Fifty microlitres of UCHT1 and 50 ul of liposomes (1 in 5 dilution) were added followed by 100 ul of the original culture supernatant containing 10% FCS and PHA. Control cells were incubated with medium alone, antibody alone, unconjugated MTX-liposomes, and antibody plus conjugated liposomes containing buffer only. Cells were harvested at 24hr and 48hr.

6.3 RESULTS

6.3.1 Incubation of cells with protein A liposomes at 37°C

PBL labelled with SUV(CF)-PA or G&M-FITC were incubated at 37°C in order to promote internalization. This initiated a rapid decline in the number of positive cells. Using the FITC label, approximately 50% of cells which were originally positive remained so after 60 min (Fig. 6.1). Liposome-labelled cells showed a similar rapid fall reaching its nadir of 50% after 30 min. This could be explained by internalisation of fluorophore with subsequent dilution in the cytoplasm and weakening of the fluorescent signal or alternatively, shedding of the complexes into the extracellular medium where they would be undetected by FACS. The experiments were therefore repeated using liposomes containing much higher concentrations of CF, thereby increasing the likelihood
Modulation of lymphocyte fluorescence at $37^\circ$C using 40 mM CF liposomes. Peripheral blood lymphocytes were labelled with anti-CD3 antibody and FITC-conjugated goat anti-mouse antibody (×) or protein A liposomes (−−−) containing 40 mM CF. Unbound antibody and liposomes were washed off and the cells were incubated at $37^\circ$C. The percentage of fluorescence positive cells at various time intervals was measured by flow cytometry. The graph is representative of two experiments.
Modulation of lymphocyte fluorescence at 37°C using 200 mM CF liposomes. Peripheral blood lymphocytes were labelled with anti-CD3 antibody and FITC conjugated anti-mouse antibody or protein A liposomes containing 200 mM carbofluorescein. Conditions and measurements were as in Fig 6.1.
of intracellular fluorescence being detected by the cytometer. Cells were labelled with liposomes containing 200 mM CF and incubated at 37°C. The opposite effect was now seen (Fig. 6.2). The number of positive cells was 25% at the start and increased to 46% at 60 min.

The difference in behaviour is explained by the degree of self-quenching in each of these species of liposome. CF liposomes, 200 mM, were considerably less fluorescent than those containing 40 mM CF although they contained approximately five times as much fluorophore (Table 6.1). Forty mM CF liposomes are relatively unquenched (74.9%) compared to those containing 200 mM CF which are highly quenched (98.4%). As incubation with 200 mM CF liposomes at 37°C proceeded, the increase in the number of fluorescent cells was therefore due to unquenching of liposomes. This is inconsistent with shedding of surface bound antibody and suggests internalization of CF followed by cytoplasmic dilution and unquenching of fluorescence.

An alternative explanation is that the increase in cell fluorescence is simply due to loss of CF from membrane bound liposomes without entry of the fluorophore into the cell. This would lead to unquenching of the liposomes and a gradual increase in brightness. However, further analysis of the location of this fluorescence using a mixture of activated papain and DDT (ZZAP) showed this interpretation to be incorrect. PBL
### Table 6.1

**Effect of carboxyfluorescein concentration on liposomal fluorescence**

<table>
<thead>
<tr>
<th>Concentration of CF in liposomes</th>
<th>Fluorescence units Before Triton-X</th>
<th>Fluorescence units After Triton-X</th>
<th>Percentage quenching</th>
</tr>
</thead>
<tbody>
<tr>
<td>40mM</td>
<td>114</td>
<td>454</td>
<td>74.9</td>
</tr>
<tr>
<td>200mM</td>
<td>35</td>
<td>2104</td>
<td>98.4</td>
</tr>
</tbody>
</table>

The fluorescence of two preparations of liposomes containing different concentrations of carbofluorescein (CF) were compared. Intact liposomes were lysed by addition of Triton-X to release the fluorophore and abolish quenching. The percentage quenching is calculated by:

\[
\text{Percentage quenching} = \left( \frac{\text{Fluorescence before Triton-X}}{100} \right) \times 100 - \left( \frac{\text{Fluorescence after Triton-X}}{100} \right)
\]
Table 6.2

Removal of Membrane-bound Liposomes by Enzyme

<table>
<thead>
<tr>
<th>Cells</th>
<th>Incubation</th>
<th>% positive cells</th>
<th>Relative Fluorescence Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) +Ab + SUV-PA</td>
<td>none</td>
<td>72</td>
<td>92</td>
</tr>
<tr>
<td>+ SUV-PA</td>
<td>none</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>b) +Ab + SUV-PA</td>
<td>60 mins at 4°C</td>
<td>72</td>
<td>93</td>
</tr>
<tr>
<td>after enzymes</td>
<td>6</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>c) +Ab + SUV-PA</td>
<td>60 mins at 37°C</td>
<td>33</td>
<td>72</td>
</tr>
<tr>
<td>after enzymes</td>
<td>25</td>
<td>58</td>
<td></td>
</tr>
</tbody>
</table>

PBL coated with anti-CD3 antibody and protein A bearing liposomes containing 40mM CF were analysed by flow cytometry and compared with control cells not coated with antibody (a). Cells labelled with antibody and liposome were reanalysed after 60 minutes incubation at 4°C (b) or at 37°C (c). These were treated with activated papain and dithiothreitol for 15 minutes at room temperature to remove membrane-bound liposomes and fluorescence was measured again.
were labelled with fluorescent liposomes at 4°C washed and analysed by flow cytometry giving the expected positivity of 72% (Table 6.2). The cells were divided into two fractions which were incubated at 4°C and 37°C respectively for 1 h after which they were put on ice and re-analysed. The cells kept at 4°C were unchanged whereas those incubated at 37°C were now 33% positive. Both fractions were treated with 2ZAP for 15 min at room temperature, washed at 4°C and re-examined. The fluorescence of cells held at 4°C was reduced to background (6%) whereas the majority of cells which were incubated at 37°C were still positive. The reduction from 33% to 25% presumably results from the removal of residual cell-bound liposomes.

These data suggest that the liposomes taken up by PBL at 4°C are primarily associated with the membrane while subsequent incubation at 37°C for 1 hr allows a substantial number to become internalized and no longer susceptible to enzyme stripping.

6.3.2 UV microscopy

The appearances and behaviour of PBL incubated with liposomes was studied by UV microscopy in an attempt to elucidate the mechanism of internalization. Lymphocytes incubated with fluorescent antibody undergo a well-recognized pattern of 'patching' and 'capping'. This process was readily observed in PBL labelled with UCHT1
and G&M-FITC and placed on a microscope slide. Upon warming of the slide from 4°C on the microscope stage, T cells initiated the patching and capping sequence which was largely complete in 20 min. Cells labelled with protein A liposomes containing 40 mM CF behaved in an identical fashion.

In contrast, cells labelled with 200 mM CF liposomes were very faint initially and on warming became brighter. The pattern also changed from a uniform peripheral distribution to patches of fluorescence which after 20 min was followed by a generalized 'blush' of cytoplasmic fluorescence. This is illustrated in Fig. 6.3 which shows the same T cell labelled with 200 mM CF liposomes and photographed at 10 min intervals. The sequence is consistent with the previous evidence of delivery of liposome contents into the cytoplasm with subsequent unquenching and it was found much easier to define the intracellular location of the liposomally-delivered fluorescence than that left after capping of fluorescent second-layer antibody. Moreover, the similarity in the patching and capping of cells labelled with 40 mM CF liposomes compared to those labelled by conventional indirect fluorescence suggests that the handling and fate of the different receptor-ligand complexes is the same.
Fig 6.3

Fluorescent micrographs of a single peripheral blood T-cell labelled with UCHT1 and protein A liposomes containing 200mM carboxyfluorescein. Photographs were taken at (a) time zero (b) 10 minutes and (c) 20 minutes after warming from 4°C.
6.3.3 Cytotoxicity of protein A liposomes containing MTX

The ability of protein A liposomes to deliver cytotoxic quantities of a drug specifically to T cells via the CD3 antigen was tested using MTX. UCHT1 is mitogenic to PBL and can be used both to target the protein A liposomes and to induce proliferation and thus sensitivity to MTX. Fig. 6.4(a) shows that resting PBL stimulated with UCHT1 were inhibited by targeted MTX-liposomes in proportion to the liposome dose but not by untargeted liposomes (those not conjugated with protein A). The growth index was lowest (5.8%) at the saturating concentration of antibody (2.5 ug/ml, Fig. 6.4 b). Lower antibody concentrations resulted in less cell proliferation and less cytotoxicity. Higher antibody concentrations also resulted in less cytotoxicity presumably by blocking of protein A attachment by excess free antibody.

The timing of inhibition by MTX-liposomes was studied by washing off liposomes at various time intervals after initiation of cell culture. Washing itself had a strong inhibitory effect presumably by disruption of intimate cell contact (Fig. 6c). Nevertheless inhibition of the proliferation of resting PBL by liposomes was not demonstrable until after 16 h of exposure becoming progressive thereafter. Lymphoblasts produced by an alternative mitogen (PHA) were inhibited by targeted liposomes but not by antibody
Effect of the concentration of MTX-liposomes on resting PBL stimulated with UCHT1. PBL (2 x 10^6) were cultured in triplicate microtitre wells with 50 ul of a fixed concentration of UCHT1 (2.5ug/ml) and 50ul of serially diluted protein A conjugated liposomes encapsulating 20mM MTX (■). Control cells (□) were cultured with UCHT1 and unconjugated liposomes (20mM MTX) in the same dilutions. Cells were cultured for 72 h; one uCi of ³H-dUr was added to each well for the last 6 h of culture.
Effect of antibody concentration on the cytotoxicity of MTX-liposomes. PBL (2 x 10^5) were cultured in triplicate microtitre wells with 50ul of various concentrations of UCHT1 and a fixed concentration (1 in 5 dilution) of protein A-conjugated liposomes encapsulating 20mM MTX (■). Control cells were cultured with UCHT1 alone (■) or with UCHT1 and unconjugated, MTX-liposomes (■). The percentage growth is graphed (→).

The notable proliferative effect of PA-conjugated liposomes in the absence of targeting antibody is most likely to be due to the known mitogenic effect of staphylococcal Protein A on human B cells (ref: Schrezenmeier H and Fleischer B. Journal of Immunological Methods, 1987; 105: 133-137)
Effect of exposure time on the cytotoxicity of MTX-liposomes. PBL (2 x 10^5) were cultured in triplicate microtitre wells with 50ul of UCHT1 (2.5 μg/ml final concentration) and 50ul of protein A-conjugated liposomes encapsulating 20mM MTX (■). Control cells were cultured with UCHT1 and unconjugated liposomes (■) or with antibody in the absence of liposomes (■). At various time intervals liposomes were washed-off and the cells re-cultured in IL-2 supplemented medium.
Effect of MTX-liposomes on PHA-lymphoblasts. 2 x 10^5 PHA-stimulated lymphocytes were cultured in triplicate microtitre wells with medium alone (■), antibody alone (□), liposomes containing 20mM MTX (■), antibody plus protein A bearing liposomes containing 20mM MTX (□) and antibody plus protein A bearing liposomes containing buffer (□). The final concentration of antibody was 5 ug/ml and that of liposome-encapsulated MTX was 750nM. 1 uCi of ^3^H-dUr was added to each well for the last six hours of culture.
alone, liposomes containing 20 mM MTX but without protein A or antibody with protein A liposomes containing buffer only. MTX encapsulated in protein A liposomes targeted with UCHT1 caused an 80% inhibition of $^3$H dUr incorporation at 24h and 90% inhibition at 48h (Fig. 6.4d).

6.4 DISCUSSION

Clinical use in vivo of targeted liposomes has been handicapped by problems with reticuloendothelial system uptake, instability in plasma and tumour inaccessibility (see Chapter 1). Manipulation of bone marrow ex vivo for transplantation circumvents many of these obstacles and may be an ideal application for targeted liposomes. As an antigen target for T cell depletion of bone marrow, the human T-cell receptor complex is a good potential candidate. It is lineage specific whereas previously studied antigens for liposome uptake by lymphoid cells have been class I and class II MHC (or HLA) antigens (Machy et al 1982; Huang et al 1983). These are expressed by other cell types and would therefore be inappropriate for clinical usage. In addition, there is some evidence that following antigen binding, CD3 is rapidly internalised (Kan et al 1983) and may therefore be capable of mediating the internalisation of attached liposomes. This could be of value in T-cell depletion
of bone marrow and may avoid some of the difficulties of current methods which employ bulk complement lysis techniques.

The intracellular delivery of liposome contents is difficult to demonstrate conclusively by a single technique. However, flow cytometry combined with UV microscopy and an enzyme stripping method showed clearly that surface-bound liposomes can deliver their contents into the cell cytoplasm within 30 min at 37°C.

In their study of the fate of CD3 on human T-cells after antibody-induced modulation, Kan et al showed rapid internalisation of the antigen without shedding into the extracellular medium. This was shown using methods including surface iodination of the membrane component, precipitation with antibody and analysis by SDS-PAGE. For the purpose of establishing the fate of surface membrane components after antibody binding, the use of fluorescent liposomes offers an alternative method which is both simple and quick. By indicating which receptors are endocytosed the results could have predictive value for the utility of a particular antigen as a receptor for cytotoxic liposomes.

When MTX is encapsulated in the liposomes the growth of lymphocytes is related to the liposome concentration (Fig. 6.4a) and can be completely abolished. The effect is antibody-mediated and can be partially reversed by the presence of excess UCHT1. This is presumably due to the
saturation of antigen receptors on cells and protein A-binding sites on the liposomes, conditions in which intimate liposome-cell contact is blocked. The effect is also highly specific as MTX-liposomes in the same concentration but lacking protein A produce no significant inhibition; nor is the effect due to growth inhibitory effects of liposomal lipid, protein A or antibody because targeted empty liposomes have no effect in the range of concentrations used. Indeed unconjugated liposomes have a slight but consistent proliferative effect on PBL by provision of lipid substrate or by some other mechanism (Fig. 6 a, b & c).

It could be argued that protein A-liposomes containing MTX prevent proliferation of resting lymphocytes by complexing with the targeting antibody in solution and interfering with its mitogenic activity. However a true MTX-mediated inhibition is conclusively demonstrated by the effect on lymphocytes stimulated by an alternative mitogen, namely PHA (Fig. 6.4d).

These data confirm the specific intracellular delivery of liposome contents, the feasibility of delivering cytotoxic quantities of drug via the CD3 pathway and the stability of the liposomes which are sufficiently impermeable to prevent non-specific cytotoxicity due to leakage after 72 h in culture.

In its present form the procedure is impractical from the point of view of T-cell depletion of bone marrow
or purging. A realistic if rather stringent requirement of cytotoxic liposomes is that they should produce significant growth-inhibition after endocytosis of a single ‘coat’ of target antigens on the cell surface (cf. complement-mediated lysis). Peripheral blood lymphocytes grow normally for 16 h after exposure to liposomes and mitogen, thereafter becoming progressively susceptible to MTX-liposomes (Fig. 6.4c). Since modulation of the CD3-Ti antigen complex is virtually complete in 60 min, this means that useful cytotoxicity is incapable of being produced by a single or even several rounds of liposome endocytosis. If CD3, which has a relatively high antigen density (approximately 40,000 copies per cell) with a very rapid rate of modulation, is unable to meet this specification then it is unlikely that any other single antigen will either.

A further reservation concerns MTX itself. MTX has several desirable properties with respect to encapsulation of cytotoxic compounds in antibody-directed liposomes. It is highly water-soluble and stable for many months in aqueous solution unlike many commonly administered cytotoxic drugs. It is active clinically against lymphoid malignancies and, encapsulated in liposomes, has proved effective in previous in vitro studies. However, it is active only against dividing cells, so unstimulated or malignant cells in G0 of the cell-cycle are unaffected. This is shown in the present
experiments by the need for prior stimulation of the lymphocytes. Following this, cells require a further 24h incubation with MTX-liposomes in order to obtain 80% inhibition and 48 hr for 90% inhibition of cell growth (Fig. 6.4 d).

An ideal agent would be rapidly cytotoxic to both resting and dividing cells. Encapsulated in liposomes and combined with the highly specific targeting conferred by monoclonal antibodies, such an agent may make feasible not only T-cell depletion but the purging from autologous bone marrow of any unwanted or contaminating cells e.g. carcinoma or residual haematological malignancy. Certain photoactivated dyes such as the sulphonated aluminium phthalocyanines have been shown to be rapidly cytotoxic and may be a more efficient class of compound for liposome encapsulation (Morgan and Gray 1989). In the present experiments, the absence of non-specific toxicity to untargeted cells holds out the possibility that stem cells and hence bone marrow engraftment would be similarly unaffected by exposure to cytotoxic liposomes.

The attachment to the liposomal bilayer of protein A (or similar ligand for immunoglobulin) as opposed to the targeting antibody itself has several advantages. It is more economical with the targeting antibody since direct coupling of liposome and antibody requires much larger and purer quantities of the latter. Direct coupling
commits those liposomes to a single antigen target whereas a single batch of protein A liposomes can be used against any antigen for which a suitable antibody is available. This offers the possibility of using a cocktail of antibodies against several antigens on the same cell thereby increasing the cytotoxic potential of a particular liposome dose. In a similar manner several low density antigens which individually may not be sufficiently numerous to mediate antibody-targeted liposomal cytotoxicity, may be combined to achieve this effect using the relevant panel of antibodies and a single batch of liposomes.

In conclusion, antibody-targeted liposomes have a useful role in the study of interactions between surface membrane components and the cytoplasmic compartment. Given agents of adequate cytotoxicity their specificity of targeting and their carrier capacity could be therapeutically exploited in the *ex vivo* manipulation of bone marrow.
CHAPTER 7

CONCLUSIONS
The anticipated enhancement of the fluorescent-antibody technique by antibody-conjugated liposomes containing carboxyfluorescein was seen with both leucocytes and erythrocytes. This is attributable to the higher loading capacity of the internal aqueous space of liposomes compared to that of antibody itself and also to the precise control which can be exerted over the concentration of encapsulated fluorophore. The latter is determined by the concentration of fluorophore in the aqueous phase and is not at the mercy of incubation times, pH, temperature and other reaction conditions as in the case of direct conjugation to antibody. This is important because small deviations in the amount of encapsulated or directly conjugated fluorophore from the optimum have a negative effect on the fluorescent signal: too little and the signal may not be maximal; too much and there may be concentration quenching.

Enhancement of the positive signal might have been counterbalanced by an increase in the negative or background control values. For the reasons discussed in the text these were comparable to the levels obtained with FITC-conjugated antibody and a log-increase in signal amplification was recorded overall.

The attachment of anti-immunoglobulin antibody to the liposomes and not specific antibody extended the range of antigens targetable by the same species of
liposomes and also allowed advantage to be taken of the signal amplification obtainable by the use of indirect immunofluorescence. Protein A-conjugated liposomes showed only weak binding to the commonly used IgG₁ subclass of surface-bound antibody whereas the attachment of anti-immunoglobulin antibody allowed binding to all IgG subclasses thereby producing a reagent with more universal staining properties. Simple as the idea of the substitution sounda no previous description was found.

The experience gained with this system could be extended to the use of other fluorophores for multicolour studies and to the use of liposomes with larger internal aqueous compartments for further signal enhancement. The production techniques are relatively easy to master. Cell labelling is no different from currently used protocols and the sensing hardware is also shared. The impressive stability of the product over many months makes it attractive both as an in-house and a commercial laboratory reagent.

The main uses of the reagent are seen as:
1. Boosting the signal from antigens already detectable by the fluorescent antibody technique but which are weak in comparison to the autofluorescence of the cells. This applies particularly to leucocytes.
2. Demonstrating the presence of low density antigens not detectable by conventional staining as shown by the example of the interleukin-2 receptor on resting peripheral blood T cells.

3. Demonstrating the presence of antigens on erythrocytes in which fluorescence is quenched by haemoglobin. Fluorescent liposomes are not seen as a substitute for routine agglutination techniques already in use for red cell phenotyping but as an adjunct in situations where the cell of interest is available in only small numbers or where it constitutes only a minority population.

An example of the latter is in the phenotyping of foetal red cells obtained from chorionic villous samples. The prediction of haemolytic disease of the newborn in a pregnancy at risk from Kell-alloimmunisation in the first trimester is reported. This lead to foetal blood sampling at 18 weeks gestation and confirmation of immune-haemolysis before the appearance of hydrops foetalis. Intrauterine transfusions maintained the foetus until a successful delivery at 36 weeks. As clinical techniques such as ultrasonography continue to develop, so does the ability to obtain biopsy material and to support these pregnancies. Diagnostic methods must keep pace and in red cell phenotyping neither standard agglutination nor conventional fluorescent
techniques are applicable to the cell numbers and cell contamination involved.

An analogous situation is the phenotyping of red cells after a delayed haemolytic transfusion reaction. Too few of the offending cells may remain in the circulation for detection by agglutination methods but it may be possible to demonstrate them by fluorescent labelling combined with UV microscopy and flow cytometry.

Red cell volume measurement is sometimes required in neonates, children and pregnant women where the use of radionuclides is precluded. Studies have been reported using non-radioactive cell markers such as biotin. The autologous biotinylated cells are re-infused into the patient and blood is sampled at intervals in the usual way. The biotin-labelled cells are detected using streptavidin-FITC and the results correlate well with the 51 Cr-labelling technique (Cavill et al 1988). Adaptations may make it possible to measure red cell survival and it would be interesting to explore the use of fluorescent liposomes as another safe alternative for these purposes.

Flow cytometry is a sensitive technique which is becoming more available in routine laboratories where it is being used for example in the typing of lymphocyte subsets, reticulocyte counting and in the measurement of platelet associated antibody. It is therefore important
to have available sensitive reagents to exploit the full diagnostic potential of these instruments.

With regard to the potential of antibody-directed cytotoxic liposomes for the ex vivo treatment of bone marrow, the results using peripheral blood T cells as targets suggested the technique was feasible in principle. The CD3 antigen on T cells was a particularly good candidate as shown by its ability to mediate the rapid endocytosis of surface-bound liposomes. This was the first description of the use of a lineage specific antigen on non-malignant, polyclonal cells as a target for antibody-directed cytotoxic liposomes. Previously used antigens have been expressed on cell lines against which either tumour specific antibodies or, more often, antibodies to class I or class II determinants (which are expressed on many other types of host cell) have been directed.

The inhibition of lymphocyte proliferation was highly specific but the kinetics of the response was too slow to be practicable. Forty eight hours of incubation at 37°C to achieve 90% growth-inhibition compares poorly with other methods of T cell depletion and adds considerably to the risk of bacterial and fungal contamination. Methotrexate is cytotoxic only to proliferating cells hence the need for prior lectin or antibody-induced mitogenesis. This would be neither feasible nor attractive in the setting of T cell
depletion where the aim is to restrict donor lymphocyte activation. The $G_0$ fraction of residual tumour or leukaemia cells would be similarly unaffected by such a cytotoxic agent. In addition the need for the liposomes to be internalised limits the choice of available target antigens to those associated with receptor-mediated endocytosis.

It could be argued that the cytotoxicity of antibody-directed liposomes would be improved by the encapsulation of drugs which are less cycle-dependent. However simple practical considerations of solubility and stability militate against many of the obvious alternatives. For example the nitrosoureas are very labile in phosphate-buffered saline at pH 7 with 5% degradation ($t_{0.95}$) occurring in 10-50 minutes at room temperature. The $t_{0.95}$ is less than an hour with the alkylating agents nitrogen mustard, chlorambucil and melphalan under the same conditions; busulphan and 4-hydroperoxycyclophosphamide have a $t_{0.95}$ of between 2 and 24 hours (reviewed by Bosanquet 1985). Liposomes encapsulating these compounds would have too short a shelf-life to be practically useful.

Papahadjopoulos (see Weinsteint 1984) proposed that the ideal cytotoxic drug for liposomal encapsulation was one which was inactive as free drug (e.g. because of poor cellular uptake) but became cytotoxic once introduced into the cytoplasm - a so-called 'endodrug'. An elegant
demonstration of the principle was reported by Heath et al (1983) using the aspartate derivative of methotrexate which is as potent an inhibitor of dihydrofolate reductase as methotrexate itself. Methotrexate-aspartate however is only 1/200th as toxic as methotrexate because of its much slower rate of influx into cells. Targeted by liposomes the drug was shown to be 18 times more effective than the drug presented in untargeted liposomes.

The main consideration behind the proposal to use liposome-dependent or endodrugs is concern over possible leakage of drug from liposomes leading to non-specific toxicity. The present author would argue from the results presented here that, with the appropriate choice of lipid, liposome-type and conjugation method, leakage is not a major problem with the procedure in its envisaged application. The major impracticality is the need for internalisation of the liposomes and the slow response rate to the drug. This makes the case for a rapidly acting agent requiring only attachment to the cell surface - what might be termed an 'exodrug'. A family of agents fulfilling the criteria of such a drug is the phthalocyanines. Liposomes could have a complementary role by virtue of their proven carrier capacity and highly specific targeting ability; harvested bone marrow is a promising target-organ by virtue of its accessibility to liposomes.
Phthalocyanines are compounds which are relatively non-toxic per se but become cytotoxic on exposure to light of specific wavelengths primarily by the generation of singlet oxygen and superoxide radical (Spikes 1986; Brasseur et al 1985). These species are able to produce oxidative damage in biological substrates such as unsaturated phospholipids, cholesterol, pyrimidines and purines. Because singlet oxygen is able to diffuse only 1000 to 2000 Å during its lifetime, photosensitising compounds have an extremely short-range cytotoxic effect which can be exerted from the cell membrane (Lindig and Rogers 1981; Grossweiner 1981).

Sulphonated alumonium phthalocyanine has many of the properties desirable for liposome encapsulation, in particular good water solubility. Studies arising from the present work have confirmed that, encapsulated in antibody-targeted liposomes, this compound is a potent and selective cytotoxic (Morgan and Gray 1989; Morgan 1989). Following targeting to cells, irradiation with red light for less than 30 minutes was able to produce greater than 95% growth inhibition with little evidence of non-specific toxicity. The incubation temperature of 4°C prevented the internalisation of the liposomes so that the effect was generated by surface binding of liposomes alone.

Using this compound, a liposomal method of T cell depletion of bone marrow could be envisaged which has the
rapidity of action of the complement-mediated lysis technique. Indeed the manner in which both methods affect cell integrity by attacking the cell membrane is very analogous. The method is independent of the ability of the antigen to be endocytosed unlike other antibody-drug and antibody-toxin conjugates which require cell penetration to be effective. It is also independent of the cell-cycle phase and could be used for purging of resting malignant cells provided one or more antibodies of sufficient tumour specificity were available.

In addition to bone marrow, other anatomical compartments such as the bladder and the peritoneum could be considered. As adjunctive treatment of ovarian and bladder cancer, photoactive liposomes could be instilled directly into these body cavities avoiding RES uptake. Exposure to light would be controlled by fiberoptic laser.

It seems that most reports of studies using liposomes end with ingenious solutions to problems which they themselves have created and the present work is no exception. Nevertheless, areas in which antibody-targeted liposomes can improve existing fluorescent techniques have been indicated and an attempt has been made to identify the problems associated with a promising in vivo application.


Ey PL, Prowse SJ, Jenkin CR. Isolation of pure IgG\textsubscript{1}, IgG\textsubscript{2a} and IgG\textsubscript{2b} immunoglobulina from mouse serum using protein A-Sepharose. Immunochemistry, 1976; 15: 429-436.

Farmer MC, Gaber BP. Liposome-encapsulated haemoglobin as an artificial oxygen-carrying system. Methods in


Fraker PJ, Speck JC. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-Tetrachloro-3a, 6a-diphenylglycouril. Biochemical and Biophysical Research Communications, 1978; 80: 849-857.


Gemke RJB, Kanhai HHH, Overbeeke MAM, Maas CJ, Bennebroek Gravenhorst J, Bernini LF, Engelfriet CP, Van’t Veer MB. ABO and Rhesus phenotyping of fetal erythrocytes in the first trimester of pregnancy.


Jeje MO, Blajchmann MA, Steeves K, Horsewood P, Kelton JG. Quantitation of red cell-associated IgG using an immunoradiometric assay. Transfusion, 1984; 24: 473-


Lindig BA, Rodgers MAJ. Rate parameters for the quenching of singlet oxygen by water soluble and lipid soluble substrates in aqueous and micellar systems. Photochem and Photobiol, 1981; 33: 627.


Martin E and Rustum YM. Toxicity and therapeutic efficacy of adriamycin administered as an intravenous infusion or entrapped in liposomes in mice. Proc Am Assoc Cancer Res 1984; 25: 267


Merry AH, Thomson EE, Rawlinson VI, Stratton F. Quantification of IgG on erythrocytes: correlation of number of IgG molecules per cell with the strength of the direct and indirect antiglobulin tests. Vox Sang, 1984; 47: 73-81.


Rochna E, Hughes-Jones NC. The use of purified 125I-labelled and anti-y globulin in the determination of the number of D antigen sites on red cells of different phynotypes. Vox Sang, 1965; 10: 675.


Rubin A, Kurman C, Biddison W F, Goldman N D, Nelson D L. A monoclonal antibody 7G7/B6, binds to an epitope on the human interleukin-2 receptor that is distinct from that recognised by IL-2 or anti-Tac. Hybridoma, 1985; 4: 911.


ABBREVIATIONS

ADM  adriamycin
ara C  cytosine arabinoside
CF  carboxyfluorescein
CVS  chorionic villous sample
DTP  dithiopyridyl
FITC  fluorescein isothiocyanate
G&H-FITC  goat anti-human FITC
GVHD  graft versus host disease
HDL  high density lipoprotein
LUV  large unilamellar vesicle
MLV  multilamellar vesicle
MDP  muramyl dipeptide
MTX  methotrexate
PA  protein A
PBL  peripheral blood lymphocyte
PC  phosphatidylcholine
PE  phosphatidylethanolamine
PG  phosphatidylglycerol
PHA  phytohaemagglutinin
PS  phosphatidylserine
RES  reticuloendothelial system
S&M-Ig  sheep anti-mouse Ig immunoglobulin
S&M-FITC  sheep anti-mouse FITC
SUV  small unilamellar vesicle
SUV-G&H  SUV conjugated to goat anti-human Ig immunoglobulin
SUV-PA  SUV conjugated to PA
SUV-S&M SUV conjugated to sheep anti-mouse Ig immunoglobulin

SPDP N-hydroxy-succinimidyl 3-(2-pyridyldithio) propionate

$T_c$ transition temperature
RELATED PUBLICATIONS

Published papers that contain some of the work included in this thesis.


Uptake of antibody directed cytotoxic liposomes by CD3 on human T cells

A. G. Gray, Janet Morgan, D. C. Linch & E. R. Huehns Department of Clinical Haematology, University College London, UK

(Accepted for publication 11 November 1987)

SUMMARY

Using polyclonal human T cells and anti-CD3 monoclonal antibodies we have shown that small unilamellar liposomes covalently coupled with protein A become bound to T cells and not to B cells and that the binding was a specific liposome-antibody-receptor interaction. Intracellular delivery of liposome contents was demonstrated by the use of encapsulated carboxyfluorescein and flow cytometry and the transfer of membrane-bound liposomal carboxyfluorescein was virtually complete in 30 min. Liposomes containing methotrexate inhibited the growth of PHA-stimulated peripheral blood lymphocytes by 90%, after 48 h incubation. Potential applications are proposed in the study of the behaviour of surface membrane components and in T cell depletion and purging of bone marrow.

Keywords cytotoxic liposomes cells bone marrow transplantation

INTRODUCTION

Liposomes can be targeted to specific cells using antibodies to cell surface antigens following which they may or may not be endocytosed (Machy, Barbet & Leserman, 1982). Previous studies have shown the uptake of antibody-directed liposomes by T and B lymphoid cell lines using MHC molecules (Machy et al., 1982; Huang, Kennel & Huang, 1983) but in order to contemplate clinical usage targeting must be restricted to antigens which are limited to specific cell types. In the context of allogeneic bone marrow transplantation, targeting of cytotoxic lymphocytes to T cells might be of value as a method of T cell depletion of marrow which effectively reduces the frequency and severity of graft-versus-host disease (Prentice et al., 1984). We have therefore investigated whether liposomes can be targeted specifically to the CD3+T complex on T cells and whether the liposome contents are internalized.

The targeting system which has been used involves the covalent attachment of protein A to the liposome membrane followed by incubation with lymphocytes which have been coated with anti-CD3 antibody in a previous step. Using this method we have studied the effectiveness of liposome-encapsulated methotrexate (MTX) in inhibiting lymphocyte growth in culture.

MATERIALS AND METHODS

Liposome synthesis

Small unilamellar vesicles or liposomes (SUV) were synthesized and covalently coupled to protein A (PA) after the method of

Leserman et al. (1980). Purified carboxyfluorescein (CF), 40 mm or 200 mm, or 20 mm MTX (Bristol Myers) was encapsulated. Approximately 20% of the available protein became coupled to liposomes as measured by radioactive counting (SUV-PA). The final concentration of liposomes was 2.5 mm (total lipid) and that of protein A 50 μg/ml.

Antibodies

UCHT1 is a monoclonal anti-CD3 antibody raised in mice. The original antibody is subclass IgG1, but IgG2b and IgG2a switch-variants of the same antibody have also been produced (Beverley & Callard, 1981). The IgG2a and IgG2a subclasses are able to bind protein A at pH 7.4 whereas the IgG1 subclass is not. Except where specifically mentioned, the IgG2a switch-variant has been used in the experiments. Saturating concentrations (> 5 μg/ml) of culture supernatant and ascitic fluid were used. Fluorescent goat anti-mouse immunoglobulin (GaM-FITC) was obtained from Nordic Laboratories.

Cell preparation

Fresh heparinized peripheral blood from healthy volunteers was diluted with cell culture medium (RPMI 1640, Gibco) and the mononuclear cells separated on a Ficoll-Hypaque gradient (Pharmacia). To remove monocytes, 5 ml of mononuclear cells at $3 \times 10^8$/ml in RPMI 1640 and 20% fetal calf serum was incubated overnight at 37°C in a plastic tissue culture flask. Non-adherent cells were removed by gently washing with RPMI 1640. PHA-lymphoblasts were obtained by incubation of peripheral blood mononuclear cells ($1 \times 10^7$/ml) for 72 h in cell culture medium containing 10% fetal calf serum and purified phytohaemagglutinin (4 μg/ml, Wellcome).
Liposome uptake by peripheral blood lymphocytes

Samples of \( 2 \times 10^5 \) cells were stained with monoclonal antibody in microtitre plates as described by Linch et al. (1982). Fifty microlitres of SUV-PA or 50 \( \mu l \) of GzM-FITC was added for 30 min at 4°C after which cells were washed three times in ice-cold medium. Flow cytometric analysis was performed on a FACS IV (Becton Dickinson). The effect of temperature on cell fluorescence was studied by placing cells labelled at 4°C with SUV-PA or GzM-FITC in a waterbath at 37°C. At specified intervals, cells were removed and the fluorescence re-analysed on the FACS.

UV microscopy

Cells labelled with fluorescent liposomes or fluorescent second layer antibody were placed on glass slides and sealed under coverslips at 4°C. They were examined immediately under a UV microscope using phase optics and a x 60 magnification oil immersion lens. Modulation of surface fluorescence was studied by allowing the chilled slides to warm on the microscope stage. The temperature of the slide was 25°C after 5 min and 35°C after 20 min. Photographs were taken at 10 min intervals using a standard exposure time of 15 s. The UV source was occluded between examinations to prevent bleaching. All prints were handled identically.

Effect of liposome-encapsulated MTX on PHA-lymphoblasts

The proliferation of PHA-lymphoblasts was assessed by measuring the uptake of triitated deoxyuridine (\(^3\)H dUr). Two hundred thousand cells per well were plated in triplicate cultures in 96-well microtitre plates. Fifty microlitres of antibody and 50 \( \mu l \) of liposomes were added followed by 100 \( \mu l \) of the original culture supernatant containing 10% FCS and PHA. Cells were cultured in a fully humidified atmosphere of 5% \( \mathrm{CO}_2 \) at 37°C. One microcurie per well of \(^3\)H dUr (Amersham) was added for the last 6 h of culture. Cells were harvested at 24 and 48 h on to fibreglass filters (Whatman) using an Automash (Dynatech). Counts were measured on a scintillation counter set for \(^3\)H.

### RESULTS

Targeting of protein A bearing liposomes at 4°C

The ability of protein A bearing liposomes to attach specifically to T cells was assessed using peripheral blood lymphocytes (PBL) which are 65-70% T cells, with B cells and non B, non T lymphocytes comprising the majority of the remainder. Because B cells do not express CD3 and hence do not bind UCHT1, they are not detected by fluorescent second layer antibody and do not attach liposomes. The non-T cell population acts as a useful internal control for any non-specific attachment of liposomes.

<table>
<thead>
<tr>
<th>Table 1. Targeting of protein A bearing liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of positive cells</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>(a) Cells + GzM-FITC</td>
</tr>
<tr>
<td>Cells + SUV-PA</td>
</tr>
<tr>
<td>Cells + UCHT1 + GzM-FITC</td>
</tr>
<tr>
<td>Cells + UCHT1 + SUV-PA</td>
</tr>
<tr>
<td>(b) Cells + UCHT1 + (IgG1) + GzM-FITC</td>
</tr>
<tr>
<td>Cells + UCHT1 + (IgG1) + SUV-PA</td>
</tr>
<tr>
<td>Cells + UCHT1 + (IgG2b) + GzM-FITC</td>
</tr>
<tr>
<td>Cells + UCHT1 + (IgG2b) + SUV-PA</td>
</tr>
<tr>
<td>Cells + UCHT1 + (IgG2a) + GzM-FITC</td>
</tr>
<tr>
<td>Cells + UCHT1 + (IgG2a) + SUV-PA</td>
</tr>
</tbody>
</table>

(a) Peripheral blood lymphocytes were incubated with anti-CD3 antibody (UCHT1) and FITC-conjugated goat anti-mouse antibody (GzM-FITC) or protein A bearing liposomes containing 40 nm carboxyfluorescein (SUV-PA). Negative controls were cells incubated with the second layer alone. Fluorescence was measured by flow cytometry in a FACSV IV (Becton Dickinson) and the results expressed as the percentage of fluorescence positive cells and the relative fluorescence intensity.

(b) Peripheral blood lymphocytes were incubated with IgG1, IgG2b and IgG2a switch variants of the anti-CD3 antibody UHT1. The ability of these antibodies to bind GaM-FITC and SUV-PA was compared.

Flow cytometric analysis of PBL labelled with UCHT1 antibody and protein A bearing liposomes (SUV-PA) containing 40 mm CF at 4°C is shown in Table 1a. The results are given as the number of fluorescence positive cells and the relative fluorescence intensity (peak channel number). The negative control in each case is cells incubated with the fluorescent layer alone, i.e. without UCHT1, and the positive control is cells incubated with UCHT1 and conventional second layer antibody labelled with FITC (GaM-FITC).

Protein A bearing liposomes attach to approximately equal numbers of PBL (65-70%) as Gz-M-FITC when UCHT1 is present. When UCHT1 is omitted the cells become negative and the small number of positives is again approximately equal in both cases.

Protein A liposomes therefore bind to cells labelled with antibody. The 30% or so of PBL which are not fluorescent are the B cells and non-B, non-T lymphocytes which, though ontologically closely related to the T cells, do not bear the targeting antibody. To exclude the possibility that T cell binding was a non-specific interaction between cell-bound antibody and liposomes, we compared the IgG1 and IgG2b switch variants of the antibody. Although the IgGl subclass of UCHT1 is present on the cell surface as indicated by its ability to bind GaM-FITC (Table 1b), it is unable to bind protein A liposomes. However the IgG2a and IgG2b variants are able to bind liposomes and GzM-FITC equally well and to bind the latter as effectively as the IgG1. These data demonstrate that the interaction of protein A liposomes with T cells is solely by means of protein A-antibody bridging.

Incubation of cells with protein A liposomes at 37°C

PBL labelled with liposomes or Gz-M-FITC were incubated at 37°C in order to promote internalization. This initiated a rapid
The experiments of fluorescent phores showed a rapid decline in the number of positive cells. Using the FITC label, approximately 50% of cells which were originally positive remained so after 60 min (Fig. 1). Liposome-labelled cells showed a similar rapid fall reaching its nadir of 50% after 30 min. This could be explained by internalization of fluorophore with subsequent dilution in the cytoplasm and weakening of the fluorescent signal or alternatively, shedding of the complexes into the extracellular medium where they would be undetected by the FACS. The experiments were therefore repeated using liposomes containing much higher concentrations of CF, thereby increasing the likelihood of intracellular fluorescence being detected by the FACS. Cells were labelled with liposomes containing 200 mM CF and incubated at 37°C. The opposite effect was now seen (Fig. 2). The number of positive cells was 25% at the start and increased to 46% at 60 min.

The difference in behaviour is explained by the degree of self-quenching in each of these species of liposome. CF liposomes, 200 mM, were considerably less fluorescent than those containing 40 mM CF although they contained approximately five times as much fluorophore (Table 2). Fluorescence was fully released after lysis with Triton-X and the percentage quenching was calculated using the formula:

\[ \% \text{ quenching} = 100 - \frac{\text{Fluorescence before Triton-X}}{\text{Fluorescence after Triton-X}} \times 100 \]

Thus 40 mM CF liposomes were relatively unquenched (74.9%) compared to those containing 200 mM CF which were

---

Table 2. Effect of carboxyfluorescein concentration on liposomal fluorescence

<table>
<thead>
<tr>
<th>Concentration of carboxyfluorescein in liposomes</th>
<th>Before adding Triton-X</th>
<th>After adding Triton-X</th>
<th>Percentage quenching</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 mM</td>
<td>114</td>
<td>454</td>
<td>74.9</td>
</tr>
<tr>
<td>200 mM</td>
<td>35</td>
<td>2104</td>
<td>98.4</td>
</tr>
</tbody>
</table>

Table 3. Removal of membrane-bound liposomes by enzyme

<table>
<thead>
<tr>
<th>Cells</th>
<th>Incubation</th>
<th>% positive cells</th>
<th>Relative fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) +Ab+SUV-PA</td>
<td>None</td>
<td>72</td>
<td>92</td>
</tr>
<tr>
<td>+SUV-PA</td>
<td>None</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>(b) +Ab+SUV-PA</td>
<td>60 min at 4°C</td>
<td>72</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>after enzyme</td>
<td>6</td>
<td>33</td>
</tr>
<tr>
<td>(c) +Ab+SUV-PA</td>
<td>60 min at 37°C</td>
<td>33</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>after enzyme</td>
<td>25</td>
<td>58</td>
</tr>
</tbody>
</table>

Peripheral blood lymphocytes coated with anti-CD3 antibody and protein A bearing liposomes containing 40 mM CF were analysed by flow cytometry and compared with control cells not coated with antibody (a). Cells labelled with antibody and liposome were reanalysed after 60 minutes incubation (b) at 4°C or at (c) 37°C. These were treated with activated papain and dithiothreitol for 15 min at room temperature to remove membrane-bound liposomes and fluorescence was again measured.
Uptake of liposomes by T cells

Fig. 3. Fluorescent micrographs of a single peripheral blood T cell labelled with UCHT1 and protein A liposomes containing 200 mM carboxyfluorescein. Photographs were taken at (a) time zero, and (b) 10 min and (c) 20 min after warming from 4°C. See text for details.

Fig. 4. Effect of liposome encapsulated methotrexate on PHA-lymphoblasts. 2 x 10^5 PHA-stimulated lymphocytes were cultured in triplicate microtitre wells with: (■) medium alone, (■) antibody alone, (■) liposomes containing 20 mM methotrexate, (■) antibody plus protein A bearing liposomes containing 20 mM methotrexate, and (□) antibody plus protein A bearing liposomes containing buffer (Ab+SUV-PA). The final concentration of antibody was 5 µg/ml and that of liposome-encapsulated methotrexate was 750 nM. 1 µCi of 3H-dUr was added to each well for the last 6 h of culture.

(highly quenched (98-4%).) As incubation with 200 mM CF liposomes at 37°C proceeded, the increase in the number of fluorescent cells was therefore due to unquenching of liposomes. This is inconsistent with the shedding hypothesis and suggests internalization of CF followed by cytoplasmic dilution and unquenching of fluorescence. An alternative explanation is that this increase in cell fluorescence is simply due to loss of CF from membrane bound liposomes without entry of the fluorophore into the cell. This would lead to unquenching of the liposomes and a gradual increase in brightness. However, further analysis of the location of this fluorescence using a mixture of activated papain and DTT (ZZAP) showed this interpretation to be incorrect. PBL were labelled with fluorescent liposomes at 4°C, washed and analysed on the FACS giving the expected positivity of 72% (Table 3). The cells were divided into two fractions which were incubated at 4°C and 37°C respectively for 1 h after which they were put on ice and re-analysed. The cells kept at 4°C were unchanged whereas those incubated at 37°C were now 33% positive. Both fractions were treated with ZZAP for 15 min at room temperature, washed at 4°C and re-examined. The fluorescence of cells held at 4°C was reduced to background (6%) whereas the majority of cells which were incubated at 37°C were still positive. The reduction from 33% to 25% presumably results from the removal of residual cell bound liposomes. These data suggest that the liposomes taken up by PBL at 4°C are primarily associated with the membrane while subsequent incubation at 37°C for 1 h allows a substantial number to become internalized and no longer susceptible to enzyme stripping.

UV microscopy
The appearances and behaviour of PBL incubated with liposomes was studied by UV microscopy in an attempt to elucidate the mechanism of internalization. Lymphocytes incubated with
fluorescent antibody undergo a well-recognized pattern of 'patching' and 'capping'. This process was readily observed in PBL labelled with UCHT1 and GzM-FITC at 4°C and placed on a chilled slide. Upon warming of the slide on the microscope stage, T cells initiated the patching and capping sequence which was largely complete in 20 min. Cells labelled with protein A liposomes containing 40 mM CF behaved in an identical fashion (data not shown). In contrast, cells labelled with 200 mM CF liposomes were very faint initially and on warming became brighter. The pattern also changed from a uniform peripheral distribution to patches of fluorescence which after 20 min was followed by a generalized 'blush' of cytoplasmic fluorescence. This is illustrated in Fig. 3 which shows the same T cell labelled with 200 mM CF liposomes and photographed at 10 min intervals. The sequence is consistent with the previous evidence of delivery of lipidosome contents into the cytoplasm with subsequent unquenching and it was found much easier to define the intracellular location of liposomally delivered fluorescence than that left after capping of fluorescent second-layer antibody. Moreover the similarity in the patching and capping of cells labelled with 40 mM CF liposomes compared to those labelled by conventional indirect fluorescence suggests that the handling and fate of the different receptor-ligand complexes is the same.

**Cytotoxicity of protein A liposomes containing MTX**

The ability of protein A liposomes to deliver cytotoxic quantities of a drug specifically to T cells via the CD3 antigen was tested using MTX. A 20 mM solution of MTX was encapsulated by measuring the uptake of the radioactive substrate 3H-deoxyuridine. PBL were stimulated with PHA for 72 h and then incubated with UCHT1 and liposomes. Control cells were PHA lymphoblasts incubated with medium alone, antibody alone, liposomes containing 20 mM MTX but without protein A or antibody with protein A liposomes containing buffer only. It was seen (Fig. 4) that MTX encapsulated in protein A liposomes targeted with UCHT1 caused an 80% inhibition of 3H dUrd incorporation at 24 h and 90% inhibition at 48 h. Control cells were not inhibited, in particular those incubated with untargeted liposomes containing MTX. This confirms the specificity of the targeting and the stability of the liposomes which are sufficiently impermeable to prevent non-specific cytotoxicity due to leakage after 48 h in culture.

**DISCUSSION**

Clinical use in vivo of targeted liposomes has been handicapped by problems with reticuloendothelial system uptake, instability in plasma and tumour accessibility (Weinstein, 1984). Manipulation of bone marrow ex vivo for transplantation circumvents these obstacles and may be an ideal application for targeted liposomes. As an antigen target, the human T-cell receptor complex is a good potential candidate. It is lineage specific whereas previously studied antigens for liposome uptake by lymphoid cells have been class I and class II MHC (or HLA) antigens (Machy et al., 1982; Huang et al., 1983). These are expressed by other cell types and would therefore be inappropriate for clinical usage. In addition, there is some evidence that following antibody binding, CD3 is rapidly internalized (Kan, et al., 1983) and may therefore be capable of mediating the internalization of attached liposomes. This could be of value in T cell depletion of bone marrow and may avoid some of the difficulties of current methods which employ bulk complement lysis techniques.

Our studies have shown that protein A liposomes attach to T cells in a manner which is rapid and entirely antibody dependent. The intracellular delivery of liposome contents is more difficult to demonstrate conclusively by a single technique. However, flow cytometry combined with UV microscopy and an enzyme stripping method clearly showed that surface-bound liposomes can deliver their contents into the cell cytoplasm within 30 min at 37°C. When MTX is encapsulated in the liposomes the inhibition of cell growth confirms specific intracellular delivery of liposome contents and demonstrates the feasibility of delivering cytotoxic quantities of drug via the CD3 pathway.

In their study of the fate of CD3 on human T cells after antibody-induced modulation, Kan et al. showed rapid internalization of the antigen without shedding into the extracellular medium. This was done using methods including surface iodination of the membrane component, precipitation with antibody and analysis by SDS-PAGE. For the purpose of establishing the fate of surface membrane components after antibody binding, we suggest that the use of fluorescent liposomes and flow cytometry offers an alternative method which is both simple and quick. By indicating which receptors are rapidly endocytosed the results could also have predictive value for the utility of a particular antigen as a receptor for cytotoxic liposomes.

MTX has several desirable properties with respect to encapsulation of cytotoxic compounds in antibody-directed liposomes. It is highly water-soluble, stable for many months in aqueous solution, is active clinically particularly against lymphoid malignancies and, encapsulated in liposomes, has proved effective in previous in vitro studies. However, it is active only against dividing cells, so unstimulated or malignant cells in G0 of the cell-cycle are unaffected. This is shown in the present experiments by the need for prior lectin-stimulation of lymphocytes which is obviously impractical from the point of view of T cell depletion of bone marrow. Nevertheless, the absence of non-specific cytotoxicity to untargeted cells, holds out the possibility that stem cells and hence bone marrow engraftment would be similarly unaffected. This has prompted our investigation of agents which are rapidly cytotoxic to both resting and dividing cells. Encapsulated in liposomes and combined with the highly specific targeting conferred by monoclonal antibodies, such an agent may make feasible not only T cell depletion but the purging from autologous bone marrow of any unwanted or contaminating cells, e.g. carcinoma or residual haematological malignancy.

The attachment of the liposomal bilayer of protein A (or similar ligand for immunoglobulin) as opposed to the targeting antibody itself has several advantages. It is more economical with the targeting antibody since direct coupling of liposome and antibody requires much larger and purer quantities of the latter. Direct coupling commits those liposomes to a single antigen target whereas a single batch of protein A liposomes can be used against any antigen for which a suitable antibody is available. This offers the possibility of using a cocktail of antibodies against several antigens on the same cell thereby increasing the cytotoxic potential of a particular liposome dose. In a similar manner several low density antigens which individually may not be sufficiently numerous to mediate antibody
targeted liposomal cytotoxicity, may be combined to achieve this effect using the relevant panel of antibodies and a single batch of liposomes.

In conclusion, antibody-targeted liposomes carrying fluorescent, cytotoxic or metabolically active compounds have a useful role in the study of interactions between surface membrane components and the cytoplasmic compartment which could be therapeutically exploited in the ex vivo manipulation of bone marrow.

ACKNOWLEDGMENTS
This work was funded by a Medical Research Council research fellowship. We wish to thank Dr R. C. Hider for advice and support and Belinda Bather for typing the manuscript.

REFERENCES
Enhanced fluorescence in indirect immunophenotyping by the use of fluorescent liposomes

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(Received 2 November 1988, revised received 16 January 1989, accepted 14 February 1989)

Small unilamellar liposomes were optimised for cell phenotyping by indirect immunofluorescence. This involved selection and covalent attachment to the liposome of a polyspecific ligand for cell-bound antibody. For this purpose sheep anti-mouse antibody was preferred to protein A because of its ability to attach to cell-bound IgG1 as well as IgG2 at physiological pH. The maximally fluorescent concentration of encapsulated carboxyfluorescein was determined to be 20 mM and liposomes thus comprised gave up to a nine-fold increase in mean cell fluorescence when compared with sheep anti-mouse antibody conjugated to fluorescein isothiocyanate. There was no parallel increase in background fluorescence. Liposomes retained their targeting and fluorescence properties after 3 months storage. They could be sterilised and were as versatile in use as FITC-antibody conjugates.

Key words: Fluorescent liposome; Indirect immunofluorescence

Introduction

Drug-containing liposomes have been used to target cytotoxic agents to specific cell populations in vitro as defined by reactivity with monoclonal antibodies (Leserman et al., 1981; Gray et al., 1988). Specific targeting has been demonstrated by the use of liposome-encapsulated fluorochromes but this technique has not been optimised for use as a probe in indirect phenotype analysis. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin is the most commonly used probe for phenotyping with monoclonal antibodies and for fluorescence-activated cell sorting. It is ideal for high density antigens but it is less satisfactory when antigens are present at less than a few thousand per cell. Many important receptors are present at such low numbers and an improved fluorescent detection system is required. Liposomes, by virtue of their relatively large internal aqueous space, can carry increased numbers of fluorescent molecules but this is only of value if the signal due to non-specific binding is not increased pari passu. We report here a technique which permits liposomes to be used as a second layer in indirect immunofluorescence with a significantly higher signal-to-noise ratio than can be achieved with FITC anti-mouse immunoglobulin. The fluorescent liposomes can be sterilised, are stable and as versatile as FITC-antibody conjugates.

Materials and methods

Antibodies

UCHT1 is a CD3-specific monoclonal antibody raised in mice. The original antibody is subclass...
IgG1 (Beverley and Callard, 1981) but IgG2b and IgG2a switch-variants have also been produced (Smith et al., 1986). Leu 3a was obtained from Becton Dickinson. UCHT1, UCHT2, UCHT4, LFA-2 and sheep anti-mouse IgG antibody were kindly provided by Dr. P. Beverley. Sheep anti-mouse IgG antibody was absorbed against human immunoglobulin immobilised on a column and purified by affinity column chromatography using immobilised mouse immunoglobulin. Conjugation with fluorescein isothiocyanate was carried out as described by Hudson and Hay (1980).

**Liposome synthesis**

Small unilamellar liposomes or vesicles (hence SUV) were synthesised and covalently coupled to protein A (Pharmacia) or sheep anti-mouse antibody after the method of Barbet et al. (1981). The main steps were as follows: liposomes were composed of dipalmitoyl phosphatidylcholine (Sigma); dipalmitoyl phosphatidylethanolamine (Calbiochem) derivatised with SPDP (Sigma) to form dipalmitoyl phosphatidylethanolamine pyridyl dithiopropionate; and cholesterol (BDH). The lipids were dissolved in chloroform methanol in a molar ratio of 66:1:33 and applied to the walls of a round-bottomed flask by rotary evaporation. 3 ml of Hepes buffer pH 7.4 containing 20 or 40 mM purified carboxyfluorescein (CF, Kodak) were added and the mixture vortexed until all the lipid was hydrated. The suspension was transferred to a water-cooled vessel at 41°C in the cabinet of a probe sonicator (MSE). Sonication was carried out at power setting Mark 8 for 45 min. The resulting suspension was centrifuged at 1000 x g for 20 min to remove tungsten particles from the tip of the probe and then applied to a short column of Sephadex G-50 (Pharmacia) in order to separate the encapsulated from the unencapsulated CF.

Protein A and sheep anti-mouse IgG antibody were derivatised with SPDP giving an average of five pyridyl dithiopropionate groups per protein molecule. Activation of the proteins by dithiothreitol (DTT) was carried out at pH 7.4 and the free thiol-bearing protein added immediately to the separated liposomes at room temperature. After coupling for 24 h, liposomes conjugated to protein were separated from free protein on a short column of Sepharose 6B. Carboxyfluorescein was purified free of hydrophobic contaminants by gel filtration on LH 20 (Pharmacia) before encapsulation in liposomes. Approximately 20% of available protein became coupled to liposomes as measured by radioactive counting. The final concentration of liposomes (lipid) was 2.5 mM, that of protein 1.3 μM and that of antibody 0.4 μM.

**Cell preparation and labelling**

Fresh heparinised peripheral blood from healthy volunteers was diluted with cell culture medium and the mononuclear cells separated on a Ficoll-Hypaque (Pharmacia) gradient. Monocytes were removed by an overnight plastic-adherence step. Aliquots of 2 x 10^5 cells were reacted with monoclonal antibodies in microtitre plates as previously described (Linch et al., 1982) using 50 μl of liposomes or 50 μl of FITC-conjugated sheep anti-mouse antibody as the second layer. Both first and second layer incubations were for 30 min at 4°C with three washes in ice-cold medium after each incubation.

**Flow cytometry**

Cells were analysed on a FACS IV (Becton Dickinson) flow cytometer using a 488 nm light source from an argon laser (300 mW). For each analysis an average of 10000 cells were counted. The fluorescent level at which cells were determined to be positive was established by visual inspection of the histogram of negative (control) cells such that less than 5% appeared in the positive zone. The fluorescence intensity per cell (as the channel-index number) was recorded using a linear amplifier and the mean cell fluorescence (MCF) of the cell population was calculated as the sum of the products of the number of cells per channel and the channel-index number divided by the total number of cells recorded:

$$MCF = \frac{\text{sum of (cells per channel × channel-index number)}}{\text{total number of cells}}$$

**Results**

**Choice of ligand**

SUV encapsulating 40 mM CF were coupled to protein A (SUV-PA) or sheep anti-mouse Ig anti-
TABLE I
TARGETING OF LIPOSOMES TO UCHT1-COATED PBL

Figures are the mean percentage values from experiments using two different batches of SUV-PA and SUV-SaM on PBL from healthy volunteers.

<table>
<thead>
<tr>
<th>Fluorescent second layer reagent</th>
<th>First layer antibody</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antibody subclass</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG1</td>
<td>IgG2a</td>
</tr>
<tr>
<td>FITC-SaM</td>
<td>7</td>
<td>67</td>
</tr>
<tr>
<td>SUV-PA</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>SUV-SaM</td>
<td>5</td>
<td>66</td>
</tr>
</tbody>
</table>

body (SUV-SaM) and compared with FITC-labelled sheep anti-mouse IgG antibody (FITC-SaM) for their ability to bind to peripheral blood lymphocytes (PBL) previously incubated with the three available switch-variants of the CD3 monoclonal antibody UCHT1 (Table I). The percentage of positive cells was similar with the three types of fluorescent label (approximately 65%) but SUV-PA failed to bind to PBL coated with the IgG1 subclass of antibody at pH 7.4. Staining with the fluorescent layer alone gave comparably negative background levels with each type.

Concentration of CF and F/P ratio

The concentration of CF giving maximally fluorescent SUV was determined by generating a family of liposomes identical in all but CF concentration and measuring the fluorescence of an aliquot. The maximum signal was reached at 20 mM CF (Table II). Although more CF is contained in the other liposomes they are less fluorescent due to the increasing effect of concentration quenching as demonstrated by lysis of the liposomal membrane with Triton-X. Quenching was calculated according to the formula:

\[ Q = 100 - \frac{\text{fluorescence before Triton-X treatment}}{\text{fluorescence after Triton-X treatment}} \times 100\% \]

Liposomal stability

Liposomal retention of fluorescein was tested in conditions known to promote liposome permeability, namely the presence of serum and a temperature of 37°C. A suspension of SUV-PA was incubated in tissue culture medium containing 10% fetal calf serum for 1 h after which unencapsulated CF was separated from encapsulated CF by gel filtration on a column of Sephadex G-50 (Pharmacia). A small amount of Triton-X was added to each fraction of eluate to release encapsulated CF and the amount of fluorescence in the encapsulated peak was expressed as a percentage of the total fluorescence present. Only 0.6% of liposomal CF leaked out under these conditions.

Retention of targeting ability was assessed by comparison of the staining performance of freshly made SUV-PA with that of the same batch of liposomes after 3 months storage at 4°C in the dark. FITC-conjugated anti-mouse Ig antibody was used as a reference standard and the same batch stored at -20°C was used for the phenotyping 3 months later. At that time there was no difference in the percentage of positive cells between the two fluorescent labels and the relative fluorescence intensity of the liposomes compared with the FITC antibody-labelled cells was unchanged at 103% of its original value. Retention of CF in liposomes was not affected by cell attachment after 3 days (data not shown) but labelled cells may be conveniently preserved for longer

TABLE II
DETERMINATION OF MAXIMALLY FLUORESCENT LIPOSOMES

The fluorescence of liposomes containing various concentrations of CF was measured. CF was released from liposomes by detergent lysis using Triton-X and the percentage of fluorescence quenching was calculated (see text for formula).

<table>
<thead>
<tr>
<th>Concentration of CF in liposomes (mM)</th>
<th>Relative fluorescence Before addition of Triton-X</th>
<th>After addition of Triton-X</th>
<th>Percentage quenching</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>234</td>
<td>67</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>353</td>
<td>264</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>384</td>
<td>434</td>
<td>12</td>
</tr>
<tr>
<td>40</td>
<td>251</td>
<td>454</td>
<td>45</td>
</tr>
<tr>
<td>50</td>
<td>189</td>
<td>580</td>
<td>67</td>
</tr>
<tr>
<td>100</td>
<td>114</td>
<td>1508</td>
<td>92</td>
</tr>
<tr>
<td>200</td>
<td>81</td>
<td>2104</td>
<td>96</td>
</tr>
</tbody>
</table>
TABLE III
TITRATION OF LIPOSOMES COUPLED WITH SHEEP ANTI-MOUSE ANTIBODY

PBL were labelled with antibody (UCHT1) and various dilutions of liposomes coupled to sheep anti-mouse antibody (SUV-SaM). The percentage of positive cells and the relative fluorescence intensity measured by flow cytometry were compared. The optimum working dilution was established at 1/4. Cells with a 1/2 dilution were compared before \( a \) and after \( b \) fixation with 1% formaldehyde.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution of SUV-SaM</th>
<th>% positive cells</th>
<th>Relative fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells + SUV-SaM</td>
<td>Neat</td>
<td>6</td>
<td>38</td>
</tr>
<tr>
<td>Cells + Ab + SUV-SaM</td>
<td>Neat</td>
<td>65</td>
<td>249</td>
</tr>
<tr>
<td></td>
<td>1/2 ( a )</td>
<td>71</td>
<td>241</td>
</tr>
<tr>
<td></td>
<td>1/2 ( b )</td>
<td>73</td>
<td>244</td>
</tr>
<tr>
<td></td>
<td>1/4</td>
<td>75</td>
<td>217</td>
</tr>
<tr>
<td></td>
<td>1/8</td>
<td>75</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td>1/16</td>
<td>71</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td>1/32</td>
<td>66</td>
<td>164</td>
</tr>
<tr>
<td></td>
<td>1/64</td>
<td>71</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>1/128</td>
<td>62</td>
<td>121</td>
</tr>
</tbody>
</table>

period by fixation with 1% formaldehyde. This is preferred to alcohol-based fixatives which may permeabilise the liposomal bilayer. Fixation itself has no significant effect on fluorescence (Table III).

Sensitivity and specificity

The preferred liposomal species was considered to be SUV containing 20 mM CF and coupled to sheep anti-mouse IgG antibody. The optimal dilution of the liposome suspension providing the maximum signal-to-noise ratio was approximately 1 in 4 although dilutions of the liposomes up to 1 in 128 gave a similar value for the percentage of peripheral blood mononuclear cells reacting with UCHT1 (Table III). The fluorescence characteristics of the 1 in 4 dilution was compared with those of a similarly optimised concentration of FITC-SaM (Table IV). The enhancement of mean cell fluorescence (calculated after subtraction of background) was increased approximately nine-fold in the case of UCHT1 and from 3.1 to 7.8 times with antibodies against a range of other lymphocyte antigens. The percentage of positive cells was the same with each antibody by either technique suggesting that the increase in sensitivity was not at the expense of specificity. This was confirmed by comparison of the background staining due to SUV-SaM with that of FITC-SaM. In four experiments using two different liposome preparations the MCF was 20.1 ± 4.4 compared with 26.1 ± 1.9 with FITC-SaM. Similarly, in an experiment comparing the autofluorescence of the lymphocytes with the fluorescence of the cells after incubation with SUV-SaM in the absence of UCHT1 (autofluorescence of cells plus non-
specific binding of label) it was found that at the optimal dilution (1/4) non-specific binding of SUV-SaM added only minimally to the autofluorescence (Table V, 15.4 versus 14.5).

Fig. 1 is a representative FACS histogram of PBL incubated with and without UCHT1 using either FITC-SaM (Fig. 1a) or SUV-SaM (Fig. 1b) as the second layer. The SUV-SaM clearly results in enhanced brightness of the positive cells without a parallel increase in background staining.

**Table V**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution of SUV-SaM</th>
<th>Mean cell fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells alone</td>
<td>0</td>
<td>14.5</td>
</tr>
<tr>
<td>Cells + SUV-SaM</td>
<td>Neat</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>1/2</td>
<td>16.1</td>
</tr>
<tr>
<td></td>
<td>1/4</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>1/8</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>1/16</td>
<td>15.1</td>
</tr>
<tr>
<td></td>
<td>1/32</td>
<td>14.4</td>
</tr>
</tbody>
</table>

**Discussion**

Targeting of liposomes to cells has been achieved in the past by covalent attachment to the liposomal bilayer of antibody itself or protein A (Leserman et al., 1980; Gray et al., 1988). However, both approaches have serious limitations in their applicability. Direct attachment of antibody has the disadvantage of committing a suspension of liposomes to a single antigen target creating the need for a different liposome preparation for each antigen. The protein A system relies upon the affinity of this staphylococcal protein for the Fc portion of the immunoglobulin molecule. Cells are incubated with targeting antibody and the excess is washed off prior to addition of the liposomes which are therefore uncommitted or polyspecific. The disadvantage is that protein A does not bind to all subclasses of mouse antibody at pH 7.4. At this pH it has only low affinity for IgG1, a commonly used subclass of mouse monoclonal (Table I). Another potential disadvantage is that protein A may bind to endogenous surface (human) IgG expressed on non-targeted cells, e.g., B lymphocytes. Protein G, also derived from bacteria and recently purified, binds to a wider range of immunoglobulin species but also has poor affinity for mouse IgG1 (Bjorck and Kronvall, 1984). We therefore reasoned that a better ligand to be coupled to liposomes would be polyclonal anti-mouse immunoglobulin and we show here that sheep anti-mouse antibody can be covalently coupled to liposomes using a bifunctional cross-linking agent without loss of reactivity. This liposomal species detects IgG1 antibodies as well as IgG2a and IgG2b.

The amount of CF which can be encapsulated in liposomes is dependent on the volume of the internal aqueous space and the solubility of the CF salt. However, high concentrations result in only weakly fluorescent liposomes due to the phenomenon of concentration quenching. Measurements on a family of liposomes encapsulating different concentrations of CF (Table II) showed that the maximally fluorescent liposome contained approximately 20 mM CF. In a previous study liposomes with only half this concentration of CF were selected (Truneh et al., 1987). However, these liposomes (which were directly coupled to the
targeting monoclonal antibody) were evaluated at concentrations of only 1, 10 and 50 mM CF. Since small variations around the optimal CF concentration have a critical effect on fluorescence, it is essential to study liposomes with small incremental differences in the concentration of the fluorophore.

With 20 mM CF the \( F/P \) ratio of liposomes coupled to SaM is 60 whereas FITC-conjugated antibody can carry only up to four molecules of fluorescein (\( F/P \) ratio = 4) without affecting the affinity of the antibody. In comparative tests with FITC-SaM the mean cell fluorescence (MCF) of PBL incubated with various antibodies was 3.1–8.9-fold higher with SUV-SaM. On the FACS histogram (Fig. 1), this is illustrated by the clear shift to the right of the fluorescent peak of the liposome-labelled cells. That this enhancement was not achieved at the expense of increased background staining is shown by comparison of the cellular fluorescence after incubation with either label in the absence of first layer antibody. The MCF is very similar in both and close to the autofluorescence of the cells.

The theoretically possible 15-fold increase in MCF predicted by comparison of the \( F/P \) ratios was not observed with any of the antibodies tested. Since antibody-conjugated liposomes are large (approximately 50 nm) when compared with FITC-antibody conjugates, some of the shortfall may be explained by steric hindrance around the antigen epitopes on the cell-bound antibody. It also needs to be explained why some antibodies, e.g., UCHT1 gave a consistently higher MCF enhancement factor than others, e.g., UCHT4. It may be that the epitopes on the monoclonal antibodies differ in their accessibility to sheep antimuscle antibody when it is linked to FITC compared with liposomes. At the level of the cell surface antigen it is also conceivable that adjacent membrane structures and the configuration or clustering of cell surface antigens could interfere with the binding of the larger antibody-coupled liposomes.

Previous attempts to boost the fluorescent signal with carrier structures have been hampered either by concentration quenching resulting in weak emissions (Shapiro, 1985) or a parallel increase in background giving a reduced signal-to-noise ratio (Kieran and Longenecker, 1984). Liposomes combine the advantage of a large internal aqueous space with the ability to encapsulate a precisely optimised concentration of fluorophore thus avoiding the possibility of quenching. The low background signal from these highly fluorescent liposomes may be attributable to several factors. A potential cause of non-specific attachment to cells is interaction of the antibody component with Fc receptors on the cell surface. However, coupling of antibody to liposomes using SPDP occurs by way of amino groups many of which are present in the Fc portion of the Ig molecule. These bonds probably result in a tethering of the Fc portion to the liposome membrane such that it is no longer fully available for Fc receptor binding, unlike the same portion of the molecule in FITC conjugates which remains capable of such interactions. In the case of the FITC conjugate the introduction of an isothiocyanate group produces an increased net negative charge on the antibody protein. This does not occur in the case of the liposome conjugate where antibody and label are separated and this difference may affect their respective non-specific binding to the cell membrane. Furthermore, the purified CF exists in a compartment which is completely separated from the cell membrane by the liposomal bilayer. This eliminates the possibility of interaction with surface protein and carbohydrate structures and also prevents partitioning of the fluorophore into the cell membrane.

As well as minimal non-specific attachments the choice of lipid and coupling method also ensures the remarkable stability of the probe. The use of saturated lipids with a relatively high transition temperature and the inclusion of cholesterol combine in reducing the permeability of solutes across the membrane (Papahadjopoulos et al., 1971). The coupling method is such that satisfactory levels of antibody attachment are achieved without exposing the liposome to oxidising agents or detergents and without the risk of homopolymerisation of the protein or aggregation of the liposomes. All of these factors combine to produce a probe which shows no significant deterioration in either targeting or fluorescence after at least 3 months. Cells can be examined by flow cytometry at any convenient time after labelling by simple
fixation in formaldehyde and sterile sorting can be performed since the probe readily passes through a 0.22 μm filter.

The high density antigens studied here are readily detectable by conventional immunofluorescence methods but preliminary results with low density antigens indicate that structures at present only detectable by radiolabelling or rosetting methods can be shown by fluorescence using the liposome technique. Applications can be envisaged in cell phenotyping for leukaemia diagnosis and in the detection of early activation antigens. Liposomes are very versatile in terms of how much and what sort of material can be encapsulated. Thus the principle could be extended to provide even higher levels of fluorescence by using liposomes with larger internal aqueous volumes; e.g., multilamellar or reverse phase vesicles, and fluorophores with different emission spectra could be encapsulated for multicolour fluorescence work. We have demonstrated that the versatility of fluorescent liposomes can be extended further by the attachment of a polyspecific ligand such as an antispecies antibody rather than a target-specific monoclonal antibody or protein A.

References


